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

II. Transition in erythropoietin sensitivity during development

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SUMMARY

The erythroid colony-forming technique has been used to study the erythropoietin (Ep) sensitivity of CFU-E during ontogenesis. Evidence is provided which indicates that an age-related decreasing Ep sensitivity of CFU-E occurs as the animal develops. In addition, an intermediate Ep-sensitive CFU-E population represented by the 2-day-old neonate is present during the transition from foetus to adult. The Ep sensitivity of the foetal, neonatal and adult CFU-E populations is shown to be an intrinsic property of these populations.

INTRODUCTION

In an earlier report (Rich & Kubanek, 1976) a difference in hepatic and adult erythroid colony-forming units (CFU-E) was described with respect to erythropoietin (Ep) requirement. It was postulated that the change from foetal to adult erythropoiesis might be accompanied by a transitional Ep-sensitive phase in the neonatal animal.

As a first stage in understanding the role of Ep during the ontogeny of the mouse, the CFU-E technique was used to study hepatic erythropoiesis (Rich & Kubanek, 1979). It was shown that during this period the Ep sensitivity of the CFU-E population remains constant throughout the second half of gestation, indicating that although CFU-E numbers change, Ep sensitivity appears to be an intrinsic property of the cell.

In this communication the Ep sensitivity of the CFU-E populations as the animal develops is described in detail. It will be shown that the major transitional phase from foetus to adult is accompanied by an intermediate Ep sensitivity of the CFU-E. In addition, perturbation experiments indicate that the Ep sensitivity, although a relative phenomenon, is an intrinsic property of a particular CFU-E population.

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Animals

Inbred CBA/Ca mice were used for these studies. Fetuses were obtained as described by Rich & Kubanek (1979). For experiments involving neonatal animals, all pregnant mice from one batch were put into a single cage and left to give birth under normal husbandry conditions. It was found that by this procedure the incidence of one of the mothers killing her young was considerably reduced. Neonates of 2, 8 and 15 days of age were used. Adult female mice were employed at an age of 8–12 weeks.

Preparation of suspensions

Preparation of foetal liver and adult bone marrow and spleen cell suspensions have been described elsewhere (Rich & Kubanek, 1979). However, femora from 2-day neonates as well as all spleens were homogenized using a loose-fitting glass homogenizer. A single cell suspension of marrow from 8- and 15-day neonatal femora was produced by flushing out the marrow with medium using a 22- or 25-gauge syringe needle.

Erythropoietin (Ep)

Two types of Ep were employed, namely that obtained from anaemic sheep plasma supplied commercially by Connaught Laboratories (Ontario, Canada), the Step III preparation, and that from human urine. The latter had a specific activity of 140 units/mg of protein and was prepared as described by Iscove, Sieber & Winterhalter (1974).

Erythroid colony-forming technique

A modified methyl cellulose method was used (Iscove et al. 1974). This modified method has been described elsewhere together with the criteria for CFU-E and BFU-E evaluation (Rich & Kubanek, 1979). For velocity sedimentation studies, however, the technique was scaled down by half and 0.5 ml of the mixed components were plated in Replica Dishes (Sterilin, England; Greiner, West Germany).

Irradiation of cell suspension

Two ml of the original foetal liver or adult bone-marrow cell suspensions were dispensed into plastic tubes and irradiated using a Stabilipan gamma-irradiator and Duplex diosmeter at 12 mA and 250 kW with a Cu filter. A distance of 45 cm separated the source from the cells, the latter receiving 24.5 rad/min. After irradiation the suspensions were thoroughly mixed, diluted to the plating dilution and added to the other, previously mixed culture components as quickly as possible to avoid changes in physical conditions.
Velocity sedimentation procedure

The Staput method of Miller & Phillips (1969) modified using a foetal calf serum gradient of 5%, 15% and 30% in Hanks balanced salt solution (BSS) was used. Cell suspensions were prepared in Hanks BSS containing 3% foetal calf serum and 2% L-glutamine. A concentration of $112.5 \times 10^6$ cells contained in 20–25 ml was loaded into an 11.5 cm diameter glass chamber (Glassbauerei, Weil am Rhein, W. Germany). After 3–5 h the cone volume was discarded and 20 fractions each of 30 ml were collected into 50 ml plastic tubes (Falcon Plastics, Becton-Dickenson, W. Germany). The tubes were centrifuged at 1200 rev/min for 10 min at 4 °C, after which the supernatant was withdrawn and the cells resuspended in 1 ml Hanks BSS. The cell count of each fraction was determined using a Coulter Counter.

Statistical analysis of results

All Ep-dose-response values were normalized as follows. An arbitrary Ep dose was chosen and the mean CFU-E value for this dose calculated for all dose responses for a particular tissue. Using this value, the individual values for this particular Ep dose were recalculated and the difference factor obtained. The colony values for the complete dose response were multiplied by the factor specific for that dose response. Linear regressions were then calculated by the method of least squares. Polynomial regressions were also calculated using this method. The probability of association was obtained from the correlation coefficient ($r$) (Bailey, 1959). Testing the significance of differences between dose-response parameters was performed by regression analysis, whereby regression line intercepts and regression coefficients were compared.

RESULTS

Transition in erythropoietin sensitivity from foetus to adult

(a) Using Step III erythropoietin

To investigate whether a transitional Ep-sensitive CFU-E population existed during postnatal development, Ep-dose responses for 2-, 8- and 15-day neonatal bone marrow and spleen were performed. The results were then compared with Ep-dose-response curves for CFU-E 14-day foetal liver and adult bone marrow and spleen by regression analysis. It was found that both 2-day neonatal bone marrow and spleen exhibited an Ep-dose-response curve which could be interspersed between that for foetal liver and adult bone marrow or spleen. Eight- and 15-day neonatal organs exhibited adult CFU-E Ep sensitivity. Figure 1 shows the parallel displacement to a less sensitive CFU-E population as erythropoiesis passes from the hepatic stage at 14 days of gestation to the adult phase seen in the bone marrow. Similar parallel displaced curves are found for 14-day foetal liver and 2-day neonatal and adult spleen, but are not shown here. These
Fig. 1. Dose-response curves for 14-day foetal liver, 2-day neonatal bone marrow and adult bone marrow CFU-E plotted as the percent response from the maximum stimulating Ep dose as a function of log Ep Step III concentration.

14-Day foetal liver  
\( y = 166.5 + 60.9(x); \)  
\( r = 0.95; P < 0.001 \)

2-Day neonatal bone marrow  
\( y = 143.7 + 57.6(x); \)  
\( r = 0.96; P < 0.001 \)

Adult bone marrow  
\( y = 111.9 + 57.0(x); \)  
\( r = 0.93; P < 0.001 \)

results were obtained using the Step III Ep preparation. Since at higher than optimal Ep doses a decrease is seen due to impurities in the preparation, linear regressions have been employed and the CFU-E values evaluated as percent from the maximum stimulated Ep concentration. The regression coefficients for all these dose-response curves indicate that the slopes of the linear regressions are very similar. This is supported by comparing the regression coefficients; the dose-response curves are parallel to each other \( P < 0.05 \). In addition, further regression analysis indicates that each parallel dose response is, within 95% confidence limits, horizontally displaced from the others. Thus, the CFU-E giving rise to each of the Ep-dose-response curves represents a different population differing in their 50% Ep values by 0.012 U/ml for 14-day foetal 0.024 U/ml for 2-day neonatal bone marrow and 0.082 U/ml for adult bone marrow.
Fig. 2. Dose-response curves for 14-day foetal liver, 2-day neonatal bone marrow and adult bone marrow CFU-E plotted as the percent response from the maximum stimulating Ep dose as a function of log purified human urinary Ep concentrations. If linear regressions instead of polynomial regressions are calculated the following dose response parameters between the given Ep concentrations are obtained.

**14-Day foetal liver** (from 0-00313 to 0-1 U/ml)  
(y = 167·8 + 63·3x;  
r = 0·98; P < 0·001)

**2-Day neonatal bone marrow** (from 0·125 to 0·1 U/ml)  
(y = 141·7 + 67·6x;  
r = 0·91; P < 0·05)

**Adult bone marrow** (from 0·025 U/ml to 0·5 U/ml)  
(y = 115·2 + 66·1x;  
r = 0·97; P < 0·001)

**(b) Using human urinary erythropoietin**

Since the above results could be caused, in part, by the degree of purity of the Step III Ep preparation, a similar set of experiments were performed using Ep purified from human urine which produced a plateau in the dose-response curve at high Ep concentrations. The normalized results, shown in Fig. 2, are again plotted as the percentage from the maximum stimulating Ep concentration; in this case, the mean plateau value. The results are plotted as polynomial regressions. The calculated 50% Ep values from these curves are 0·014, 0·043 and 0·1 U/ml for 14-day foetal liver, 2-day neonatal bone marrow and adult bone marrow respectively. If, however, only the linear portions of the regressions are considered, the 50% Ep values are 0·014 U/ml for 14-day foetal liver, 0·048 U/ml for 2-day neonatal bone marrow and 0·1 U/ml for adult bone
Table 1. Change in concentration and absolute CFU-E of splenic erythropoiesis during the development of the mouse

<table>
<thead>
<tr>
<th></th>
<th>17-Day prenatal spleen</th>
<th>19-Day prenatal spleen</th>
<th>2-Day neonatal spleen</th>
<th>8-Day neonatal spleen</th>
<th>15-Day neonatal spleen</th>
<th>Adult spleen</th>
</tr>
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<tr>
<td>No. of experiments</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>CFU-E/10^6 cells</td>
<td>25</td>
<td>43</td>
<td>477</td>
<td>326</td>
<td>68</td>
<td>9</td>
</tr>
<tr>
<td>CFU-E/organ</td>
<td>46</td>
<td>257</td>
<td>10116</td>
<td>194207</td>
<td>34416</td>
<td>10738</td>
</tr>
</tbody>
</table>

Table 2. Change in concentration, absolute and total CFU-E of myeloid erythropoiesis during the development of the mouse

<table>
<thead>
<tr>
<th></th>
<th>2-Day neonatal bone marrow</th>
<th>8-Day neonatal bone marrow</th>
<th>15-Day neonatal bone marrow</th>
<th>Adult bone marrow</th>
</tr>
</thead>
<tbody>
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<td>No. of experiments</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>CFU-E/10^6 cells</td>
<td>159</td>
<td>258</td>
<td>340</td>
<td>759</td>
</tr>
<tr>
<td>CFU-E/organ</td>
<td>782</td>
<td>2035</td>
<td>17561</td>
<td>80302</td>
</tr>
<tr>
<td>CFU-E/animal*</td>
<td>0.13 × 10^6</td>
<td>0.34 × 10^6</td>
<td>2.92 × 10^6</td>
<td>13.38 × 10^6</td>
</tr>
</tbody>
</table>

* Calculated from one femur being 6% of the total bone marrow (see text).

marrow. Comparison of regression coefficients in this case shows that the dose-response curves are parallel ($P < 0.05$) and they are significantly horizontally displaced from each other ($P < 0.05$). Thus, as the animal matures, different and transient CFU-E populations occur exhibiting decreased Ep sensitivity.

Transition in erythropoiesis

Tables 1 and 2 show CFU-E concentrations and absolute CFU-E values for pre-, neonatal and adult spleen and neonatal and adult bone marrow. Maximum splenic CFU-E values are obtained at about the 8th day of life, thereafter declining to a constant but low level. Myeloid CFU-E increases continuously throughout the neonatal period and reaches maximum numbers in adult life. The number of CFU-E/animal have been calculated by assuming that one femur is approximately 6% of the total bone marrow (Smith & Clayton, 1970).

Effect of perturbation on the erythropoietin-dose-response curve

A set of experiments were performed in order to test whether mixing different cell suspensions would affect the Ep sensitivity. The experiments were carried out as follows: $1 \times 10^5$ irradiated (850 rad) adult bone marrow cells per ml were mixed with $1 \times 10^5$ normal 14-day foetal liver cells per ml, and the Ep-dose-response curve so obtained was compared to the Ep-dose-response curve for $0.5 \times 10^5$ normal 14-day foetal liver cells per ml (the usual cell dose plated).
Fig. 3. Dose-response curve for normal 14-day foetal liver cells mixed with irradiated adult bone marrow cells ($y = 142.6 + 53.2(x); r = 0.95; P < 0.001$) and normal adult bone marrow cells mixed with irradiated cells ($y = 115.8 + 62.6(x); r = 0.97; P < 0.001$) as a function of log Ep concentration. Shaded area represents the dose-response curve for normal 14-day foetal liver and adult bone marrow cells (see Fig. 1).

Fig. 4. Velocity sedimentation profiles for adult bone marrow and 13-, 14- and 15-day foetal liver CFU-E.
The reverse experiment was also performed in which $4 \times 10^5$ irradiated (850 rad) 14-day foetal liver cells per ml were mixed with $4 \times 10^5$ normal adult bone marrow cells per ml. The resulting Ep-dose response was compared to that for normal adult bone marrow cells plated at a cell concentration of $2 \times 10^5$/ml. The results are shown in Fig. 3. There is no significant ($P < 0.05$) change in the regression coefficients for the dose response obtained using the mixed cell suspension compared with the normal cell suspensions. It should, however, be noted that at all Ep concentrations an increase in CFU-E values was obtained.

Figures 4 and 5 show the velocity sedimentation profiles for CFU-E and BFU-E for adult bone marrow and 13-, 14- and 15-day foetal liver. There is a distinct difference in the sedimentation velocities between adult bone marrow and foetal liver for both CFU-E and BFU-E. Single or pooled fractions from three different positions of the velocity sedimentation profiles for adult bone marrow or 14-day foetal liver were tested for their retention of Ep sensitivity and compared with unfractionated bone marrow or foetal liver CFU-E in Fig. 6. Points are the mean values of three experiments. The Ep-dose responses for the unfractionated cell suspensions are essentially parallel. In addition, there is little or no variation between separated cell suspensions or the unfractionated cell suspension.

**DISCUSSION**

The results presented here lead to the conclusion that as the animal develops from the foetus to the adult, CFU-E populations occur which show a specific
and intrinsic erythropoietin (Ep) responsiveness. This hypothesis has been postulated previously (Rich & Kubanek, 1976) and results have been summarized elsewhere (Kubanek, Heit & Rich, 1978; Rich, Heit & Kubanek, 1978, Rich 1978).

The ontogeny of haemopoiesis and erythropoiesis in particular is a process of orderly and sequential transitions, initiated in the yolk sac. Migration of haemopoietic stem cells to the liver initiates haemopoiesis in this organ (Johnson & Moore, 1975). Later, erythropoiesis occurs in the spleen and finally in the bone marrow (Tables 1, 2).

Parallel to the sequential changes of haemopoiesis from the foetal liver to the bone marrow is an age-related decreasing Ep sensitivity of CFU-E. Concentrations and CFU-E values per organ during this developmental period are fluctuating continually. Thus for comparison of Ep-dose-response curves results had to be normalized and presented as the percentage of the maximal stimulating Ep concentration.

Fig. 6. Dose-response curves for CFU-E as a function of log Ep concentration performed on velocity sedimentation fractions from separated 14-day foetal liver and adult marrow cells. Three fractions from each separated haemopoietic tissue were taken corresponding to the sedimentation velocity (mm/h) given in the diagram and compared with CFU-E from unfractionated foetal liver or adult bone marrow. The 50% Ep values are as follows:

<table>
<thead>
<tr>
<th>Fractionated</th>
<th>Unfractionated</th>
<th>mm/h</th>
<th>U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-day foetal liver</td>
<td>14-day foetal liver</td>
<td>12.57</td>
<td>0.029</td>
</tr>
<tr>
<td>14-day foetal liver</td>
<td>14-day foetal liver</td>
<td>9.86</td>
<td>0.022</td>
</tr>
<tr>
<td>14-day foetal liver</td>
<td>14-day foetal liver</td>
<td>7.26</td>
<td>0.017</td>
</tr>
<tr>
<td>Adult bone marrow</td>
<td>Adult bone marrow</td>
<td>11.97</td>
<td>0.111</td>
</tr>
<tr>
<td>Adult bone marrow</td>
<td>Adult bone marrow</td>
<td>7.92</td>
<td>0.104</td>
</tr>
<tr>
<td>Adult bone marrow</td>
<td>Adult bone marrow</td>
<td>5.77</td>
<td>0.123</td>
</tr>
</tbody>
</table>
The Ep-dose-response curves are represented in a similar fashion to that used by Camiscolli & Gordon (1971) for the polycythaemic mouse bioassay and by de Klerk, Hart, Kruiswijk & Goudsmit (1978) for the in vitro bioassay of serum Ep levels. Recently, Hågå & Falkanger (1979) have described the in vitro assay of Ep using newborn mouse livers less than 24 h old as a source of CFU-E. Figure 2 shows that early foetal liver CFU-E also provide an extremely sensitive, easily reproducible in vitro bioassay for different Ep preparations using either fresh or cryopreserved cells (unpublished data). By keeping the Ep preparation constant and assuming that Ep acts by the same mechanism in all these cell populations, changing the target cell provides a series of parallel curves from which the displacement between the linear sections of the dose response provides an estimation of the Ep sensitivity of one cell population compared to another. Erythropoietin sensitivity in this case is a relative phenomenon since it may change under different in vitro conditions and with different strains of mice. Thus 14-day foetal liver CFU-E are more sensitive to Ep than adult bone marrow CFU-E. In addition, the change from foetal to adult erythropoiesis is not only accompanied by a change in organ sites but also by a transition in Ep sensitivity as shown in the example of 2-day neonatal bone marrow. This phenomenon is not dependent on a particular Ep preparation but is equally expressed using the commercially prepared Step III or a more purified human urinary Ep. Therefore, during ontogenesis, different Ep-sensitive CFU-E populations exist. However, the possibility that this is due to differences in the age structure of the precursor compartment at different times seems to be excluded since colonies from foetal and adult erythropoietic tissue are morphologically identical (Rich, 1978). Thus the Ep sensitivity of the CFU-E is an inherent property which changes during development.

That the Ep sensitivity is a stable and intrinsic property of a particular CFU-E population has been shown under several different conditions.

During pregnancy, splenic erythropoiesis is greatly stimulated with a 40-fold increase in CFU-E. This natural perturbation did not change the Ep sensitivity of adult CFU-E at any time point during this stimulated period (Rich & Kubanek, 1979). It can be concluded that the Ep sensitivity of adult CFU-E remains stable during a period of considerable stimulation and changing composition of the erythropoietic tissue. In 1974, Gregory, Tepperman, McCulloch & Till demonstrated that under extreme perturbation the Ep sensitivity of the CFU-E population was displaced in both the bone marrow and spleen. These results are in apparent contradiction to those presented here. One reason for this discrepancy could be the type of perturbation used. However, subjecting mice to extreme haemorrhage did not shift the Ep-dose-response curve (unpublished data). Therefore the difference in results still remains unclear.

Retention of parallelism without displacement of the Ep-dose-response curves was found when living and lethally irradiated haemopoietic cell suspensions of different Ep sensitivities were mixed together. This again shows that
CFU-E erythropoietin sensitivity changes

Ep responsiveness is an intrinsic property of the cells. Since a greater number of CFU-E are stimulated at all Ep concentrations in these mixed suspensions, this implies that radiation may cause the release of a factor into the medium capable of stimulating CFU-E. This could be similar to the erythropoietic stimulating factor released when macrophages are treated with silica. This factor is similar, if not identical, to Ep as shown by its effect in polycythaemic and bled mice and its dose-dependent stimulation in the polycythaemic mouse bioassay (Rich, Heit & Kubanek 1980). These observations lend further support to that proposed by Gruber, Zucali & Mirand (1977), namely that macrophages are producing and/or storing Ep.

Velocity sedimentation studies indicate that CFU-E from 13- or 14- or 15-day foetal liver are a larger cell than that from adult bone marrow. The CFU-E from various fractions of the velocity sedimentation profiles from either 14-day foetal liver or adult bone marrow show a retention of their specific Ep sensitivity and are not significantly different to the respective unfractionated cell suspension. This implies that the whole foetal liver or adult bone marrow CFU-E population is homogeneous with respect to their Ep sensitivity and the difference is not due to a different composition of the erythroid precursor compartment.

The velocity sedimentation profile of BFU-E derived from foetal liver also shows a shift to a larger size when compared to adult bone marrow BFU-E. This is similar to that found by Johnson & Metcalf (1978) for GM-CFC. However, both adult bone marrow and foetal liver BFU-E require the same high Ep concentrations for growth in order to achieve maximum stimulation and show similar colony morphology, even though their velocity sedimentation profiles are different. This observation would imply that BFU-E's may only require Ep for differentiation.

Our results have shown that foetal liver CFU-E are more sensitive to Ep than adult bone marrow CFU-E and that during the transition from the foetus to the adult, CFU-E populations occur (in both femur and spleen) which exhibit a transitional Ep sensitivity. In addition, the Ep sensitivity shown in vitro by a specific CFU-E population is an intrinsic property of that population. Earlier work by Jacobson, Marks & Gaston (1959) and Lucarelli et al. (1968) working with foetal erythropoietic tissue, as well as Lucarelli, Howard & Stohlman (1966) and Carmena, Howard & Stohlman (1968) employing the neonate, concluded that either regulatory mechanisms operating in the adult were different from those in the foetus or early neonatal life, or that foetal erythropoiesis was regulated autonomously. However, Bleiberg & Feldman (1969) also speculated that foetal liver cells, although being Ep-dependent, are regulated by very low levels of Ep. Other experiments by Rencricca, Howard, Kubanek & Stohlman (1976) showed that the repopulating capacity of foetal liver cells was greater than 10-day neonatal bone marrow which in turn was greater than adult bone marrow. This shows that the same phenomenon can be obtained under
different conditions and supports the proposition of transitional Ep-sensitive CFU-E populations.

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REFERENCES

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