STUDIES *IN VITRO* ON THE MATURATION OF ERYTHROBLASTS IN NORMAL AND PATHOLOGICAL CONDITIONS

BY

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During the past few years we have been studying the mechanism of the maturation of erythroblasts by observing the survival *in vitro* of bone marrow cells. By this means the maturation of erythroblasts of adults and children, both normal and pathological, has been followed in physiological and various experimentally modified conditions. We believe that this method furnishes more accurate information on the maturative activity of normal and pathological bone marrow than morphological studies of biopsy specimens recorded by means of maturation curves or indices.

Method

Our method consists in the introduction of 1–2 ml. of aspirated heparinized bone marrow into small Carrel flasks, the marrow being obtained from the sternum in adults and from the diaphysis of the long bones in children. Equal amounts of heparinized plasma from the same patient and of Ringer, or, preferably, Tyrode solution, are added. A small quantity of the suspension is immediately removed for erythrocyte and reticulocyte counts. The latter are performed on films stained with brilliant cresyl blue. We determine the percentage of reticulocytes relative to the erythrocytes, and the maturation curve according to Heilmeyer (dividing the reticulocytes for the sake of convenience) into three groups only: (1) Group "O," corresponding to orthochromatic erythroblasts; (2) Group "A," or reticulocytes, Types I and II; (3) Group "B," or reticulocytes, Types III and IV of Heilmeyer. Erythroblast counts are also made on films stained with May-Grünwald-Giemsa. Both the percentage relative to the erythrocytes and the maturation curve (after Pontoni) of the erythroblasts are determined.

Immediately after the removal of the specimens required for counting the culture flasks are incubated at 37° C. Further specimens are removed for counting at intervals of six or twelve hours, up to the 96th hour or later. All these operations are performed under conditions of bacteriological sterility.

After counting, the percentages of erythroblasts and reticulocytes are converted into absolute values by using the known absolute erythrocyte counts, and, for ease of inspection, the data are charted, times being represented on the abscissae and cell counts on the ordinates. Besides the graphs showing the absolute values, it is also useful to draw curves of relative values, such as the maturation curve after Pontoni (1936) and the reticulocyte curve after Heilmeyer (1942).

Under our experimental conditions the maturation of erythroblasts and reticulocytes continues, but their proliferation comes to an untimely end. We can therefore evaluate the approximate maturative rhythm of erythroblastic tissue from a study of our maturation curves.

It would, however, be a mistake to assume that the events occurring *in vitro* are necessarily identical with those in the living organism. Conditions *in vitro* differ fundamentally from those in the body in the following respects. The conditions of both anabolism and catabolism are dissimilar. There is an absence of nervous and hormonal control (with the exception of hormonal or other factors contained in the culture medium). In cultures mature elements persist, whereas in the living organism they enter the circulating blood. The normal mechanism of the destruction of blood is absent.

In experiments carried out by this technique normal and pathological erythroblasts show distinct differences in their behaviour, although they live in an identical medium. This fact permits the conclusion that any such differences observed *in vitro* depend on intrinsic differences in cellular constitution. Moreover, by means of this method we are able to modify the culture medium, and thus to study directly the effects of chemical, physical, or hormonal agents on bone marrow cells.

Results

A. Normal Bone Marrow.—This section summarizes the results in adults and children of various ages of Astaldi and Bernadelli (1945, 1946), and of Astaldi and Reggiani (1946a, b).

FIG. 1.—Maturation of erythroblasts in culture of normal adult bone marrow. Note progressive relative decrease of basophil and polychromatic, and increase of orthochromatic, forms.

The maturation curves (Fig. 1) show that, while polychromatic erythroblasts predominate in the early stages of culture, their number declines from the 18th to the 20th hour, at which stage it equals that of the orthochromatic cells. Also basophilic erythroblasts are becoming less numerous at the same time. Towards the thirtieth hour orthochromatic cells begin to predominate.

The absolute number of orthochromatic erythroblasts increases at an early stage, sometimes as soon as the sixth hour, and reaches a maximum at 12 to 18 hours, more rarely at 24 hours. Later it declines progressively to fall below the initial level at 72 hours.

Coincident with the decrease in the orthochromatic erythroblasts, a moderate increase in the most immature reticulocytes (Group "A") is observed.

The fall in the number of reticulocytes of Group "A" is followed by a rise in those of Group "B," which are generally considered as approaching full maturity; later, however, these also disappear (Fig. 2).

These results show that maturation is possible *in vitro*: basophil erythroblasts become polychromatic, then orthochromatic, and eventually, by loss of their nuclei, reticulocytes. Finally, the reticulocytes mature. The duration of the different phases of maturation is: 18–24 hours for the basophil-polychromatic, and 12–24 hours for the polychromatic-orthochromatic stages; about 24 hours for the transformation from orthochromatic erythroblasts to reticulocytes of Group "B," and 24–36 hours for the final maturation to adult erythrocytes. The cycle of complete maturation thus requires about 100 hours *in vitro*.

Orthochromatic erythroblasts mature partly to reticulocytes of Group "A," but also to those of Group "B," or directly to adult erythrocytes. When stained with brilliant cresyl blue, they can often be seen to contain fragments of granule-filamentous substance, in consequence of an asynchronism between nuclear and cytoplasmic maturation; these elements, following the elimination of their nuclei, will later appear as reticulocytes of Group "B" or as adult erythrocytes.

The work of Tolentino (1947) on erythroblasts and reticulocytes in children of different ages gives results more or less similar to those observed in adults, as far as infants and children of pre-school

FIG. 2.—Maturation of reticulocytes in culture of normal adult bone marrow: Group "O," orthochromatic erythroblasts; Group "A," reticulocytes with reticular granule-filamentous substance; Group "B," reticulocytes with filamentous and granular substance.

FIG. 3.—Maturation of orthochromatic erythroblasts in bone marrow cultures from normal children showing also delay in extrusion of nucleus in neonates. Normal maturation in older children.

and school ages are concerned. In neonates up to one month old, there is a normal maturation of the reticulocytes, accompanied by a delay in the maturation of the orthochromatic erythroblasts: values of 25% of the observed maximum are still found at the end of the experiments (Fig. 3).

This slow maturation might perhaps explain the great frequency of erythroblastæmia at this age when there is an abnormal peripheral demand for erythrocytes, as in haemolysis, haemorrhage, or cyanosis. It thus becomes unnecessary to postulate the presence of an extramedullary haemopoiesis.

B. Erythroblastoses.—In this group of diseases we have chosen cases of Cooley's anaemia, congenital haemolytic disease, and infantile kala-azar, all of which are accompanied by erythroblastosis and haemolysis.

Cooley's Anaemia.—The first investigations of Fieschi and Astaldi (1946) were followed by those of Astaldi and Reggiani (1946a, b), and unpublished ones by Astaldi and Tolentino. These have shown that erythroblasts mature normally down to the orthochromatic stage. In more serious cases, as in that published by Astaldi and Reggiani, a tendency

maturation was still evident in circulating erythroblasts (Fig. 5); it seems therefore that these erythroblasts are useless for the production of adult circulating erythrocytes.

We have called the phenomenon of delayed extrusion of the nucleus "nuclear deficiency" for morphological reasons; it may, however, originate in a more complex protoplasmic deficiency. It has proved particularly evident in the most serious cases; in slighter cases, marrow erythroblasts have, on the other hand, shown a behaviour not far from normal (Fig. 4, Cases III and IV).

It is a noteworthy peculiarity that erythroblasts which do not eliminate their nuclei show a complete maturation of the cytoplasm: in fact, while getting older, they lose those remnants of the basophil substance which can normally be demonstrated in many orthochromatic erythroblasts by suitable stains like brilliant cresyl blue.

Haemolytic Disease of the Newborn.—We have so far investigated two cases of this disease following *in vitro* the behaviour of medullary and cir-

FIG. 4.—Maturation of orthochromatic erythroblasts in bone marrow cultures from patients with Cooley's anaemia, and delayed extrusion of nucleus in some cases.

FIG. 5.—Culture of immature erythrocytes from blood of first severe case of Cooley's anaemia showing gross delay in extrusion of nucleus of orthochromatic erythroblasts. Normal maturation of reticulocytes.

culating erythroblasts and reticulocytes. As in the case of Cooley's anaemia we have also been able to extend our study to the circulating elements (Astaldi and Tolentino, 1946). This is obviously impossible in normal subjects.

Fig. 6 shows that we found a decrease in the number of erythroblasts to one-fifth of the initial value in one case, and to one-third in the other, both towards the 48th hour. The reticulocytes of Group "A" in the first case diminished progressively, disappearing completely as early as the 30th hour, while those of Group "B" had disappeared at 70 hours. In the second case the corresponding figures were 48 and 84 hours.

On the whole we may say that erythroblasts mature normally, compared with the values obtained in normal neonates. Further, circulating erythroblasts may mature completely, as we observed *in vivo* in one of the two cases, in which there was a remarkably high number of erythroblasts in the peripheral blood. It follows that the passage of nucleated elements into

cases, compared with the other, reproducing the clinical and haematological differences, appears to show that there may be a superadded marrow failure, accounting for a delayed maturation.

Leishmaniasis.—We have observed that maturation proceeds as usual from basophil to orthochromatic erythroblasts, and that the reticulocytes, too, mature normally. On the other hand, there appears to be an obstacle to the extrusion of the nuclei in orthochromatic erythroblasts, when these are compared with normal elements (Fig. 7). This difficulty does not seem to be connected either with the inhibitory action of the spleen, or with the number of parasites present in the bone marrow: even in such cases it seems to be a function of the clinical and haematological condition

FIG. 6.—Normal maturation of orthochromatic erythroblasts in cultures from two children with haemolytic disease of the newborn.

of the patient, and a result of exhaustion of the bone marrow in consequence of the severe and long-continued haemolysis. Failure of orthochromatic erythroblasts to extrude their nuclei ("nuclear deficiency") which has been evident in

all our cases of Leishmaniasis, may possibly lead to their accumulation in the bone marrow; if so, an increased percentage of orthochromatic erythroblasts in maturation curves may actually be an indication of a check in maturation.

FIG. 7.—Maturation of orthochromatic erythroblasts in bone marrow cultures from four children with leishmaniasis showing delay in extrusion of nucleus. (The delay was greatest in the most severe cases.)

C. Pernicious Anaemia. — Astaldi, Baldini, and Frugoni (1946a, b, 1948) studied the explanted bone marrow of patients with pernicious anaemia, comparing the maturative rhythm of megakaryoblasts with that of normoblasts. In this way they were able to demonstrate that the evolution from the basophilic to the orthochromatic stage is slower for the megakaryoblast than for the normoblast, the whole process requiring not less than three

FIG. 8.—Maturation of erythroblasts in bone marrow cultures from a case of pernicious anaemia showing delayed maturation from basophil to orthochromatic stage, corrected by addition of liver extract.

days for the former, but not more than two days for the latter (Fig. 8).

D. Effects of Various Agents.—Our method is suitable for the study of temperature and various agents on cells surviving *in vitro*.

Temperature.—The influence of temperature on the process of maturation of erythroblasts and reticulocytes has been studied in several experiments. In these it was found that, within certain limits, an increase of temperature above 37° C. accelerates the process, but at temperatures above 42° C. there appear signs of cellular damage, such as lysis. A decrease of temperature inhibits maturation, which practically ceases below 20° C.

Liver Extracts.—Astaldi, Baldini, and Frugoni have demonstrated an acceleration in the maturation of megaloblasts in pernicious anaemia, following the addition of liver extracts to the culture medium. The evolution from the basophil to the orthochromatic stage appeared to be quicker in the presence of extract than in the controls (Fig. 8).

Spleen Extracts.—Tolentino and Lombroso (1947) found a delay in the maturation of normal erythroblasts following the addition to the cultures of pathological human spleen extract from a case of lipoidosis. A delay in the maturation of erythroblasts was also observed in another case, in which both the cells and spleen extract were obtained from the same patient with Cooley's anaemia.

Niacin.—Niacin may have an accelerating influence on the maturative rhythm of megaloblasts surviving *in vitro*, as shown by the work of Astaldi and Baldini (1948). In contrast to the action of liver extracts, however, the addition of niacin does not lead to a normoblastic transformation of the megaloblasts.

Colchicine.—Astaldi, Mauri, and Tolentino (1948) have described a delaying action of colchicine on the maturation of normal bone marrow cells *in vitro*.

Summary

The method we have described and adopted has proved useful in the study of the maturation of erythroblasts and reticulocytes. The following applications have been of practical value. Standard values of the duration of maturation *in vitro* for normal subjects at various ages have been obtained.

We have been able to point out that some apparently sound and authoritative theories in the dynamics of haematology may stand in need of revision; thus our demonstration of possible delays at the orthochromatic stage is in contrast to the accepted view (Fieschi, 1946, and Rohr, 1940) that inhibition of the maturation of red cell precursors always leads to a "shift to the left" in the medullary formula. The significance of maturation curves or indices becomes thereby less clear cut.

We have been able to show that marrow failure may express itself in "nuclear deficiency" of orthochromatic erythroblasts, and to formulate a hypothesis explaining the various phases of the clinical and haematological picture found in Cooley's anaemia, haemolytic disease of the newborn, and leishmaniasis. It has been possible to demonstrate the influence on the maturation of erythroid cells of various factors applied *in vitro*.

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REFERENCES

- Astaldi, G. (1944). *Bol. Soc. Med. Chir. Pavia*, **58**, 1249.
 — and Baldini, M. (1948). *Acta Vitaminologica*, **2**, 78.
 — and Frugoni, C., jr. (1946). *Bol. Soc. ital. Biol. sper.*, **22**, 1162.
 — (1946). *Ibid.*, **22**, 1165.
 — (1948). *Haematologica*, **31**, 265.
 — and Bernadelli, E. (1945). *Bol. Soc. ital. Biol. sper.*, **20**, 824.
 — (1946). *Minerva med.*, Torino, **37**, 109.
 — Mauri, C., and Tolentino, P. (1948). *Rev. belge de path. & méd. Expér.*, **19**, 101.
 — and Reggiani, G. (1946). *Bol. Soc. ital. Biol. sper.*, **22**, 481.
 — (1946). *Ibid.*, **22**, 714.
 — and Tolentino, P. (1946). *Atti. III° Congr. Trarf. Sangue*, Milan, 307.
 — (1948). *Haematologica*, **32**, 13.
 — In the press.
 Fieschi, A. (1946). *Semeiologia del midollo osseo*. Garzanti, Milan.
 — and Astaldi, G. (1946). *La culture in vitro del midollo osseo*. Tip. del Libro, Pavia.
 Heilmeyer, L. (1942). *Blutkrankheiten*. Berlin.
 Pontoni, L. (1936). *Haematologica*, **17**, 883.
 Rohr, K. (1940). *Das menschliche Knochenmark*. Thieme, Leipzig.
 Tolentino, P. (1947). *Atti XVIII° Congr. It. Pediatria*, Pisa.
 — and Lombroso, C. (1947). *Bol. Soc. ital. Biol. sper.*, **23**, 81.