Review of Methods for Studying Maturation of Human Erythroblasts in Vitro: Evaluation of a New Method of Culture of Cell Suspensions in a Clot-Free Medium

By LAWRENCE BERMAN AND EDWARD R. POWSNER

With the technical assistance of Mary L. Davis and Marie Fly

Our purpose in this paper is to describe a method for studying maturation of human erythroblasts in vitro. A procedure for studying proliferation in the same experimental model will be presented in another paper. The need for inquiring into maturation and proliferation separately is illuminated by results of assays of erythropoietic agents in different laboratories, especially those assays in which reticulocytosis, erythrocytosis and erythroid hyperplasia occurred without an associated increase of hemoglobin, and with the appearance of red cells of decreased size. It is reasonable to suppose that at least two types of factors exist, one having its main effect on proliferation and the other on maturation. It is not known whether or not some of their actions have direct effects on the erythroblastic cells.

A confusing aspect of the literature is the variety of technics of cell culture which were often described without explanations of why they were selected. Since such information has great bearing on interpretation of results, it is necessary to present a review of methods in use, after which we will give an account of the development of our own in vitro model. Finally, we will discuss the results of challenging the model with various types of cell-plasma relationships. Specific details of technic are given in the Appendix.

Review of Methods in Use

Each of the large number of reported methods has its own superficially subtle but significant variations which affect the behavior of cells. Fieschi and Astaldi, Plum, Reiser and Lajtha have reviewed parts of the subject. This discussion supplements theirs and emphasizes points having a connection with the method we have developed. Techniques of marrow cell culture are of two main types: (1) plasma clot methods in which fragments of marrow tissue are grown in clots of human, animal, or mixed plasmas, overlaid by a gas phase which is usually air, or a fluid phase composed of diluted serum or plasma or nutrient medium; and (2) cultures of discrete, disassociated cells in fluid medium, in which the cells are allowed to sediment in standing vessels or in which they are kept in motion by various means. In stationary cultures, the density of the cell population and the depth of medium are important factors, whereas the effects of these factors can be reduced or eliminated by keeping the cells in motion, as in roller tubes or with stirrers, or by agitating the cell suspensions by means of turbulence created by streams of gas flowing through the medium.
METHODS FOR STUDYING HUMAN ERYTHROBLAST MATURATION

TABLE 1.—Absolute Numbers of Erythroblasts in Cell Suspension Cultures Prepared by Methods A, B, C, and D

<table>
<thead>
<tr>
<th>Pro- and Baso-philic cells</th>
<th>Polychromatophilic cells</th>
<th>Orthochromic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>0</td>
<td>135</td>
<td>40</td>
</tr>
<tr>
<td>24</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>72</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>96</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Method A: Stationary cultures in 16 x 150 mm. test-tubes containing 1 ml. of culture material.
Method B: Roller cultures in 16 x 150 mm. test-tubes containing 1 ml. of culture material.
Method C: Stationary cultures in T-9 flasks (Kontes Glass Co.) containing 1 ml. of culture material.
Method D: Roller cultures in individual 10 x 75 mm. test-tubes containing 0.25 ml. of culture material (Method described in Appendix).

In their report of a trial of three in vitro systems, Swan, Reisner and Silverman stated, "Most of the published reports have dealt with suspension cultures of the Osgood and Brownlee type, a method of tissue culture which has been criticized by many workers in this field." The validity of these criticisms of cell suspension methods based on the principles of Osgood and Brownlee was not discussed.

The personal experience of one of us (L. B.) with a variety of tissue culture methods demonstrates a point on which tissue culturists would agree, namely, the superiority of the classic plasma clot methods for some purposes and the cell suspension methods for others. For example, if morphologic study of myeloid or stromal cells is an objective, the highly successful methods of Fieschi and Astaldi can be used to reveal exquisite detail, or a simpler Beckman beaker technic described by Berman et al. can be used to advantage. If the objective is to study large masses of cultivated human marrow cells and their potentialities for transformation to malignant or heteroploid variants, the microbiologic methods applied to marrow cells by Berman et al. and Ruddle et al. can be used. The maturation studies of human erythroblasts pursued by workers of the Italian school, Littia, Swan et al., and others are based on the original technic of Osgood and Brownlee. Furthermore, marrow cell suspension cultures have been used effectively in studies of heme and DNA synthesis by marrow cells in vitro.

Some important assumptions have been made by students of in vitro models of marrow. There is no doubt that in various types of cultures the relative or absolute counts of orthochromic cells increase while those of the other types of erythroblasts decrease during incubation. This is illustrated in reports of researchers of the Italian school who used a system of plasma clots submerged in fluid medium. It is also demonstrated in table 1 (taken from protocols in our series of trials of different types of cell suspension methods). Usually it is assumed that such changes are reflections of maturation of erythroblasts and when this is observed it is regarded as an activity of the miniature sample of the erythron. Some have gone much further in concluding that such a sample depicts changes which are quantitatively similar to those occurring in vivo. Let us examine these assumptions.

*The original method proposed by Osgood and Muscovitz required complicated apparatus serving the functions of lung, kidney and circulation. A similar and even more elaborate apparatus was designed by Phimm, but in each case the apparatus was abandoned in favor of a simplified version, one being the method of Osgood and Brownlee, which may be regarded as the forerunner of practically all the cell suspension methods in present use.
The models are not samples of the erythron alone; they are samples of the part of the marrow organ which can be obtained by aspiration. They contain leukocytic elements and stromal cells and, in most cases, large numbers of erythrocytes. Stromal elements and reticulum cells are under-represented in such material. The use of actual fragments of marrow tissue partly overcomes this deficiency, except that various particles may differ greatly in their cellular constituents. At any rate, when maturation is determined by various premises it is not a study of erythroblasts alone, but also a study of all the possible effects of incubating the other types of cells in the system. An example of the influence of nonerythroid cells is made evident by electron microscopy of the iron cycle in bone marrow which reveals how reticulum cells play a role in the transfer of iron-containing material to the erythroblasts. In the case of proliferation, the number of reticulum cells in the sample may determine the extent and duration of active generation of erythroblasts; this is a point which appears to have been emphasized only by the Italian workers.

Mitotic activity among erythroblasts is prominent in plasma clot cultures, as attested by numerous illustrations in the reports of Astaldi and Mauri. A common belief about cell suspension methods is that mitotic activity does not occur, or when it does, it is of negligible extent. This belief is shared by Matoth et al. and Astaldi, although others would disagree. Such differences of opinion cannot be resolved without knowing how the problem of proliferation was studied. If conclusions were based on mitotic indexes they may be invalid because the mitotic index is only an indication of the incidence of mitotic figures at any moment of study. It is not, by itself, a measure of mitotic rate, that is, the number of cells entering into mitosis per unit of time. On the other hand, Astaldi, who has an awareness of the need for studying mitotic rates, has found that proliferative activity in suspension cultures of the Astaldi and Tolentino type is rapidly attenuated. Researchers in Italy have tried to take advantage of this by using a plasma clot system for studying proliferation and a cell suspension system for studying maturation as isolated phenomena. Unfortunately, in plasma clot systems where mitotic activity is prominent, maturation of erythroblasts also occurs.

Up to the time of development of our present cell suspension system, if one wanted cultures in which both proliferation and maturation occurred in maximal degrees, he would have been wise to select some form of plasma clot technic. In fact, Matoth et al. recently described another plasma clot technic because they considered this to be an advantage. In the clot-free cell suspension system we have developed there is also both proliferation and maturation of erythroblasts going on at the same time but we do not agree that this is always desirable.

The apparently irreconcilable differences of opinion concerning the extent of proliferation in cell suspension cultures are more easily understood in the light of the gradient principles enunciated by Osgood and Krippaehne. They showed that the density of the cell population multiplied by the depth of the medium is a constant peculiar to the type of cell. Consequently we may expect different results in the cases of cells kept at the bottoms of vessels in deep layers of medium, or cells in Carrel flasks under a thin layer of medium or, as in our method, with cells under conditions in which gradients are upset by placing the cell suspensions in roller tubes. The generally accepted view that erythroblasts proliferate in plasma clot systems but do not proliferate in fluid cell suspension systems is a shibboleth which should be discarded. Mitotic activity in our cell suspension cultures proceeds vigorously for 16 to 18 hours and is quantitatively greater than that observed by Astaldi and Mauri in their plasma clot cultures. The important point is that the interpretation of data depends a great deal on understanding the exact details of the culture method as well as the method of analysis of results, that is, the parameters selected for study.

Differential counts of erythroblasts have been used as parameters of maturation. In addition, morphologic changes have been the basis for determining effects of metabolites on pathologic cells, as in numerous studies of pernicious anemia. Both plasma clot and cell suspension systems yield...
such information but the advantage of the latter is that absolute counts of nucleated cells can be determined. Total nucleated cell counts are made and with differential counts on smears the absolute counts of the various maturation stages can be calculated. In some instances, the total erythrocyte count rather than nucleated cell count has been used, as in the manner of counting platelets by the indirect method.8,10 An assumption made by investigators of the Italian school is that the disappearance of pro- and basophilic cells in cultures is due to their maturation to more differentiated forms and, likewise, the disappearance of polychromatophilic cells is due to their maturation to orthochromic forms and, finally, the disappearance of orthochromic cells is due to their denucleation. The times for disappearance of various maturation stages have been used as parameters of maturation.7,8,11,17,18 In the Astaldi and Tolentino type of cultures (thin layers of cell suspensions in Carrel flasks) there is a fall in the number of all maturation forms as incubation proceeds, except for a brief temporary rise in the number of orthochromic cells.10 This is true also of other types of stationary cell suspension cultures.33

On the contrary, in cultures prepared by our new cell suspension method there is a steady increase of orthochromic cells up to and past 48 hours. The disappearance of the cells, used as a parameter of maturation for stationary cell suspension methods, does not apply to other types of cultures. In each instance the conditions are artificial and the usefulness of deductions arrived at must be determined on the basis of whether or not they correlate with those reached by other means.

Another innocent-appearing assumption, especially cogent to the model we have developed, is that maturation of erythroblasts is indicated by the color change, polychromasia to orthochromasia. This is reasonable in the case of in vivo situations but it is more difficult to justify in the case of in vitro systems. For instance, during the first 24 hours of culture almost all of the heme synthesis takes place, as determined by isotopic studies in our cultures, and yet there is a continued accumulation of orthochromic cells during the ensuing 24 to 48 hours.9 Does the change from polychromasia to orthochromasia indicate degeneration of cells already effete at 24 hours, or does it have the same significance as that occurring in vivo? This question will be discussed in more detail later.

DEVELOPMENT OF OUR PRESENT CELL SUSPENSION METHOD

The criteria for selecting a culture system were the following: (1) convenience; (2) high quality of preparations for morphologic study; (3) easily recognized end-points; (4) applicability of the system to isotopic studies; (5) simplicity of apparatus; (6) reasonable degree of required skill; and (7) replicability of samples. Eleven different systems were examined. Over 300 cultures were evaluated. The minimal number of trials with a single method was 10. The list below gives the methods and their origins.

A. Methods for use with clotted plasma
   a. Explants of marrow tissue fragments in plasma clots
      1. Double coverslip method (Maximow60)
      2. Cultures with and without a fluid phase in stationary vessels (Fieschi and Astaldi61); (Berman et al.14)
      3. Cultures with a fluid phase in roller tubes (Woodard and Pomcrat69)

B. Methods for use in clot-free systems
   a. Explants of marrow tissue fragments
      1. Explants on glass in fluid medium in stationary tubes (Swan et al.56)
      2. Explants on glass with a thin over-layer of fluid medium in stationary Leighton tubes (Berman et al.15)
   b. Cell suspensions
      i. Cells in deep layers of medium

Our experience with these culture methods will now be discussed according to the criteria previously mentioned.

Convenience.—Plasma clot cultures were superior for examination of reticulum cells, myeloid cells and chromosomal details which are illustrated beautifully by Fieschi and Astaldi. The use of clots is often difficult and time-consuming because the plasma must be balanced by an appropriate concentration of heparin which will prevent coagulation during handling but permit efficient clotting when thrombin or embryonic extracts are added. For reliable results, the heparin must be titrated in advance for each batch of human plasma or coagulating agent. Furthermore, clots of human plasma are frequently poor in transparency, firmness or resistance to lysis if the cultures are maintained after 24 hours. Fieschi and Astaldi were able to overcome this by adding chicken plasma. This introduced an unnecessary complication for studies of human cells. Finally, the preparation of films of cells requires mechanical disruption or tryptic lysis of the clot and subsequent mixing of recovered cells and clot materials prior to making the smears. We have found both these procedures capricious and unpredictable for making films of high quality. A number of otherwise excellent methods were discarded because of these considerations.

Another point to be considered was the convenience of scheduling observations. In some systems, notably those based on disappearance rates of maturation stages, it is necessary to make observations at frequent regular intervals for periods as long as 96 hours. This imposes difficulties in designing a schedule to fit the ordinary working day. For these reasons we concentrated on systems which yield useful data at 24 and 48 hour intervals of study.

Quality of permanent preparations.—For differential counts of smears of cell suspensions it is important to have material free of degenerative changes. Almost all systems gave satisfactory results for periods up to 24 hours, but only those in which the cells were kept in motion yielded consistently good material up to or after 48 hours.

End points.—It is noteworthy that in all types of systems there were similar morphologic phenomena. In cultures of tissue fragments explanted to clots it was easy to observe a shift from predominance of polychromatophilic to orthochromic cells, and with cell suspension cultures this was found to be a reflection of actual increases of orthochromic cells. In the stationary cell suspension methods the absolute increase of orthochromic cells was very brief in duration. There were three cell suspension methods in which absolute cell count increases were observed consistently. These were the systems in which the cells or cultures were kept in motion. The most easily defined

---

*A method used in the survey of procedures.
end point was the appearance of orthochromasia, or the appearance of orthochromic erythroblasts in the cultures. In agreement with Lajtha, we defined the orthochromic erythroblast as a cell with "No traces of basophilia in the cytoplasm; it has the colour of the ripe erythrocyte (and not the polychromatic erythrocyte!)." This end point was more restrictive than one based on the wide range of cell types in the polychromatophilic class. Besides, for maturation studies it was theoretically desirable to observe cells which do not undergo mitosis.

**Applicability of the system to other types of study.**—Any type of cell suspension system is easily adapted to the study of effects of soluble reagents. The most effective continuous contact between the cells and medium is attained in the nonstationary systems. Without modification of apparatus or method of culture we have studied heme synthesis in our cell suspensions in parallel with morphologic maturation studies, by means of observing the incorporation of C14-labeled glycine into hemin.

**Simplicity of apparatus.**—The complicated apparatus used by Osgood and Muscovitz and that used more recently by Plum were found to be unnecessary for studying maturation. The former was replaced by the simple vaccine vial technic of Osgood and Brownlee. Our own experience with the Plum apparatus obtained from the original manufacturer and made according to Plum's specifications proved unwieldy in our hands mainly because of the delicate glassware required. Furthermore, results obtained later by Clemmesen and Plum with a simplified apparatus consisting of a bottle supplied with an aerator tube were practically identical with those yielded by the original apparatus. All the other systems we investigated can be set up with inexpensive items of standard laboratory equipment.

**Skill required.**—The skill needed for harvesting data was acquired relatively slowly with plasma clot systems in which only shifts of differential counts were observed. Less skill was needed in the use of cell suspension systems where absolute cell counts were used. In the case of methods in which the parameter is the increase of erythrocyte counts after 2, 3 or 5 hours of incubation a very high degree of skill was required to insure the necessary accuracy of observations. Clemmesen and Plum specify the use of a special mechanical shaker for insuring even suspension of cells, and the engagement of persons with a high degree of accuracy in counting, with precautions that they must be left totally undisturbed during the counting procedure. We were unable to duplicate their small coefficients of variation in erythrocyte counts.

**Replicability of samples.**—There are very few reported data concerning the consistency of results obtained in replicate cultures of different types performed in one laboratory. Three different culture systems were finally selected for further study on the basis of the criteria already discussed. These were (1) deep marrow cell suspensions in stationary large tube vessels from which successive samples were taken, (2) deep marrow cell suspensions in large roller tubes from which successive samples were taken, and (3) marrow cell suspensions in small individual roller tubes, the entire contents of each being used as a sample for observation. Duplicate runs of 6 or 7 marrow cultures

*The method described in the Appendix.
TABLE 2.—Average Per Cent Differences Between Two Replicate Cultures in Serially Sampled Cell Culture Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>% difference*</th>
<th>Number of observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Stationary large tubes</td>
<td>48.3</td>
<td>55</td>
</tr>
<tr>
<td>2. Large roller tubes</td>
<td>32.4</td>
<td>63</td>
</tr>
<tr>
<td>3. Small individual roller tubes</td>
<td>8.5</td>
<td>52</td>
</tr>
</tbody>
</table>

*Mean of \(|A - B| \div (A + B)/2 \times 100.
†Method described in Appendix.

were made by each of these methods. Observations were made at intervals of 0, 6, 12, 24, 30, 48, 54, 72, 78 and 96 hours. The average percentage differences of absolute nucleated cell counts among replicate samples was determined (table 2). The stationary and large roller tube methods showed relatively large variations. The small roller tube method, by comparison, yielded more consistent and reasonably accurate sampling results.

The method finally selected for maturation studies, described in detail in the Appendix, consists of incubating marrow cell suspensions in small roller tubes. There is a continuous shift from a predominantly polychromatophilic cell population to one composed mainly of orthochromic cells. During 48 hours of culture there is a steadily increasing absolute count of orthochromic cells. Since the actual number of orthochromic cells accumulating in the cultures is determined in part by the size of the original erythroblast inoculum and by the number of orthochromic cells present at the beginning, the counts are expressed as the number of accumulated orthochromic cells per 1000 total erythroblasts at zero hour of the culture. As with other methods of analysis, there are certain assumptions inherent in our system.

In this type of cell suspension culture the total erythroblast count remains fairly steady between 6 and 72 hours of incubation, while the polychromatophilic cell count falls and the orthochromic cell count rises. We think this means that there is a maturation of the cell population, realizing that this is affected by proliferation of basophilic and polychromatophilic erythroblasts, denucleation of orthochromic cells and probably other factors as well. The most important assumption is that the color change, polychromasia to orthochromasia, is not merely some kind of degeneration having no analogy in vivo, but is actually due to maturation of the cell. An overriding reason for use of the increase of orthochromic cells as a parameter is that it correlates with observations on heme synthesis made simultaneously in the same system. However, we observed that heme synthesis reaches a maximum at a time when the conversion to maximum values for orthochromic cells is only about half completed. The reasons for this time relationship are discussed elsewhere; there are theoretical reasons for using the parameter.

Synthesis of heme is, of course, not the only biochemical event related to the effectiveness of the erythropoietic function of marrow. The conversion of erythroblasts from the polychromatophilic to the orthochromic state is another event which must take place before erythrocytes become available. Under normal conditions only a very small fraction of cells reaching the
circulation are in polychromatophilic condition. In fact, departure from this is a sign of disturbed, usually accelerated, formation of erythrocytes. Therefore, a study of the dynamics of conversion of cells from polychromatophilic to orthochromic stages is an important part of the problem of maturation. In spite of inherent difficulties presented by in vitro models, evaluations of the accumulations of orthochromic cells may have validity because they are determined by the net capacity of the model to form these cells.

The practical question to be resolved is whether or not this parameter is of value in distinguishing different types of cells or different types of plasmas. This can be met in part by confronting the model with challenges in the form of cultures of erythroblasts about which our clinical experience has given us insight, selected from patients with various types of anemias or other conditions, especially if the same problems were studied by others using different approaches.

Accordingly, we set up a series of cultures of control material from the marrow of patients with normal hematologic findings. This was compared with the only other available similar series reported by Lajtha.\textsuperscript{33} In addition, we challenged the model with studies of erythroblasts and plasmas from patients with iron deficiency anemia, thalassemia, azotemia and erythrocytosis. There are very few reports of more than several examples of any one type of cell-plasma system involving normoblasts or plasmas from anemic or polycythemic individuals. When possible, our findings will be compared with those of others who have used different methods. We have accumulated enough data for statistical analysis in the case of iron deficiency anemia, but other experiments are presented as challenges to determine whether or not our in vitro model provides a means for distinguishing among various kinds of cell-plasma relationships.

**RESULTS OF EXPERIMENTS WITH THE NEW IN VITRO MODEL**

*Normal cells in normal plasma.*—Twenty-three cultures have been completed as control material to be used in future studies. Figure 1 shows a series of maturation curves derived from our experiments, and from Lajtha’s\textsuperscript{33} series of 23 normal systems. In each, the shifting character of the relative numbers of various maturation forms was revealed. In our roller tube cultures which yielded good morphologic detail up to 96 hours, the shift continued until practically all the cells were orthochromic.

Counts of accumulated orthochromic cells are shown in figure 2. Unlike the situation in Lajtha’s stationary cultures in which the increase is of very short duration, our roller tube cultures displayed steadily increasing counts up to and past 48 hours.

When orthochromic cells were studied by means of differential counts of erythroblasts, the two methods of cell suspension culture appeared to yield the same kinds of results, but when counts of accumulated orthochromic cells were made they did not. This is shown in figure 3.

*Iron deficiency anemia.*—Astaldi, Mauri and Salera\textsuperscript{7} used a stationary cell suspension technic to study erythroblasts from a patient with essential hypochromic anemia. Their parameter was the rate of decrease of cells in the system. They concluded that maturation was better in normal than in the patient’s serum.

In our experiments, the erythroblasts of iron deficiency (FeD) also showed greater maturation in medium containing normal plasma than was the case for medium containing FeD plasma (table 3). The usual interpretation of such results is that the relatively poor maturation of FeD cells is due to an inadequacy of the FeD plasma for supporting
maturation, rather than to a defect in the cells. This conclusion is based on the observation that maturation of FeD cells, as determined by the rate of increase of cells in our culture system, is improved when the cells are incubated in normal plasma medium.

However, if we compare these results with those derived from our experiments in which normal cells were incubated in FeD plasma, as well as from our control series
METHODS FOR STUDYING HUMAN ERYTHROBLAST MATURATION

Fig. 3.—Changes in orthochromic cells in two different cell suspension culture systems. Left: percentages of orthochromic cells in the erythroblast population (23 normal cultures in Berman and Powsner series; 23 normal cultures in Lajtha series). Right: accumulated orthochromic cells per 1000 erythroblasts of the original inoculum (23 normal cultures of Berman and Powsner series; data derived from 14 normal cultures of Lajtha series).

Table 3.—Accumulated Orthochromic Erythroblasts per 1000 Erythroblasts in Original Inoculum in Cultures of Cells from Patients with Iron Deficiency (FeD) in Iron Deficiency and Normal (N) Plasma

<table>
<thead>
<tr>
<th>Culture No.</th>
<th>FeD cells in FeD plasma</th>
<th>FeD cells in N plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr.</td>
<td>48 hr.</td>
</tr>
<tr>
<td>270</td>
<td>42</td>
<td>222</td>
</tr>
<tr>
<td>256</td>
<td>15</td>
<td>133</td>
</tr>
<tr>
<td>246</td>
<td>31</td>
<td>164</td>
</tr>
<tr>
<td>236</td>
<td>81</td>
<td>242</td>
</tr>
<tr>
<td>233</td>
<td>12</td>
<td>48</td>
</tr>
<tr>
<td>220</td>
<td>99</td>
<td>269</td>
</tr>
<tr>
<td>215</td>
<td>138</td>
<td>168</td>
</tr>
<tr>
<td>210</td>
<td>89</td>
<td>130</td>
</tr>
<tr>
<td>203</td>
<td>231</td>
<td>345</td>
</tr>
<tr>
<td>Means</td>
<td>90.4</td>
<td>173.6</td>
</tr>
</tbody>
</table>

*Based on the mean differences of individual cultures at times specified: t(24 hr.) = 1.97, p = .05; t(48 hr.) = 4.79, p = .0005.

in which both cells and plasmas were normal, additional tentative deductions can be made (table 4). This experience showed that when normal cells were cultured in FeD plasma their maturation was not inhibited. This suggests that conclusions made from experiments without controls may be oversimplified or actually misleading. This is so because FeD cells responded to contact with normal plasma in such a way that their maturation was increased, whereas normal cells were not affected by exposure to FeD plasma. In other words, FeD cells seemed to be stimulated by factors which were absent or deficient in FeD plasma but present in normal plasma, but normal cells did not appear to be dependent on such factors. The experiments indicated a difference between FeD and normal plasmas with respect to their effects on maturation, but in addition there was also a difference between FeD cells and normal cells with respect to their potentialities for maturation in vitro.

Thalassemia.—The work of Astaldi, Tolentino and Sacchetti, who used a method
in which the parameter of maturation was the life span or decrease of various maturation forms, is one of the few instances in which results were compared with those derived from control cultures of normal cells in patient's serum or plasma. These authors studied an unspecified number of cultures of normal cells in normal serum, 21 cultures of thalassemia cells in thalassemia serum, seven with thalassemia cells in normal serum and three with normal cells in thalassemia serum. They observed that maturation of thalassemia erythroblasts was the same in thalassemia and normal serum (in both instances retarded). Furthermore, maturation of normal cells was not affected by contact with thalassemia serum.

We set up two experiments with thalassemia cells in normal serum. The results were compared with those obtained with normal cells in the two thalassemia plasmas, and with the 23 control cultures of normal cells in normal plasma. The results are shown in figure 4B. Although the number of observations is small it is noteworthy that in our system, in which the increase of orthochromic cells is the sign of maturation, the conclusions reached are the same as those derived by Astaldi et al.,11 who used a system in which the sign of maturation is the decrease of various maturation forms. Specifically, both cell culture systems pointed to an intrinsic defect of maturation of thalassemia cells which was not overcome by exposing them to normal medium. They also showed that thalassemia plasma did not contain factors which inhibited maturation of normal cells. This is in contrast to the situation in experiments with iron deficiency anemia cells, in which their delayed maturation was brought to normal by placing them in normal plasma (table 3 and fig. 4A).

**Azotemia.**—Markson and Rennie38 reported their use of a stationary cell suspension system of the Osgood and Brownlee type42 in studies of maturation of erythroblasts of patients with hemolytic and post-hemorrhagic anemias tested in serum from nine patients with uremia. Crossed experiments with cells from uremia patients were not reported. Using changes in differential counts as the parameter, they found that maturation was significantly less in uremic than in normal serum in 24 hour cultures. Examination of older cultures was unsatisfactory because of rapid degeneration of cells. They concluded that uremia serum inhibited maturation of marrow normoblasts. These were, of course, normoblasts from patients with accelerated erythropoiesis. Sacchetti54 studied marrow tissue explants in plasma clots by the method of Fieschi and Astaldi,8 with marrow from 6 patients with uremia. The tissue was exposed to medium containing the patients' or normal serum and the differential counts of erythroblasts during 72 hours of incubation were compared. Since a higher differential count value for orthochromic cells was reached in cultures with normal serum medium, they reached a similar conclusion.

We have completed studies of erythroblasts from three patients with azotemia. The cells were cultured in azotemia and normal plasma and the results were compared with those of our cultures of normal cells in the azotemia plasmas, as well as with those of our control series of 23 cultures of normal cells in normal plasma. Our experiments also indicated that azotemia plasma had an inhibitory action on the maturation of cells

### Table 4.—Accumulated Orthochromic Erythroblasts per 1000 Erythroblasts in Original Inoculum in 48-Hour Cultures of Cells and Plasmas from Patients with Iron Deficiency (FeD) Anemia and Normal Cells and Normal Plasmas (N)*

<table>
<thead>
<tr>
<th></th>
<th>FeD cells in FeD plasma</th>
<th>FeD cells in N plasma</th>
<th>N cells in FeD plasma</th>
<th>N cells in N plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of observations</td>
<td>10</td>
<td>10</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td>Means</td>
<td>173.6</td>
<td>304.7</td>
<td>242.5</td>
<td>266.0</td>
</tr>
<tr>
<td>Column number</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

*Based on difference of means for columns indicated $t(4 - 1) = 2.39$, $p = .013$; $t(2 - 1) = 2.12$, $p = .025$; $t(4 - 3) = 0.39$, $p = .50$; $t(2 - 4) = 0.687$, $p = .50$. 
METHODS FOR STUDYING HUMAN ERYTHROBLAST MATURATION

Fig. 4.—Mean values of accumulated orthochromic cells in experiments with various types of cell-plasma relationships. (A) Iron deficiency anemia; (B) thalassemia; (C) azotemia; (D) erythrocytosis.

from individuals without azotemia. The additional observations were that azotemia plasma inhibited maturation of normal cells and that azotemia cells matured more rapidly when the medium was normal plasma (fig. 4C). The retardation of maturation in these cultures was an attribute of the plasma, not the cells.

Erythrocytosis.—With a stationary cell suspension method in which the parameter of maturation was survival time, Bernardelli, Rondanelli and Gorin studied erythroblasts from a patient with polycythemia vera, in normal and polycythemia serum. Experiments with normal cells were not reported. They concluded that maturation of polycythemia cells was the same in the two sera.

In our experiments with erythroblasts of three patients with erythrocytosis, the cells were cultured in erythrocytosis and normal plasma and the results were compared with those from cultures of normal cells in erythrocytosis plasma, as well as with our controls consisting of 23 cultures of normal cells in normal plasma (fig. 4D). The findings are quite different from those seen in experiments with iron deficiency anemia, thalassemia and azotemia. In erythrocytosis, the plasma appeared to have an accelerating effect on maturation of cells, especially in the first half of the incubation period. In view of what is known about erythropoietic factors in plasma from patients with erythrocytosis this is not unexpected. However, the relatively rapid maturation of erythrocytosis cells in normal plasma, which is suggested by our observations, is worthy of further study.

DISCUSSION

One difference between stationary (Lajtha) and roller tube (Berman and Powsner) cell suspension methods is that, either because of disintegration
or denucleation of cells, the accumulation values of orthochromic erythroblasts decrease after six hours in the former, but do not in the latter (fig. 3). In any system in which the total number of cells is not constant, the differential counts at various times of culture are not comparable because both the numerator and denominator of the fraction are changing. In our system, the count of total erythroblasts, which is the denominator of the fraction, is fixed.

In the Astaldi and Tolentino maturation system\textsuperscript{a} the rate of maturation of erythroblasts is deduced from observations on the survival times of various cell forms, the basic assumption being that the disappearance of relatively undifferentiated cells results from their transformation into the more differentiated or mature cell types. Then, as compared with the results of culturing normal cells in normal serum, any delay of disappearance of the various maturation forms is interpreted as evidence of retarded maturation. On the other hand, in the system we have developed, the net maturation of the population of erythroblasts is determined by observing the increase of orthochromic cells during a 48 hour period of incubation. Superficially this may appear to be the inverse of the approach used by Astaldi and his co-workers, but it is equally informative and probably less cumbersome because of the shorter period of observation required. As we have shown, different methods for studying maturation of human erythroblasts in vitro lead to similar deductions, provided that the characteristics of the in vitro models are well understood and that the proper parameters are selected.

When we examined previously reported methods on the basis of the criteria already mentioned, we found that none were completely satisfactory. The method we have described satisfied the requirements best. It was shown to be capable of distinguishing between different cell-plasma relationships, and the challenges presented to the model yielded information in accord with clinical knowledge of the conditions tested. Hence, it may have a worthwhile application to the investigation of factors affecting maturation of human erythroblasts in vitro.

A major limitation of any in vitro model for studying maturation is that it can demonstrate only direct effects on the erythroblasts. If there are indirect maturation effects in vivo, mediated through the action of humoral factors on a nonmyeloid organ or tissue, in vitro technics cannot be expected to expose them. Furthermore, in studying maturation effects of plasma or humoral factors it may be necessary to use more than one type of erythroblast population in the experiments. It is conceivable that an active factor may not affect normal cells in the same way as it affects cells from patients with anemia or other abnormal conditions. For example, in the case of hypoplastic and aplastic anemias, in which erythropoietic factor is present in plasma in normal or increased concentrations,\textsuperscript{a} severe anemia

METIIOI)S
FOIl STUDYIN(; HUMAN EBYT1IROBLASI MATULIATION

is nevertheless present. Whether this is due to lack of responsiveness of erythroblasts or to that of more primitive precursors such as reticulum cells is a question which might be resolved by in vitro studies, provided that it is possible to obtain a sufficient number of erythroblasts and reticulum cells from aplastic anemia marrows. On the other hand, even normal erythroblasts seem to have undergone accelerated maturation in the few experiments we have completed with plasma from patients with erythrocytosis. It will be necessary to find out whether or not normal erythroblasts are the proper objects for study in all types of testing of humoral agents.

The chief reasons for recommending our method as compared with other methods are its relative convenience, replicability and high quality of preparations for morphologic study. Another important reason, not discussed in this paper, is that the same system can be used without modification for investigating proliferative activity of erythroblasts.

SUMMARY

Characteristics of various tissue and cell culture methods for studying maturation of human erythroblasts were reviewed and the basic assumptions required in their use were examined.

The relationship of maturation and proliferation of erythroblasts in different types of cultures was considered.

Criteria for selection of an in vitro model for studying maturation were established. A new clot-free culture system in roller tubes was found to be superior to those previously described because of convenience, replicability and adaptability to studies of proliferation.

The results of challenging the new culture system with various types of cell-plasma relationships indicated that it was effective in distinguishing among erythroblasts and plasmas of iron deficiency anemia, thalassemia, azotemia and erythrocytosis, and that the deductions arrived at were similar to those derived from the use of less convenient in vitro systems in which different parameters of maturation were used.

Experience with the new method suggests that it may be useful in the study of humoral factors affecting maturation of human erythroblasts.

The chief limitation of the proposed method, as for other in vitro methods, is that only direct effects of humoral agents on erythroblasts can be studied.

SUMMARIO IN INTERLINGUA

Es passate in revista le caracteristicas de varie methodos de histo-cultura e de cultura cellular in le studio del maturation de erythroblastos human. Le premissas fundamental que es requirite in lor uso es examinate.

Le relation inter maturation e proliferation de erythroblastos in varie typos de cultura es examinate.

Criterios pro le selection de un modello al uso in le studio del maturation in vitro es establite. Esseva trovate que un nove systema de cultura sin coagulo, utilisante tubos revolvente, es superior al systemas previemente describite,
tanto ab le puncto de vista de su simplicitate como etiam ab le punctos de vista de su replicabilitate e de su adaptabilitate a studios de proliferation.

Le resultatos de testar le nove systema de culturation con varie typos de relation de cellulas a plasma indicava que illo es efficace in distinguier inter erythroblastos e inter plasmas de anemia a deficientia de ferro, thalassemia, azotemia, e erythrocytosis, e que le deductiones assi facite eseva simile a illos obtenite per le uso de minus convenibile systemas de studio in vitro in que differente parametros de maturation eseva usate.

Le experientias con le nove methodo suggere que illo va possibilemente esser de valor in le studio de factores humoral que affice le maturation de erythroblastos human.

Le major limitation del methodo hic proponite—un limitation que etiam characterisa le altere methodos de studio in vitro—es que solmente directe effectos de agentes humoral super le erythroblastos pote esser investigate per illo.

APPENDIX

Technic.—Marrow was aspirated from the sternum with the Illinois needle, from the posterior ilium with the Bierman needle and from the anterior iliac crest with either needle. Prior to their use, syringes and needles had been rinsed with sterile heparin solution. Five to 10 ml. of marrow material were obtained at each aspiration, but to obtain a sufficient volume multiple aspirations were necessary. A fresh needle and syringe were used for each aspiration.

After smears were made, five ml. or less of the aspirated material were placed in each of one or more test tubes containing 10 ml. of Hank’s balanced salt solution. The tubes were centrifuged at 700 x g for 10 minutes. The supernatant diluted plasma was removed and its volume noted. Fresh sterile Hank’s solution was then added to restore the volume to that of the original marrow specimen.

Estimation of the cellularity of the material was based on inspection of smears and the size of the buffy layers of the centrifuged material. All specimens of a cellularity corresponding to approximately 15,000 or more nucleated cells per cu.mm. were pooled in a siliconized bottle and mixed thoroughly. This material was referred to as reconstituted marrow.

Plasma was prepared from blood obtained by venipuncture in syringes rinsed with heparin solution. The medium was composed of equal volumes of this plasma and Hank’s balanced salt solution.

Reconstituted marrow was mixed with an equal volume of the appropriate media and 0.25 ml. quantities of the mixtures were placed in the bottoms of 10 x 75 mm. siliconized Pyrex test tubes. These were inserted into 16 x 150 mm. sterile Pyrex test tubes closed with sulfur-free, white rubber stoppers. The stoppered tubes and their contents were incubated at 37°C. in a revolving drum which turned at one-fifth revolution per minute. The axes of the tubes were parallel to that of the drum, which was inclined 7 degrees above the horizontal. Cultures were prepared in quadruplicate for each combination of cells and medium and period of incubation.

Immediately following the required period of incubation, or without incubation in the case of zero-hour samples, the contents of each culture tube were thoroughly mixed. Two smears were made and set aside. With two white cell diluting pipets, one for both sides of a standard Neubauer ruled hemocytometer, four total nucleated cell counts were completed. At a later time, the smears were stained and a tally was kept of each type of erythroblast seen during a count of 500 to 1000 leukocytic cells. The number of each type of erythroblast per cu.mm. of cultured cell suspension was then calculated. The values for each of the quadruplicate tubes of a set were totaled.

*Prepared according to the method of Syverton, Scherer and Elwood.57
METHODS FOR STUDYING HUMAN ERYTHROBLAST MATURATION

In a typical experiment, marrow and plasma were obtained from both the subject under study and a normal individual of the same blood group, within the same hour. A portion of reconstituted marrow from each individual was mixed with medium containing the subject's own plasma and a second portion was mixed with medium containing the plasma of the other individual. There was thus a set of quadruplicate tubes for each of the four possible combinations of marrow and plasma incubated for each of the specified periods, usually 24 and 48 hours, or in some cases early in our investigation for 72 hours as well, in addition to the zero-hour samples made up in the same way.

The parameter used in the study of maturation was the increase of orthochromic cells during incubation. Since the actual number of orthochromic cells accumulating in the cultures was determined in part by the size of the original erythroblast inoculum and the number of orthochromic cells present at the beginning of the experiments, the counts of orthochromic cells were expressed as follows:

\[
O = \frac{O_{xhr} - O_{ohr}}{T_{ohr}} \times 1000
\]

where \(O\) = Accumulated orthochromic cells at \(x\) hours per 1000 total erythroblasts at zero hour of the culture; \(O_{xhr}\) = Orthochromic cells per cu.mm. at \(x\) hour of incubation; \(O_{ohr}\) = Orthochromic cells per cu.mm. at zero hour of experiment; and \(T_{ohr}\) = Total erythroblasts per cu.mm. at zero hour of experiment.

Criteria for classification of subjects.—To qualify as normal the subject must have had normal hematologic laboratory findings including hemoglobin and hematocrit values, erythrocyte, reticulocyte, leukocyte, platelet and differential counts and myeloid:erythroid ratio of bone marrow. In addition, the following diseases were specifically excluded by history and laboratory studies: diabetes, uremia, cirrhosis of the liver, hepatitis, jaundice of any cause, pernicious anemia, malignancy and acute or chronic infections. In the case of patients hospitalized for trauma, usually fractures, marrow and plasma were obtained only after the injury had healed and when the patient was ready for discharge from the hospital.

To qualify as examples of iron deficiency anemia the subjects must have had hypochromic microcytic anemia without elevated or normal serum iron concentration, without evidence of stainable iron in bone marrow and without failure to respond to iron therapy. Certain findings were considered confirmatory: low serum iron concentration, history and laboratory evidence of prolonged blood loss, response to iron therapy. When associated disease might have been regarded as the possible cause of the anemia, low serum iron concentration and response to iron therapy were considered adequate evidence that iron deficiency was the major factor in the anemia.

Thalassemia subjects had familial hypochromic microcytic anemia with elevated serum iron concentration, stainable iron in bone marrow and shortened red cell survival time.

Azotemia subjects were patients with chronic renal disease and blood urea nitrogen levels over 90 mg. per cent.

Erythrocytosis subjects included patients with classic primary disease of long standing associated with leukocytosis, thrombocytosis and a normal arterial oxygen saturation. In addition, some subjects had secondary erythrocytosis without leukocytosis, an associated reduced arterial oxygen saturation and evidence of organic or functional pulmonary disease.

REFERENCES


3. —, —, and ——: L’effetto dell’estran
epatico sul tessuto megaloblastico
sopravvivente in vitro. I. L’effetto sue
processi citogenetico ed evolutivo.
Soc.ital.biol.sper.Boll. 22:1162–1184,
1946.

4. —, —, and Bernardelli, E.: Crisi reticulo-
citaria negli esplanti midollari di
anemico pernicioso all’inizio del tra-
tamento massivo. Soc.ital.biol.sper.
Boll. 20:824–826, 1945.

5. —, —, and ——: Il gettito reticolocitario stu-
diato con la cura in vitro del
midollo osseo. Minerva Med. 1:109–114,
1946.

6. —, —, and ——: La valutazione
dell’attivita proliferativa delle
cellule midollari. Studio di un “test stat-
tistico”.” Haematologica 33:583–
629, 1949.

7. —, —, and Salera, U.: Études “in vitro”
sur la maturation et la proliferation
des erythroblastes de l’anémie hypo-
chrome essentielle. Rev. d’Hématolo-

8. —, —, and Tolentino, P.: L’influence
de la colchicine sur la maturation
des erythroblastes et des réticulocytes.
Étude sur la moelle osseuse in vitro.
Rev. belge path.med.exper. 19:101–
114, 1948.

9. —, —, and Troiano, M.: Re-
searches on the biology of erythroblast
in hookworm anemia. Comptes rend.
d.troisième Congres d.l.Soc.Int.
Europeenne d’Hemat., pp. 542–545.
Edizioni Mediche e Scientifiche, Rome,
1951.

10. —, —, and Tolentino, P.: Studies in vitro
on maturation of erythroblasts in
normal and pathological conditions.

Pavia, Tipografia del Libro, 1951.

12. Berman, L.: Technics used in the study
of aspirated sternal marrow. Am.J.

13. —, —, and Stulberg, C. S.: The Detroit
strains of human epithelial-like cells
from non-leukemic peripheral blood.

14. —, —, and Ruddle, F. H.: Long-term
tissue culture of human bone mar-
row. I. Report of isolation of a
strain of cells resembling epithelial
cells from bone marrow of a patient
with carcinoma of the lung. Blood

15. —, —, and ——: Human cell culture.
Morphology of the Detroit strains.

16. Bernardelli, E., Mele, V. and Bani, M.:
Experimental research on the pro-
liferative and differentiative activity
of the erythroblast in chronic ery-
thermia, the chronic form of Di
617, 1956.

17. —, —, and Gorini, P.: Ricerca sull’at-
tivita proliferativa e differenziativa
dell’eritroblasto della policitemia vera.

18. —, —, and Zani, P.: Sulla biologia del
genaloblasto dell’anemia nutrizionale.

19. Bessis, M.: Editorial. At the level of
ten Angstroms. Blood 13:410–412,
1958.

20. Biernacki, H. K. and Kelly, K. H.: Multi-
ple marrow aspirations in man from
posterior ilium. Blood 11:370–374,
1956.

throleukemia. Blood 6:261–269,
1951.

22. Russo, L., Pozza, G., Eriani, S., Fava,
P. L. and DeMicheli, E.: Ricerche
sul comportamento “in vitro” del tes-
suto megaloblastico in presenza di
differenti fattori attivi sui processi
di maturazione cellulare. Recentia

the nature of Castle’s hemopoietic

24. Clemmesen, J., Espersen, T. and Plum,
C. M.: In vitro study of bone mar-
row. III. Erythropoiesis in vitro of
sternal marrow from cases of per-
nicious anemia and lymphatic leuko-
sis under therapy. Blood 3:155–164,
1948.

25. —, —, and Plum, C. M.: A simplified
method for in vitro examination of the
erythropoiesis of bone marrow applied
to cases of pernicious anemia and

26. Feinmann, E. L., Sharp, J. and Wilkin-
son, J. F.: Observations on the be-
METHODS FOR STUDYING HUMAN ERYTHROBLAST MATURATION


28. — and Bernardelli, E.: La modif-

29. — and Bernardeili, E.: La modifi-

30. Franco, J. and Arkun, S. N.: Transformation in vitro de inoelies megalo-

31. Lajtha, L.: The culture of bone mar-


34. —: Bone marrow culture. Methods in Medical Research In press.

35. Linarzi, L. R. and Bedinger, P. L.: A modified and improved sternal punc-

36. Linman, J. W. and Bethell, F. H.: The plasma erythropoietic stimulating fac-
tor. Observations on circulating ery-

37. Linman, J. W. and Long, M. J.: Ery-
throcyte osmotic fragility of rats re-
ceiving the thermostable plasma ery-

38. Markson, J. L. and Rennie, J. B.: The anaemia of chronic renal insufficiency; the effect of serum from azotemic patients on the maturation of norm-
oblasts in suspension cultures. Scott-


40. Maximow, A.: Uber die Entwicklung argyrophiler und kollagener Fasern in Kulturen von erwachsenem Säug-

41. Nieweg, H. O.: The effect of liver ex-
tract in cultures of megaloblastic bone marrow. Scandinav.J.Clin. & Lab.In-
vest. 5:195-197, 1953.


44. — and Muscovitz, A. N.: Culture of human bone marrow. Preliminary re-

45. Parreira, F.: A accao "in vitro" da vita-
mina B12 ácido fólico e uracilo sobre a medula ossea de dois casos de ane-


47. Plum, C. M.: Some investigations of erythropoiesis in human bone-mar-


49. —: In vitro study of bone marrow. II. Studies of erythropoiesis. Blood, Spe-

50. Rambach, W. A., Alt, H. L. and Cooper, J.A.D.: The mode of action and na-
ture of a heat stable plasma ery-

51. Reisner, E. H.: The nature and signifi-
cance of megaloblastic blood forma-


Review of Methods for Studying Maturation of Human Erythroblasts in Vitro: Evaluation of a New Method of Culture of Cell Suspensions in a Clot-Free Medium

LAWRENCE BERMAN, EDWARD R. POWSNER, Mary L. Davis and Marie Fly