The ontogeny of erythropoiesis in the mouse detected by the erythroid colony-forming technique

I. Hepatic and maternal erythropoiesis

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SUMMARY

Employing the erythroid colony-forming technique, it is shown that throughout hepatic erythropoiesis in the mouse, the CFU-E population remains sensitive to erythropoietin. Maximum stimulation was achieved during this period using an erythropoietin concentration of 0.075 units/ml. The peak in the CFU-E concentration occurs between the 11th and 12th day while absolute values show a maximum on the 14th day of gestation. These results are discussed in terms of changing cell populations, both of erythropoietic precursors and hepatocytes from which it is concluded that at no time during foetal erythropoiesis does the CFU-E population change or become unresponsive to erythropoietin. The BFU-E population follows closely that of the CFU-E, but declines about 24 h earlier on the 16th day of gestation.

The effect of the foetus on the mother was also studied during the second half of pregnancy. During this period of natural perturbation both femoral and, in particular, splenic erythropoiesis are increased. However, during this time an erythropoietin concentration of 0.3 units/ml was required to maximally stimulate the CFU-E population derived from these tissues. The fact that both adult and foetal erythroid tissue maintain a rather constant requirement for erythropoietin for their growth in vitro, indicates that it is an intrinsic property of the cells. It is concluded that increased maternal erythropoiesis is due to an increased oxygen requirement causing hypoxia due to the growing foetus.

INTRODUCTION

Pregnancy in the mouse lasts between 20 and 21 days. During this time, erythropoiesis in both the growing foetus and the pregnant mother undergoes changes not only in the site of erythropoiesis but also in its homeostatic regulation. Foetal haemoipoiesis appears between the 7th and 8th day of gestation when erythropoiesis is initiated in the yolk sac (Snell & Stevans, 1966; Metcalf & Moore, 1970). Immature erythroblasts enter the foetal circulation from the yolk sac at about the 9th day and continue to differentiate into nucleated erythrocytes (Craig & Russell, 1964; de la Chapelle, Fantoni & Marks, 1969; Marks &

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Rifkind, 1972) which can be detected in the circulation until about the 14th to 15th day of gestation (Fantoni et al. 1969; Kubanek, unpublished results). Between the 10th and 11th day, erythropoiesis is initiated in the foetal liver by migration, seeding and proliferation of pluripotential stem cells from the yolk sac (Johnson & Moore, 1975). Hepatic erythropoiesis continues until just after birth.

During development of the foetus, the pregnant mother responds by increasing erythropoiesis primarily in the spleen which is erythropoietically dormant under steady-state conditions, the increase reaching a maximum between the 12th and 15th day of pregnancy (Fowler & Nash, 1968; Fruhman, 1968). Fruhman (1968) postulated that the observed increase in erythropoiesis especially in the spleen was the result of the rapidly growing foetus and associated tissues, causing a greater demand for oxygen by the mother. Regulation of erythropoiesis by the mother has been postulated to be independent of that occurring in the foetus since by hypertransfusion or starving the mother, thus causing a decrease in maternal erythropoiesis, foetal erythropoiesis carried on at a regular rate of red cell production; that is, change in maternal erythropoietin (Ep) levels has no effect on foetal erythropoiesis (Jacobson, Marks & Gaston, 1959; Lucarelli et al, 1968).

However, the effect of exogenously added erythropoietin to organ and suspension cultures of mouse yolk sac and foetal liver cells respectively was shown by Cole & Paul (1966) to have profound effects. Whereas explanted yolk sacs did not respond to erythropoietin, as measured by the incorporation of $^{59}$Fe into haem, foetal liver cells were stimulated but only until a decrease was observed. It was postulated that after the 14½-day, foetal liver erythropoietic cells lost their responsiveness to erythropoietin (Cole & Paul, 1966).

In experiments employing the erythroid colony-forming technique (Stephen- son, Axelrad, McLeod & Shreeve, 1971; Iscove, Siber & Winterhalter, 1974), it was shown that under the same conditions, 14-day foetal liver CFU-E responded maximally to 0.075 u./ml of erythropoietin, while adult bone marrow CFU-E required 0.4 u./ml for maximum stimulation (Rich & Kubanek, 1976). This difference pointed to the interesting possibility that during the ontogeny of the mouse the erythropoietin sensitivity might change in response to changing demands and/or sites of erythropoiesis. A detailed investigation was therefore undertaken in order to study the response of early erythropoietic precursor cells to erythropoietin in the foetus and simultaneously in naturally perturbated and stimulated maternal erythropoiesis during pregnancy.

**METHODS AND MATERIALS**

**Animals**

Foetuses were obtained by placing 20 female CBA/Ca mice 8–12 weeks old in cages for 3 weeks in order to produce an anovulatory cycle by ‘overcrowding’. Ovulation was then induced by placing two males and two females in a cage
Hepatic and maternal erythropoiesis

separated by a partition for 24 h. After this time the partition was removed, the morning after (a period of 12 h) being designated day 0 of gestation.

Preparation of suspensions

All mice were killed by cervical dislocation. Foetuses were removed aseptically from pregnant mice and placed into cold Hanks's balanced salt solution. After dissecting out the foetal livers, they were put into a 1 ml syringe containing alpha medium (Stanners, Eliceiri & Green, 1971) without ribosides or deoxyribosides, but containing 20 mM L-glutamine, 5% foetal calf serum and 100 mg each of penicillin and streptomycin (Flow Laboratories, Bonn, West Germany, supplied alpha medium and L-glutamine). The pooled livers were passed through needles of decreasing diameter into plastic tubes (Falcon Plastics, Becton Dickinson, West Germany) containing 2 ml of alpha medium. After a short time on ice to allow debris to settle, the exact volume was measured and foetal liver suspensions made up to a specific volume. For 11- and 12-day foetal livers, five to ten organs were suspended in not more than 2 ml of medium. For 13- and 14-day foetal livers, two organs were suspended in 1 ml of medium, while organs from later days of gestation were suspended as one organ/ml of medium.

Femora from the mothers were cut to a length of 9 mm from the distal end. The proximal end was fitted into a 22-gauge needle connected to a syringe containing 1 ml of alpha medium and the marrow flushed through the bone three times (Fruhman, 1964) into a plastic tube containing cold alpha medium. After all the marrows had been flushed out, the crude suspension was allowed to settle for a short time and the exact volume measured by withdrawing the suspension through a 25-gauge needle. Adult bone marrow suspensions were usually made up so that one organ was suspended in 1 ml of medium.

Spleens also obtained from the mothers were first homogenized in a loose-fitting homogenizer and after a few minutes on ice to allow large particles to settle, the suspension was decanted into a plastic tube and small particles then allowed to settle. The exact volume was measured and the suspensions were made up so that a maximum of three spleens were suspended in 5 ml of medium.

Suspensions prepared in the above manner were all single cell suspensions as seen in the haemocytometer. However, nucleated cells were regularly counted using a Coulter Counter Model B with a lower threshold of 16 and an upper threshold of 108 at 1/4 amplification and 1/2 aperture current.

Erythroid colony-forming technique

The methyl cellulose modification (Iscoe et al., 1974) of the erythroid colony-forming technique was employed using a standardized procedure previously described by Rich & Kubanek (1976). In essence, a total volume of 2.5 ml was made up consisting of alpha medium, 30% foetal calf serum, erythropoietin Step III (Connaught Laboratories, Canada; Lot 3005-1 containing 300 units in 91 mg) dissolved in alpha medium, alpha-thioglycerol (end concentration,
I. N. RICH AND B. KUBANEK

1 x 10^-4M) diluted in alpha medium, cell suspension previously diluted to the required concentration and 0.8% of a 2% methyl cellulose (Serva, premium grade, 4000 cps) stock solution prepared in alpha medium. The components were mixed in plastic tubes using a Vortex mixer and 1 ml was dispensed into each of two 35 mm Petri dishes (Greiner Plastics, West Germany).

Due to the fact that to obtain BFU-E colonies, more than ten times as much erythropoietin is required than for CFU-E colonies, the above method was scaled down so that only one quarter of the above quantities were used. Using this method, 0.2 ml were plated in multiwell tissue culture plates (Falcon Plastics, Becton Dickinson, West Germany). Like CFU-E, BFU-E multiwell plates were incubated at 37 °C in 5% CO₂ and an approximately 98% humidified atmosphere.

Aggregates of cells counted between 36 and 48 h of incubation were considered CFU-colonies if the aggregates contained eight or more tightly packed cells which would quickly stain positive for haemoglobin using benzidine solution (Cooper et al. 1974). Usually, however, CFU-E colonies were counted without staining or fixing with glutaraldehyde (Cooper et al. 1974). In considering BFU-E colonies counted after 10 days of incubation, the following basic criteria were used: (a) aggregates consisted of at least one core of cells, usually red in colour, (b) the aggregates contained more than 200 cells, (c) aggregates of cells similar in form to CFU-E colonies on the periphery which stain in a positive manner with benzidine (see Fig. 1). In this way it was easy to distinguish BFU-E colonies from small granulocytic/macrophage colonies consisting of much larger cells formed under the same conditions and probably due to the presence of colony stimulating factor (CSA) in the erythropoietin preparation and/or the foetal calf serum used. It was found, however, that using 11-day foetal liver cells, no aggregates of cells similar to CFU-E colonies were observed in the periphery. These colonies were extremely compact and, in comparison to background colonies, were much larger. These were therefore considered 11-day foetal liver BFU-E colonies (Fig. 2).

RESULTS

Using the culture conditions described previously, erythropoietin dose–response curves were performed using 0.5 x 10^6 foetal liver cells/ml.

Figures 3 and 4 show the number of CFU-E/10^6 cells plated as a function of erythropoietin concentration for the 11th, 12th, 13th, 14th, 15th and 16th days of hepatic erythropoiesis. The dose response curves depicted in Fig. 3 were performed at a later date and using a different batch of foetal calf serum than those shown in Fig. 4. However, since the linear regression parameters for 13- and 14-day foetal liver cells are similar in both figures, a continuity in the results obtained can be assumed.

The high incidence of colony growth obtained from early foetal liver
Fig. 1. Thirteen-day foetal liver BFU-E as seen in the plate after 8 days of incubation with $2 \times 10^6$ cells/ml and 40 u./ml erythropoietin. The colony consists of a compact core with many CFU-E like colonies in the periphery (magnification ×80).
Fig. 2. Eleven-day foetal liver BFU-E after 8 days of incubation with $2 \times 10^6$ cells/ml and 40 u./ml erythropoietin. This colony consists of a very tight ball of cells with no CFU-E-like colonies on the periphery (magnification $\times 50$).
Hepatic and maternal erythropoiesis

Fig. 3. Dose-response curves for CFU-E foetal liver growth as a function of log erythropoietin concentration. All foetal liver cells suspensions plated at 0.5 x 10^5/ml. 11-day (r = 0.83; P < 0.05); 12-day (r = 0.92; P < 0.001); 13-day (r = 0.96; P < 0.001); 14-day (r = 0.96; P < 0.001).

cells has allowed colony counts to be obtained using erythropoietin concentrations from 0.00156 u./ml to 0.075 u./ml. Spontaneous colony formation was observed amounting to about 20 CFU-E colonies/10^6 for 11- and 12-day foetal liver cells and between 10 and 12 CFU-E colonies/10^6 for 13- and 14-day liver cells.

With the exception of 11-day foetal liver cells which show a maximum at 0.025 u./ml in the dose–response curve, a maximum erythropoietin stimulation is found at 0.075 u./ml for all later days of hepatic erythropoiesis. Instead of a definite plateau being obtained, an almost instant decrease is seen when higher concentrations of erythropoietin (Step III) are employed. This effect is almost certainly due to the presence of unspecific toxic substances in the erythropoietin preparation (Stephenson & Axelrad, 1971), since by further purification a plateau occurs (Iscove et al. 1974; Iscove & Sieber, 1975). Using a more purified erythropoietin preparation derived from human urine a dose-response on 14-day foetal liver cells was performed using the same foetal calf serum batch as that for 11-day foetal liver (Fig. 3). It is apparent (Fig. 5) that even though the concentration of CFU-E is increased over the whole erythropoietin dose range, the dose producing 50% stimulation of CFU-E is the same as that using the
commercially obtained Step III preparation, namely 0.014 u./ml. In contrast, however, to the latter preparation, the purified substance produces a plateau ranging from 0.075 u./ml to 4.00 u./ml, the highest concentration employed.

The progression of hepatic erythropoiesis is shown in Figs. 6 and 7. Figure 6 illustrates that four distinct phases of growth occur. The first, between the 11th and 13th day of gestation, appears to be one of very rapid growth with the organ increasing in cellularity by a factor of over 25, that is, an approximate doubling time of 12 h. Between the 13th and 15th day a doubling time of about 24 h occurs followed by a levelling off by the 17th day. Finally, there is a slight decrease between the 17th and 19th day of gestation.

The absolute or total of CFU-E/organ (Fig. 6) increases up to the 14th day of gestation, followed by a decrease to the 17th day, but never quite reaches zero, even on the 19th day of gestation. The increase in cellularity seen in the foetal liver, together with the changing cell populations, results in the decrease seen after the 12th day in the CFU-E concentration. (Fig. 6).

In contrast to the CFU-E population, the BFU-E population shows a constant maximum erythropoietin stimulating dose of 4.0 u./ml from the 11th to the
Hepatic and maternal erythropoiesis

16th day of gestation, which is the same dose as required for maximum growth of BFU-E from adult bone marrow. Furthermore, a similar pattern in the frequency and absolute number of BFU-E during hepatic erythropoiesis is observed when compared with CFU-E. The BFU-E concentration values for 11- and 12-day foetal liver are not significantly different, but it would appear by comparison of Fig. 7 with Fig. 6 that the peak in the BFU-E concentration would perhaps occur between the 10th and 12th day of gestation. It is however clear that a decline to almost zero levels is seen in the BFU-E concentration by the 16th day of gestation, approximately 24 h before the decline of the CFU-E population.

The apparent dilution effect of the CFU-E concentration as the cellularity of the foetal liver increases would also be applicable to the BFU-E population. It is of interest to note here that comparison of concentration and absolute numbers of CFU-E and BFU-E indicate that the BFU-E population represents about 1% of the CFU-E population throughout the 11- to 16-day hepatic period, assuming the same plating efficiency of both cell populations.

The effect of the foetus on the mother is shown in Figs. 8 and 9 for bone marrow and spleen. The upper panels of these diagrams illustrate the change in cellularity of the bone marrow and spleen over the same period (11–19 days),

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Fig. 5. Dose-response curve for CFU-E foetal liver growth as a function of log erythropoietin concentration of Step III and a more purified preparation from human urine. 14-day-old foetal liver cell suspensions were plated at 0.5 × 10⁶/ml.

- . . . . . , Purified Ep; - - - - - - , Step III Ep.
as that described for hepatic erythropoiesis in the foetus. The number of cells/organ is elevated in both bone marrow and spleen above the normal (control) adult animals. The spleen, in particular, doubles in cellularity (over $200 \times 10^6$ compared to $100 \times 10^6$ cells/organ) from the 11th to about the 15th day of pregnancy. After the 15th day, a gradual decrease to near-normal levels is observed.
The effect of pregnancy on erythropoiesis measured by the CFU-E technique is shown in the middle and lower panels of Figs. 8 and 9. The CFU-E concentration of the bone marrow decreases from the 11th to the 14th day and then declines more sharply to the 19th day. The effect on the absolute CFU-E values does not vary to any great extent from normal bone marrow. The effect on splenic erythropoiesis is more pronounced. From the 11th to the 14th day of
pregnancy, splenic CFU-E concentrations seem to follow those of bone marrow but remain at much higher levels (between 10- and 40-fold) than normal spleen. A similar pattern of events is seen for the absolute splenic CFU-E which decrease to almost normal values by the 19th day of pregnancy.

Figure 10 shows a scatter graph for the erythropoietin dose-response curves...
Fig. 9. The effect of pregnancy on splenic erythropoiesis in the mouse. (a) Total number of cells/organ, plotted on a log scale. (b) Number of CFU-E/10^6 cells plated. (c) Number of CFU-E/organ. All parameters as a function of days of gestation. Continuous horizontal line indicates the mean value of normal (control) values throughout the period of investigation (vertical bars: mean ± standard deviation).

from the 11, 13th and 15th days of pregnancy in the maternal spleen compared with that for normal adult spleen. These normalized results are expressed as the percentage of the CFU-E response from the maximum stimulating Ep dose. On examination of the curves it is seen that they are parallel to the normal adult
splenic erythropoietin response, indicating that neither the cell nor its mechanism of response to erythropoietin has changed. In addition, the erythropoietin concentration required to produce maximum colony formation remains at 0·3 u./ml.

DISCUSSION

During hepatic erythropoiesis, the foetal liver is growing very rapidly with a doubling time of about 12 h between the 11th and 13th day, decreasing to 24 h from the 13th to the 15th day followed by an even slower rate until the 17th day of gestation. The most frequent erythropoietic cell type up to about the 13th day is the pronormoblast, the number of which steadily declines to the 19th day (Silini, Pozzi & Pons, 1976; Tarbutt & Cole, 1970; Kubanek, Bock, Bock & Heit, 1975); the basophilic erythroblasts show a maximum on the 15th day while the polychromatic and orthochromatic erythroblasts peak on the 16th day of gestation (Tarbutt & Cole, 1970). It has also been shown by Paul, Conkie & Freshney (1969) and Tarbutt & Cole (1970) that during early hepatic erythropoiesis, 55–70% of all cells belong to the erythropoietic series. However, during the last 5–6 days of hemopoiesis in the foetal liver the number of hepatocytes also increases (Silini et al. 1967; Paul et al. 1969). The pattern observed
for the CFU-E during hepatic erythropoiesis would appear to be similar to that of the pronormoblast.

The pattern of appearance of the BFU-E in the foetal liver follows that of the CFU-E fairly closely, although the peak of the BFU-E concentration cannot be positioned exactly during early hepatic development. The earlier decline in BFU-E on the 16th day is about 24 h before that seen by the CFU-E population. Furthermore, the BFU-E population represents about 1% of the CFU-E population during this period suggesting that within the erythropoietic hierarchy, the CFU-E is derived from this earlier BFU-E.

Throughout hepatic erythropoiesis, with the exception of the 11th day, the maximal erythropoietin sensitivity of the foetal liver CFU-E remains at 0.075 u./ml. This would suggest that changes in the concentration of erythropoietin in the foetal circulation during this period are not the main determining factor for the rate of red cell production, since the CFU-E are capable of responding to extremely low doses of erythropoietin. BFU-E derived from foetal liver have a similar high requirement for erythropoietin to grow in vitro as BFU-E derived from adult marrow. This may imply that BFU-E growth is governed by a factor independent of erythropoietin.

Recent reports by Zucali, McGarry & Mirand (1977c) and Zucali, McDonald, Gruber & Mirand (1977b) have indicated that foetal liver cells in culture are capable of producing an erythropoietic stimulating factor which reaches maximum production between the 14th and 15th day of gestation, after which production decreases. Gruber, Zucali & Mirand (1977) have implicated the Kupffer cell or macrophage of the foetal liver capable of storing if not also producing erythropoietin. If this is so, then it may be envisaged that internal hepatic, local concentrations of erythropoietin, however small, would, in fact, be high concentrations for the cells responding to it in the immediate vicinity of the erythropoietin-producing and/or storage cells. Whether the minimum erythropoietin concentration in vitro reflects the actual minimum erythropoietin concentration in vivo can only be assumed. Nevertheless, the fact remains that throughout hepatic erythropoiesis, the CFU-E continue to respond to erythropoietin in vitro, with the decreased absolute response reflecting the decreased CFU-E concentration.

In 1966 Cole & Paul reported that whereas yolk sac erythroblasts did not incorporate $^{59}$Fe into haem in response to exogenous erythropoietin, foetal liver cells did, but only until the 14th day of gestation. They postulated that up to the 14th day erythropoietin production increased; thereafter it was in excess and the erythropoietic cells which up to this time had incorporated $^{59}$Fe, failed to do so. This was interpreted as a loss in potential for erythropoietic stimulation. However, Cole, Regan, White & Cheek (1975) demonstrated that CFU-E could be obtained until at least the 16th day of gestation, this being correlated with the response of foetal liver cells suspensions to erythropoietin as measured by the rate of haem synthesis. These authors not only proposed that erythroid
colony formation was associated with high erythropoietin levels in the circulation of the foetus, but that this also corresponded with the greatest demand for erythrocytes. In addition, the rapid decrease in CFU-E after the 16th day was associated with a loss in erythropoietin sensitivity. The difference between these results and those presented here are difficult to interpret.

Jacobson et al. (1959) and Lucarelli et al. (1968), using mice and rats respectively, showed that if the mother is subjected to polycythaemic conditions by hypertransfusion or starvation, foetal erythroid production continued in a normal manner. It was therefore postulated that either adult and embryonic erythropoiesis were controlled by different mechanisms or, that embryonic erythropoiesis, although being erythropoietin dependent, is regulated by very low levels of endogenous erythropoietin since such concentrations could be present even in the polycythaemic mouse (Bleiberg & Feldman, 1969). As shown in Figs. 3 and 4, foetal liver cells are, in fact, extremely sensitive to low erythropoietin concentrations. It is therefore concluded that at no time during hepatic erythropoiesis does the CFU-E population cease to respond to erythropoietin.

The observations of Bleiberg & Feldman (1969) imply that mouse adult bone marrow cells are less sensitive to erythropoietin than foetal liver cells. Rich & Kubanek (1976) have shown that normal adult bone marrow CFU-E require five times as much erythropoietin than foetal liver CFU-E to achieve maximum stimulation in vitro. This same requirement for erythropoietin is also observed for bone marrow or spleen taken at any time during the second half of pregnancy in the mother. Despite increased splenic erythropoiesis and changing erythroid composition seen during this naturally perturbated phase, a change in erythropoietin sensitivity of the CFU-E population is not observed. These findings indicate that erythropoietin requirement for growth of CFU-E is an intrinsic property of the adult or foetal erythroid tissue and not due to a different composition of these tissues.

The pattern shown by the changing splenic cellularity during the second half of pregnancy in the mother is in good agreement with the change in spleen weight described by Fruhman (1968) when, on the 12th day, both these parameters have doubled in comparison with normal animals. The CFU-E population appears to reach a maximum on or about the earliest day measured, namely day 11 of pregnancy, while the peak in the morphologically identifiable erythroblasts has been shown to occur on the 12th day (Fowler & Nash, 1968). The femoral cellularity is also increased above normal levels during this time. Concentrations of CFU-E parallel those of the spleen, again with an apparent peak occurring at about the 11th day of pregnancy. Fruhman (1968) has shown that the nucleated erythroblasts peaked at between the 9th and 12th days although the $^{59}$Fe incorporation decreased throughout pregnancy, an effect considered to be due to preferential uptake of the isotope by the growing foetus (Fruhman, 1970). The fluctuation in concentration and absolute values of CFU-E between the 11th and 13th day of pregnancy despite the relatively stable cellularity of
the organs during this period is unclear, although Fowler & Nash (1968) have pointed out that maternal erythropoiesis and litter size are directly related to each other.

During pregnancy, a disproportional increase in plasma volume and red blood cells results in a decreased haematocrit causing anaemia. Despite an increased red cell production, maintenance of erythropoietic homeostasis in this stress situation appears to be mainly a function of the spleen. Viewed in comparison with the total myeloid content of the animal (calculated from one femur representing about 6% of the total bone marrow as described by Smith & Clayton in 1970), the total splenic CFU-E content is of the same order of magnitude as, that calculated for the total bone marrow. That is, erythropoiesis has, in effect been doubled due to the addition of splenic erythropoiesis between the days 11 and 14 of pregnancy.

As to the mechanisms of increased maternal erythropoiesis, Fruhman (1968) suggested that the growing foetus and the associated tissues have an increasing requirement of oxygen causing hypoxia in the mother leading then to increased maternal erythropoiesis. Furthermore, as foetal growth progresses, the oxygen requirement decreases so causing a decrease in maternal hypoxia leading to a decline in erythropoiesis.

The decrease in CFU-E values observed after the 14th day of pregnancy may reflect the possibility that hypoxia in the mother is decreasing, which would be in agreement with the proposed regulation of maternal erythropoiesis (Fruhman, 1968).

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