Congenital Disorders of Erythropoiesis

Ciba Foundation Symposium 37 (new series)

In memory of the late Fred Stohlman

1976

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Ciba Foundation Symposia are published in collaboration with Elsevier Scientific Publishing Company / Excerpta Medica / North-Holland Publishing Company in Amsterdam.

Elsevier / Excerpta Medica / North-Holland, P.O. Box 211, Amsterdam

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ISBN Excerpta Medica 90 219 4041 8
ISBN American Elsevier 0-444-15185-0

Published in January 1976 by Elsevier / Excerpta Medica / North-Holland, P.O. Box 211, Amsterdam, and
American Elsevier, 52 Vanderbilt Avenue, New York, N.Y. 10017.

Suggested series entry for library catalogues: Ciba Foundation Symposia.
Suggested publisher's entry for library catalogues: Elsevier / Excerpta Medica / North-Holland.

Ciba Foundation Symposium 37 (new series)

Printed in The Netherlands by Mouton & Co, The Hague

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Chairman's opening remarks

D. J. WEATHERALL

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Fred Stohlman and his wife were killed on September 8th, 1974, as they were returning from the International Congress of Haematology in Israel. It seemed fitting that this symposium should be dedicated to Fred Stohlman because if he were alive he would be here. He made tremendous contributions to the field of the control of erythropoiesis, kinetics of erythropoiesis and, particularly important for this meeting, to making a start in our understanding of the control of fetal erythropoiesis. There is no need to repeat the extensive obituaries that describe the life and work of Fred Stohlman. Probably the best way we can remember Fred is by trying to keep the standard of presentation and discussion here at the level to which he always aspired at meetings he attended. He was a giant of a man, both physically and intellectually, and he will be missed sorely.

The time seemed ripe for a meeting of this type because work on congenital disorders of erythropoiesis has indicated that although the disorders result from many different underlying molecular defects, they have many pathophysiological features in common. It is apparent that when looking at these disorders we are dealing with two main problems: abnormal erythroid proliferation and abnormal maturation. In addition, their study is complicated by the fact that we are trying to sort out the pathophysiology at a time when profound changes are taking place in the pattern of erythropoiesis. Thus the overall rate of erythropoiesis declines markedly after birth and there is a concomitant change from fetal to adult haemoglobin and other red cell proteins at the same time. Although this makes it more difficult to disentangle the pathophysiology of these disorders, it does give us an opportunity to investigate and possibly learn more about the mechanisms controlling the switch from fetal to adult erythropoiesis, both in these disease states and in the normal infant.

As time is limited, we should restrict ourselves to three main areas. First,

we should try to define the factors that control fetal erythropoiesis and the switch from fetal to adult red-cell protein production, and to define some further questions to ask about these important problems. Secondly, and probably most important, we ought to look at the pathophysiology of disordered erythropoiesis in early childhood: the aplastic anaemias, hypoplastic anaemias, dyserythropoietic anaemias and the thalassaemias. Although we understand much about the molecular defect in the thalassaemias, we know nothing about the aetiology of the other conditions. However, in terms of the patterns of cellular proliferation and maturation, many of them seem to have features in common. We ought to review the pathophysiology of each of these conditions and see if we can define what the similarities are and if these findings shed any light on the underlying aetiology of the conditions other than the thalassaemias. Finally, we ought to collate this information and see if we can translate it into any useful approaches for early diagnosis and therapy.

Cellular kinetics of erythropoiesis

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Abstract The haemopoietic system presents itself as a three-tier interlinked structure of cell populations starting with the pluripotent stem cell (with sustained self-maintenance) and followed by two 'amplifying transit' populations: the 'committed' precursors and the 'maturing end cells'. This structure implies two distinct differentiation steps (qualitative changes in genetic programming): one from the stem to the committed precursor and the second from the latter to the maturing end cells. Both the amplifying transit populations have an 'age structure' defined by the maturation rate process (elaboration to amplification of the products resulting from the genetic programming).

The system has multiple control processes: proliferation control in the stem cells, cell cycle modulation in the two transit populations, differentiation control at both steps and maturation rate controls (which affect amplification of cell numbers indirectly) in both transit populations.

Some of the controlling factors are beginning to be understood and recent techniques are yielding an increasing amount of insight into the mechanisms. Some murine hereditary anaemias and some recent radiobiological studies are particularly useful model systems for the elucidation of pathological mechanisms.

Fifteen years have elapsed since the last Ciba Foundation symposium on haemopoiesis. A model was then described for the kinetics of erythropoiesis (Lajtha & Oliver 1960) as far as it was understood then and, in general, the conclusions reached are still regarded as valid. However, our concept of erythropoiesis then was more limited than that which we hold at present. The haemopoietic stem cell was thought to be the immediate precursor of the earliest recognizable erythroid cells. The application of techniques developed since that time has given us the opportunity to study haemopoiesis comprehensively and to obtain a clearer, but still incomplete, understanding of the kinetics of erythropoiesis in particular.

It is a relatively simple matter to estimate the total daily production of red cells under equilibrium conditions from a knowledge of the red cell concentra-

tion and life span, and the total blood volume. The flow from the stem cell population into differentiating pathways can now be calculated and reasoned assumptions can be made about the proportion of these cells moving into the erythron. Thus, the amplification necessary between the stem cell and the mature erythrocyte can be calculated. We shall examine the functional identity and kinetics of the cells intervening between these two cell types.

RED BLOOD CELL PRODUCTION

Since the ability to measure stem cell numbers and proliferative state extends so far only to mouse haemopoietic tissues, calculation of red cell production in the mouse is relevant here. The life span of a red cell varies from one strain of mouse to another but it can be measured accurately in syngeneic animals by a single injection of ^{59}Fe and following the fate of the labelled red cells. The average life span is about 35 days. The red cell concentration is obtained by standard methods and the blood volume is conveniently measured by intravenous injection of a small volume of a suspension of red cells, labelled with ^{59}Fe by injection of the isotope into a syngeneic donor.

Therefore the rate of red cell loss is (in cells per day) $RV(100/L)(1/100)$, where R is the red blood cell count (in cells/ml), V is the blood volume (in ml) and L is the life span of the red blood cell (in days). Substituting appropriate values, we find the rate of loss of red cells is:

$$10^{10} \times \tfrac{3}{2} \times \tfrac{3}{100} = 4.5 \times 10^{8} \text{ cells/day}$$

Production must also be 4.5×10^8 red blood cells/day because the system is in steady-state conditions.

STEM CELL PRODUCTION

The most widely used, indeed the only basic technique for the study of pluripotent haemopoietic stem cells is that developed by Till & McCulloch (1961) involving the observation of spleen colonies in irradiated mice. The injection of haemopoietic cell suspension intravenously into mice whose own haemopoietic tissue has been totally destroyed by radiation results in the appearance of nodules on the surface of the spleen seven or more days later. Each nodule arises from a single cell (Becker *et al.* 1963) and consists of recognizable erythroid, granulocytic and megakaryocytic cells and their precursors (Wu *et al.* 1967) and also colony-forming cells (Siminovitch *et al.* 1963). Thus the cell giving rise to a colony satisfies the criteria of a pluripotent haemopoietic stem cell and one can, therefore, by counting the colonies, obtain the number of

stem cells in the injected suspension which settled, survived and proliferated in the spleen.

By a modification of the basic technique (Siminovitch *et al.* 1963) the proportion of the injected stem cells which formed colonies has been measured (i.e. the 'plating' efficiency) from which the absolute total number of stem cells in haemopoietic organs can be calculated. Since it is also possible to assess the proportion of the total marrow contained in, say, one femur from which the injected marrow was extracted (Schofield & Cole 1968; Schofield, unpublished observations), with this information the absolute number of pluripotent haemopoietic stem cells (colony-forming cells, CFC) in the mouse can be calculated.

The total of CFC in the mouse (the values referring to the C57 Bl $\times$ DBA_2 F_1 strain used in our laboratory) is thus NT/afF where N is the number of colonies in spleen, T is the total number of nucleated cells in femur (1.5×10^7), a is the number of injected bone marrow cells ($\sim 3 \times 10^4$), f is the fraction of injected stem cells (CFC) producing spleen nodules ($\sim 10\%$) and F is the fraction of total marrow in femur ($\sim 5\%$). Substituting the appropriate values, we derive a total of 10^6 CFC per mouse.

We must know the rate of production of new colony-forming stem cells in order to determine their rate of loss (for differentiation). This can be calculated by determining the proportion of stem cells in the S phase of the cell cycle. We use the technique of [^{3}H]thymidine killing, in which the cells are exposed *in vitro* for 30 min to a high concentration of [^{3}H]thymidine followed by assay of the colony-forming units (CFU) in control and in treated suspensions (Becker *et al.* 1965). CFU synthesizing DNA are killed by internal radiation from the incorporated tritium and the reduction in the number of colonies formed indicates the proportion of such stem cells. This is essentially an estimate of the labelling index of CFU. From an estimate of the length of the DNA–S phase we can calculate the number of colony-forming cells passing through the cell cycle per day (assuming the rate is constant throughout the day or that we have measured the mean rate). There is no way so far of measuring the cycle parameters of the CFC directly, since one is unable to recognize the cells. However, we have estimated the length of the CFC cycle (Schofield & Lajtha 1969), though under stress conditions, to be about six hours. In steady-state conditions, this will probably be longer. For the present calculation we shall assume that the S phase itself normally lasts six hours. Thus, the number of CFC cycling per day (i.e. the daily production of CFC) is $Nx/100D$ where N is the total number of CFC (10^6), $x/100$ is the fraction of CFC killed by [^{3}H]-thymidine (5/100) and D is the length of S phase (in h) divided by the length of observation period (i.e. 24 h) ($= 6/24$). Substituting values for these parameters, we calculate 2×10^5 CFC cycling per day.

Therefore, about 2×10^5 new CFC are produced each day and, as the total number remains constant, this is also the number leaving the stem cell population each day. Thus, 2×10^5 stem cells give rise to about 4.5×10^8 red cells each day. They are also responsible for the production of granulocytes and platelets (Wu *et al.* 1967) and probably for at least some of the lymphoid precursors also (Mekori & Feldman 1965; Moore & Warner 1971). It is not unreasonable, then, to suppose that 2×10^5 stem cells result in the production of not less than 10^9 mature cells each day. This means that about 12 duplications are necessary from the stem to the final progeny provided that efficient use is made of each cell type at every stage of development.

Referring again to the previous Ciba Foundation Symposium on Haemopoiesis we calculated, on the basis of autoradiographic studies, that there were five duplications in the erythron, since at that time we thought that the stem cell differentiated directly into the pronormoblast compartment (Alpen & Cranmore 1959). These data were obtained from human and rabbit bone marrow cells and there is no reason to modify them now. Since that time Tarbutt & Blackett (1968) have determined the kinetics of the recognizable stages in the rat. They reported a seven-doubling sequence in this cell series.

However, we calculate at least 12 doublings from the stem cell to the erythrocyte under steady-state conditions and, from evidence to which we shall refer later, we can expect the number, on demand, to be greater than this. Therefore, between the time when the cell loses its identity as a stem cell and the time when it emerges as the earliest recognizable erythroid cell, 5–10 doublings take place. We can now divide erythropoiesis into three stages which can be examined more or less independently (Fig. 1).

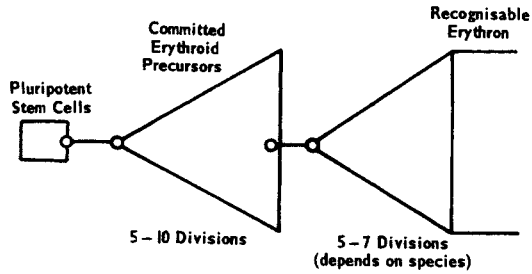

FIG. 1. This diagram illustrates the three functionally different stages of erythropoiesis. One of the progeny of the pluripotent stem cell is the cell destined to become an erythrocyte if it completes its potential differentiative course. If the late ECP is stimulated by erythropoietin it moves into the haemoglobin-synthesizing population, recognizable morphologically in the bone marrow; this population inevitably develops into erythrocytes.

ERYTHROID 'COMMITTED' PRECURSOR CELLS

Erythropoiesis can be suppressed (as judged by absence of recognizable erythroid cells in the marrow and failure of appearance of ^{59}Fe in circulating erythrocytes after an injection of the isotope) by induction of polycythaemia experimentally (Jacobson *et al.* 1957; Gurney & Pan 1958). The suppression arises because endogenous erythropoietin production is eliminated, but erythropoiesis can again be induced within a few hours of injection of erythropoietin; by 12–16 h classical pronormoblasts can be seen in the spleens of animals so treated. Clearly, in such a short time the hormone could not have induced the necessary amplification divisions (at least 5–6) from the pluripotent stem cell. Induction of erythroid differentiation, therefore, can only have been produced in an intermediate erythropoietin-sensitive precursor, which has become known as the erythropoietin-responsive cell (ERC) (Bruce & McCulloch 1964).

The evidence also indicates that these cells are committed precursors of the erythroid cells only. When a whole-body irradiated and bone-marrow-grafted mouse is made polycythaemic, the erythroid colonies are eliminated (i.e. the total colony count decreases) (Liron & Feldman 1965) and the mixed colonies are reduced in size because of failure of erythroid differentiation (O'Grady *et al.* 1967*a*,*b*; Schooley 1966). Despite the high demand for granulocytes and platelets the erythroid precursor cells in the colonies do not respond to other stimuli. Similarly, in the irradiated, bone-marrow-grafted mouse made polycythaemic by hypertransfusion microcolonies of mononuclear cells develop which can be stimulated by erythropoietin to produce full-size erythroid colonies within three days of the hormone injection (Feldman & Bleiberg 1967; Bleiberg *et al.* 1961; O'Grady *et al.* 1967*b*). The evidence suggests that the mononuclear cells in the microcolonies are ERC or pre-ERC (or both) but direct confirmation of this has not been obtained.

Lajtha *et al.* (1971) postulated, on the basis of the kinetics of spleen colony development, that the ERC has an 'age structure' and a population of cells, pre-ERC, should be interpolated between the pluripotent stem cell and the cells which fully respond to erythropoietin. Whilst the pre-ERC are committed to erythroid differentiation, they cannot respond to erythropoietin by differentiation into recognizable erythroid cells without first having 'matured' during their extensive proliferation. In other words, sensitivity to erythropoietin (in respect of differentiation) only develops in the later stages of maturation of this population (Fig. 2).

It is by no means clear what stimulates the production of the ERC but it appears that the cells continue to develop in the absence of demand for erythrocytes and even though none differentiates to the recognizable erythron. We

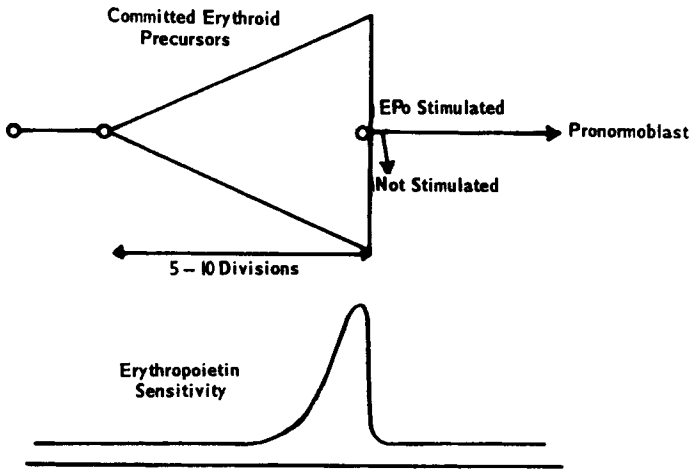

FIG. 2. The sensitivity to the action of erythropoietin for the initiation of haemoglobin synthesis is shown in relation to the stage of amplification of the cells committed to erythroid differentiation. Without erythropoietin stimulation the cells are lost to this pathway.

(Lajtha *et al.* 1969) have compared the erythropoietin-responsiveness of polycythaemic mice two hours after intraperitoneal injection of either saline or of [^{3}H]thymidine (0.8 mCi). There was, in each experiment, loss of 70–80% of the cells which could respond to erythropoietin by differentiation into haemoglobin-synthesizing cells, as a result of [^{3}H]thymidine killing. Thus 70–80% of the ERC are synthesizing DNA at any time and, therefore, virtually all of them must be in cell cycle. As there is no net increase in the ERC population, continuous production and loss of these cells must have been taking place independently of the demand for differentiation from them. Despite the functional loss of ERC we have no information to indicate what is their actual mode of disappearance.

However, the thymidine killing of ERC, by the nature of the erythropoietin assay, essentially measures only the turnover of those cells which can respond to erythropoietin within a few hours of its injection. This type of test does not indicate whether the whole amplifying population (from the pluripotent stem cells) is in the same state of cycling or not.

It must be remembered that all our assay methods are operational, therefore our definitions of cell populations are also operational. Thus, although we know that the amplifying process from stem cell to pronormoblast is represented by the erythroid committed precursor (ECP) cells, the term erythropoietin-responsive cells (ERC) refers, strictly speaking, to the 'late' part of this population.

The major role played by erythropoietin is unquestionably that of inducing the ERC to differentiate into the population synthesizing haemoglobin. That it can influence the course of the erythroblast maturation has been suggested by several workers (e.g. Finch & Coleman 1955; Lajtha & Suit 1955; Fischer 1962; Fisher *et al.* 1965). Reissmann and his co-workers have produced evidence that erythropoietin can also have an effect of amplifying the pre-ERC compartment with the subsequent production of an expanded ERC population. When administration of 5-fluorouracil (Reissmann & Samorapoompichit 1969) or Myleran (Reissmann & Samorapoompichit 1970; Reissmann & Udupa 1972) sufficient to reduce the haemopoietic precursor cell populations to low levels is followed by large doses of erythropoietin (15 units), there is no significant effect of this large dose on subsequent ^{59}Fe incorporation. However, when the mouse is challenged with a small dose (1 unit) of erythropoietin at various times after such a large 'priming' dose the response to this test dose, measured as ^{59}Fe incorporation, increases. Maximum response is obtained when the test dose of erythropoietin is given five days after the large, priming dose. At the same time, the response to one unit of erythropoietin in treated mice which had not been injected with the priming dose of erythropoietin is still low—only about 10% of the erythropoietin-primed mice. Thus, it appears that large amounts of erythropoietin, such as may normally be produced in anaemic animals, have the effect of inducing extra amplification in the pre-ERC population (Fig. 3). There is no similar increase in CFC produced by challenging with the erythropoietin and this population recovers similarly whether or not the animal has been primed.

This represents an economical system whereby, on demand for increased erythropoiesis, the increased concentrations of erythropoietin stimulate not only differentiation of late ERC but also the production of their immediate precursors. The population of erythroid-committed precursor cells acts, therefore, as an amplifying compartment to convert a relatively small number of stem cells into a large number of erythroblasts, this amplification being elastic. Recently, Wu & Lajtha (unpublished observations) chronically irradiated mice at a rate of 70 rad/day and showed that when the population of CFCs had been reduced to about 1%, little further fall occurred on continuing the radiation. This has been interpreted as a sparing of the loss of CFC into the differentiative compartment by increased amplification of the ECP population. Previously, Lamerton *et al.* (1960) showed that, even when the repopulating ability of rat bone marrow has been reduced to almost undetectably low levels by chronic irradiation, a normal output of red cells is still maintained, an observation similarly indicating increased amplification during 'transit' from stem cells to late erythroblasts.

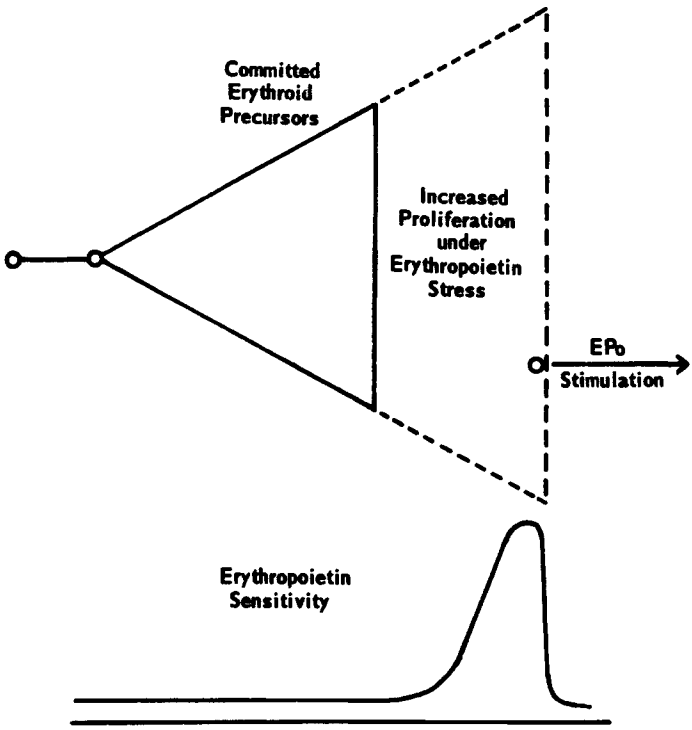

FIG. 3. The diagram illustrates the increased expansion which can be achieved in the ECP compartment under strong erythropoietin stimulation. Sensitivity to erythropoietin for the initiation of haemoglobin synthesis is shown to occur only in the later stages of the expanded compartment.

Another approach to the investigation of the erythroid precursor populations comes from *in vitro* studies. In 1971 Stephensen *et al.* published a method for the growth of erythroid colonies in plasma clot cultures and Iscove *et al.* (1973) reported similar results using methylcellulose as the support medium. The cells from which the colonies arise have been called CFU-E (erythroid colony-forming units). They are extremely sensitive to erythropoietin and do not form colonies in its absence. Colonies of up to 32 cells have been reported by Axelrad *et al.* (1973) but Iscove claims production of even larger colonies. Therefore, five or more divisions can occur in the development of the erythroid colony. The responsiveness of these cells is, however, transient and, if not stimulated within a few hours, they either die or simply become unresponsive.

Further exploitation of the plasma culture system by Axelrad *et al.* (1973) has demonstrated another erythroid precursor which appears to be a precursor of the CFU-E. It is sensitive to erythropoietin but much less so than the CFU-E.

When stimulated with large doses of erythropoietin it responds by producing several 'bursts' of erythroid colonies (i.e. groups of colonies). The cellular unit from which they arise has been called the BFU-E (erythroid burst-forming unit). The production of this population is independent of erythropoietin since there is no reduction in its numbers in the polycythaemic mouse whereas the number of CFU-E is reduced as a result of hypertransfusion.

The descendant of the BFU is a 'mobile' cell since the existence of discrete colonies separated from each other within a burst implies movement in the support medium. It is difficult to correlate the CFU-E and the BFU-E clearly with members of the erythron recognizable functionally *in vivo*. The high erythropoietin sensitivity of the CFU-E suggests close relationship with ERC whereas the reduction in the number of CFU-E in experimental polycythaemia relates the cell more closely with the pronormoblast population. The number of cells per erythroid colony does not help in ascribing the CFU-E to either population but tends to confirm the impression that it lies in the late ERC/early erythroblast region of the erythron. The BFU-E is apparently a precursor of the CFU-E in that, under strong erythropoietin stimulation, it can give rise to a burst of erythroid colonies, each of which, presumably, arises from a CFU-E. Therefore, this cell corresponds with some part of the pre-ERC population, though whether it is an earlier stage or one just preceding the ERC cannot yet be determined. Whatever the exact identity of the cells, the development of an *in vitro* system of this kind opens up the possibility of investigating the action of erythropoietin at the level of the individual cell and may well provide a situation for obtaining clearer understanding of the ECP.

One of the outstanding questions remaining to be answered is what controls erythropoietic cell production? The differentiation path is at least a two-step control, one part of which (the erythropoietin stimulation of ERC into haemoglobin-producing cells) is broadly understood. The first stage, i.e. differentiation from the pluripotent stem cell into the ECP cell, is, however, still completely unknown. Increasing the level of erythropoietin has been shown to increase CFU turnover, a finding which might be a sign of increased 'feed' (directly or indirectly) to the next compartment. Decreasing the level by induction of polycythaemia does not suppress the cycling rate of ERC although it may decrease their amplification from earlier pre-ERC. If there is a biological monitor of the size of the ECP or ERC populations we have as yet no information regarding the nature or mechanism. The humoral regulation of erythropoietic differentiation by erythropoietin affords no clue to the mode of action of the regulator of ECP size or amplification; attempts to find such a circulating regulator have been unsuccessful. On the other hand, control of CFU production is, at least largely, local: the experiments of Gidali & Lajtha (1972)

demonstrate that CFU in different parts of the body are regulated independently. A heavily-depleted (X-irradiated) population in all but one leg of a mouse does not induce increased cell production in the shielded leg.

The continuous production of ERCs even in the polycythaemic mouse also raises the question of the destiny of these cells. Certainly, if they are not stimulated to differentiate into the haemoglobin-producing cell pathway, they are functionally lost (i.e. they lose their ability to respond). This may be because the unstimulated ERC is essentially an end cell with a short life-span. It is possible that erythropoietin not only induces the differentiation step but, as a result, also enables the cell to undergo several more divisions. Without the stimulus the cell may well die. On the other hand, the unstimulated ERC may in some way adopt a new identity and perform an alternative function.

An interest has arisen during the past few years in inhibitory regulators of cellular proliferation to which the term 'chalone' has been applied (Bullough & Laurence 1960). These compounds are alleged to be produced by the mature cells of a line and to act specifically upon the precursors of that cell so that an increase in the population causes increased production of the inhibitor. This slows the production of new cells and prevents overproduction, and *vice versa*. The most intensively investigated chalone of haemopoietic cells has been that of the granulocytic series. A few reports have appeared dealing with erythrocytic chalone (Kivilaakso & Rytömaa 1971). Lord *et al.* (1974) have demonstrated specific action of an extract of erythrocytes on their precursors using a technique developed by Cercek & Cercek (1972) for measuring changes in the microstructure of the cytoplasm of the cell. With a synchronized cell population, the 'structuredness' changes dramatically at the end of G_1 phase of the cell cycle and remains low throughout S, returning to its ground state in G_2 (Cercek *et al.* 1973). Extracts of erythrocytes (but not granulocytes or lymphocytes) altered the structuredness of erythroid precursors, whilst erythrocytic extracts had no effect on the structuredness of granulocytic precursors or on phytohaemagglutinin-stimulated lymphocytes. Bateman (1974) and B.I. Lord (personal communication) have also shown by autoradiographic studies that erythrocytic extracts reduce the rate of inflow of recognizable erythroid (but not granulocytic) precursors into DNA synthesis, as demonstrated by a reduction in the labelling index. Lord also showed a reduction in the rate of increase of labelling index. The results are consistent with an impediment at the G_1–S boundary.

It is difficult to ascribe any major role to an inhibitor of erythropoiesis. Output of erythrocytes will be reduced by an inhibitor acting on the recognizable erythron if it reduces the amplification between the pronormoblast and the erythrocyte. This could be achieved by (*a*) maintenance of the maturation rate

at the normal level but an increase in the cell cycle time or (*b*) maintenance of the normal cell cycle time but with an increase in the rate of maturation. In either way the number of divisions which can occur during the maturation will be reduced and, as a result, the erythrocyte output will also be reduced. From the available data it is probable that the erythrocyte extract may act as a cell cycle modulator rather than a modifier of the maturation process. In the face of the overwhelming evidence for a major role of erythropoietin in erythroid cell regulation and of the continuous supply of ECP from the stem cell, an inhibitor such as this extract must act simply as a modifier of processes largely controlled by some other method.

In summary we can report the following advances in the understanding of erythropoiesis during the past ten years or so:—

(1) A population of erythroid committed precursor (ECP) cells exists which is derived from the pluripotent stem cells (CFC) and which precede the cells that synthesize haemoglobin.

(2) This compartment acts as an amplifier of the erythron and produces an excess of erythropoietin-responsive cells (ERC) in normal and many abnormal situations.

(3) Only the cells in the later stages of the compartment are susceptible to the differentiating stimulus of erythropoietin leading to haemoglobin production.

(4) There is continuous production of ERC whether there is a demand for differentiated cells or not.

(5) The ECP cells preceding the ERC (i.e. pre-ERC) are susceptible to higher concentrations of erythropoietin and respond by increasing the number of proliferative cycles between the pluripotent stem cell and the ERC.

(6) *In vitro* techniques have been developed for growing erythroid colonies in plasma clot or methylcellulose cultures. Two stages of erythroid precursor have been recognized: the CFU-E, which is probably the late ERC/early erythroblast stage, and the BFU-E, a precursor of the CFU-E and hence a member of the pre-ERC population.

(7) Some evidence of a specific negative feedback control has accumulated. This probably acts on the haemoglobin-synthesizing cells, reducing the amplification by reducing the number of cell cycles between the ERC and the erythron. This is achieved by increasing the cell cycle time with or without a concomitant increase in maturation rate.

Many questions regarding erythropoiesis still need to be answered, for example:—

(1) What is the stimulus inducing differentiation from the pluripotent stem cell to the early ECP cell?

(2) How is the production of this stimulus regulated?

(3) What is the fate of the ERCs which are not stimulated by erythropoietin to enter the haemoglobin-synthesizing pathway?

It is hoped that a subsequent Ciba Foundation Symposium will see the answer to such questions.

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Discussion

Ingram: I am troubled by your idea that the last step in erythropoietin stimulation is a genetic reprogramming that had happened much earlier, when the stem cell became fully committed.

Lajtha: Where the genetic reprogramming occurs is to some extent a question of semantics. I believe that it is genuine reprogramming in the '*second step* differentiation' because the erythropoietin-responsive cells (ERC) in the last stages are obviously in a fast state of proliferation, even when there is no demand for erythropoietin. A simple explanation is that these are blindly proliferating cells which die if they are not needed. That degree of cell death is the kind of luxury which the body does not as a rule afford. I feel in my bones that these cells go somewhere else! In granulopoiesis we know that the same kind of committed granulopoietic precursor cell can give rise to neutrophilic and eosinophilic granulocytes and to monocytes. So, the committed cell to some extent is not fully and specifically monophyletically committed—it still has a differentiation spectrum possible for it depending on which kind of stimulus acts on it. Another reprogramming probably occurs, switching it from one subline to another. Whether this reprogramming is truly a rearrangement of proteins on DNA or some permissive control event, I do not know. The techniques which will allow us to decide that are becoming available.

Ingram: What is the evidence to support your statement that erythropoietin has two qualitatively different effects: one on the final stages of amplification and the other on the expression of differentiation?

Lajtha: The evidence comes primarily from the work of Reissmann & Udupa (1972). When mice are hypertransfused for a long time, the overall size of the erythropoietin-responsive population eventually decreases. Previous priming, particularly early after hypertransfusion, or, even better, priming with large doses of erythropoietin after a cytotoxic insult (e.g. with busulphan [Myleran] which results in a long-term depressant effect on stem cells in general) results in an enlargement of this population, so that a challenge with the usual small test dose of erythropoietin elicits a good, almost normal, response. This stimulation with larger doses of erythropoietin has to be done fairly early (i.e. while the population is still there). In other words, this is not an erythropoietin effect on the first-step differentiation (pushing pluripotent cells into ERC); an early ERC (erythroid committed precursor cell) must still be present in the relatively

early stages. A sufficiently large dose of erythropoietin will trigger the amplification and thus the increase of population.

Weatherall: By genetic reprogramming, do you mean that a hormone is switching on different sets of genes during different stages of the cell cycle?

Lajtha: Somehow, the pattern of repression in the genome is changed (repression which requires at least one round of DNA synthesis to be 'fixed' for proper expression). That I call a differentiation step; that results in a qualitative and, as far as we know, in physiological conditions, irreversible step. To change it one would have to do some radical genetic manipulation like nuclear transplantation.

Ingram: I would have thought this 'differentiation' had already occurred before the amplification step; Fig. 1 precludes any other way for the cell to differentiate.

Lajtha: A qualitative step is certainly taken before amplification, but there are *two* such steps: first, from the stem to the committed precursor, which then undergoes some kind of 'maturation' (see p. 7) and, secondly, the qualitative change which we can observe.

Most of the terms I am using are operational definitions. The ERC are cells that respond to erythropoietin with production of pronormoblasts. A pluripotent stem cell is a cell which can form colonies in the spleen.

Najean: Did you observe the same number of stem cells and the same kinetics of population proliferation in children as in adults?

Lajtha: In the growth stage in the young, the stem cell population is not in full steady-state equilibrium; as the animal grows so does the stem cell population. Therefore, in the young animal, the turnover state of the pluripotent stem cell is always higher than in the adult animal in steady-state equilibrium.

One must be extremely careful with these turnover experiments. Several published reports are controversial because the work was not done on clean mice; one of the best ways of pushing a pluripotent stem cell into cycle is to infect the mice. The cleanliness of the mouse strain can be checked by the cycling of the pluripotent stem cells. If more than 5% of the stem cells are cycling, the strains are not clean. It is difficult to hypertransfuse newborn mice so as to get them into a suitably high polycythaemic state, because mortality rates then are extremely high. However, the amplification from the stem to the erythron seems to be smaller in the young animal than in the adult animal. Whether this is connected with the increased proliferation during growth, which is required, and the possible balance between proliferation and differentiation, I do not know. Some kind of control is built into the system: whatever the differentiation demand, the proliferative demand is satisfied first. For example, in the lethally irradiated, bone-marrow-grafted and infected mouse (even if it

has been previously bled)—so it has the maximum demand on the cell population (which has been depleted by the receipt of only a small graft) for differentiation—one will not be able to slow down its proliferation rate. One is only cutting the differentiation rate. The maximum number of cells which can be lost from the stem cells when they proliferate is 40% per cell cycle (50% loss per cell cycle would mean steady state with no recovery). This sensitive built-in control means that one cannot run down the stem cell population whatever the differentiation demand is.

Najean: This could perhaps explain why young children cannot respond as well as adults to the same stimulus, for instance a chronic haemolytic anaemia.

What do you think is the target of androgens: is it the committed cells, as suggested by their effect on the proliferation cycle and their relation with ESF production, or the uncommitted stem cell, as suggested by the delay in the clinical response and the possible efficiency on the granulocytic and platelet lines?

Lajtha: After our early experiments (Byron 1970) we thought that androgens acted directly on the committed precursor cells because we observed a larger response to erythropoietin after previous androgenic stimulation. Later, the same hormones (or at least most of them) were found to increase the cycling of the stem cells—indeed they will kick resting stem cells into cell cycle. Most of these androgens, in doses administered to the experimental animals, will stimulate the adenylate cyclase system. Once that happens, almost everything will be stimulated with the increasing or changing relative concentrations of cyclic AMP and cyclic GMP. The effect of pharmacological doses of androgens is an unspecific increase which triggers the pluripotent stem cell into cycle and increases amplification in the committed erythroid precursors. The mechanism is not known in detail, but certainly requires the cyclase system, for it can be blocked with imidazole.

Peschle: In your model for erythropoiesis, the erythroid colony-forming unit (CFU-E) is found either in the early differentiated compartment, possibly as a pronormoblast, or at the level of an ERC. We have recently observed (Peschle *et al.* 1976) that in exhypoxic polycythaemic mice the number of CFU-E in the marrow is either comparable with or higher than that in normal mice. From day 5–6 after hypoxia, these polycythaemic mice show a total suppression of erythropoiesis and few pronormoblasts in marrow smears. Thus we are inclined to accept that CFU-E are late ERC, rather than pronormoblasts. It should be mentioned, however, that the CFU-E number is reduced in transfusion-induced polycythaemic animals, which also show complete abolition of red cell production.

Lajtha: I suggested (p. 11) that the CFU-E were in the late population of the

erythropoietin-responsive cells. Depending on the techniques used, people adopt different views. With our techniques (essentially the same as that of Dr Iscove; see Iscove *et al.* 1974*a*), we see a quick response of these cells to polycythaemia induced by hypertransfusion. In that case, we are dealing with some cell that is sensitive to hypertransfusion, but the number of erythropoietin-responsive cells as such is not profoundly affected. Don't forget that these *in vitro* tests are rough 'torture chambers' for innocent cells. *A priori*, there is no reason why, when we culture haemopoietic tissue, it should not behave like perfect bone marrow. The fact that it does not means that our *in vitro* systems are extremely crude. In many instances, either the stimulated state of the cell or the culture conditions result in a 'permissive' state—the cell is allowed to survive in these wretched conditions. Conceivably, after exhypoxic polycythaemia we have stimulated the pre-ERC to a state in which it can now survive better *in vitro* and form erythroid colonies. The cell more unresponsive to erythropoietin than the CFU-E is the erythroid burst-forming unit (BFU-E) because it is a cell further back, and there is no doubt whether it is a pronormoblast or one of the precursor population.

Ingram: The BFU perplexes me; for seven days it does nothing before it manifests itself.

Lajtha: No, it doesn't do nothing. It takes some time for a colony to be recognized as such. I repeat that these *in vitro* conditions are wretched: the cell cycles are much longer than *in vivo*. The cell cycle time of the ERC population and that of the pluripotent stem cell in a mouse under stress is barely more than six hours, but in these *in vitro* conditions it is 14–16 h.

Ingram: Didn't Cooper *et al.* (1974) show that the doubling time was faster than that?

Lajtha: I have never found in an *in vitro* system a doubling time for haemopoietic tissue of much less than 12 h. An interesting aspect of the BFU is that it appears to be a cell that can move, because these colonies will be at some distance from each other, in a cluster. Since the bursts follow a Poisson distribution, we are dealing with a cell which is a potential ancestor of these clusters, and the fact that they are clusters of colonies at some distance from each other means that the first descendant is moving.

Ingram: What do you mean by movement? I find it difficult to visualize the precursors of the BFUs locomoting across the substrate, because they are likely to be much more fully differentiated towards a non-moving cell than the original moving mesenchymal cell.

Lajtha: I did not like the idea either, but it is a fact that these are clusters of colonies some distance apart and the only way they can get there is if some cells move.

Ingram: No. They could interact with something that diffuses out from the central burst, builds up in concentration and interacts with the cells that are already there. The concept of locomotion of the cells is distasteful conceptually (unless the movement is proved, an experiment which would not be difficult to do).

Lajtha: Subject to experimental proof, with the radius of these bursts as we know now there is a critical cell concentration at which the colony distribution should be random. We see this when the plates are overcrowded to a greater extent than would be predicted from the diffusion radius. The evidence as it stands is more in favour of the mobile cell. However much I share your aversion to the concept of the mobile erythroid precursor, the evidence seems to favour it.

Iscove: Perhaps it would be helpful to show some photographs of these erythroid colonies. In our culture system (Iscove *et al.* 1974*a*; Iscove & Sieber 1975), mouse bone marrow cells are immobilized in methylcellulose. Erythropoietin-dependent colonies containing 8 to 60 cells can be identified after 36 h of incubation, when they have the appearance indicated in Fig. 1. In most of these colonies growth ceases at this point. Over the succeeding 36 h, the cells accumulate haemoglobin and by 72 h of incubation many of them have become mature erythrocytes.

The proliferative activity of the CFU-E can be estimated by determining their sensitivity to the lethal effect of [^{3}H]thymidine. The cells are exposed for 20 min to the labelled nucleoside (of high specific activity) immediately after they are taken from the animal. Of the CFU-E, 70% are killed by such treatment, indicating an actively cycling population. Intriguingly, the thymidine 'kill' remains high in hypertransfused mice, but it is not higher in mice made anaemic by bleeding.

An obvious question arises: if CFU-E are already in cycle when they are plated, why is erythropoietin required in culture for colony formation? It would seem that the function of erythropoietin in culture is not simply to trigger CFU-E into cycle. However, the hormone could be necessary for subsequent divisions (or possibly survival) in culture.

There is another population of erythropoietin-dependent colonies in these cultures which increases in size through 10 to 12 days of incubation, attaining macroscopic dimensions and as many as 10 000 cells. One such colony is shown in Fig. 2 (p. 22). The large spectrum of cell sizes and the compact clusters of extremely small cells are characteristic features. Many of the cells stain positively with benzidine. Other cells are benzidine-negative, and some of these are not recognizable as belonging to the erythroid line. Many of these colonies are disperse, and it is tempting to attribute this appearance to active locomotion by early members of the clone. Many of the cells, when observed over a period