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Erythropoiesis is Distinct at Each Stage of Ontogeny

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ABSTRACT. ***In vitro*** **erythropoiesis from fetuses, newborn infants, and adults was compared in methyl cellulose cultures. Fetal and newborn blood erythroid colony formation tended to be more sensitive to erythropoietin than adult. The day of maximal colony formation was earlier in fetal than in newborn or adult cultures. The number of colonies/100 000 mononuclear cells on d 13 of culture and on the day of peak growth was highest in fetal, intermediate in newborn, and lowest in adult cultures. Burst forming units-erythroid/mL of blood on culture d 13 and the day of peak growth were similar in fetuses and newborns, and both were significantly greater than in adults. The proportional synthesis of γ-globin in fetal colonies was 2-fold greater than in newborn colonies, and 6-fold greater than in adult colonies. Thus, fetal, newborn, and adult erythroid progenitor cultures are each unique with regard to erythropoietin sensitivity, growth time course, number of erythroid colonies, and the proportion of γ-globin synthesis. (*Pediatr Res* 31: 170–175, 1992)**

Abbreviations

BFU-E, burst forming unit-erythroid
CFU-E, colony forming unit-erythroid
Ep, recombinant human erythropoietin
Ep ½ max, concentration of erythropoietin at which half-maximal growth was achieved
Ep plateau, concentration of erythropoietin beyond which no additional growth was observed
MNC, mononuclear cells

In vitro semisolid culture assays have been used extensively to characterize erythroid progenitor cells and to study erythropoiesis during normal human ontogeny. Although not morphologically identifiable, erythroid progenitor cell classes can be identified by the phenotypes of the clonal colonies that they form *in vitro*. The earliest identifiable committed erythroid progenitor cell is the BFU-E, which further differentiates into the CFU-E, which in turn produces erythroblasts.

During intrauterine development, erythropoiesis occurs first in the embryonic yolk sac, then the fetal liver, and finally in the bone marrow, which becomes the definitive hematopoietic organ in the newborn and adult. Erythroid cell cultures have demonstrated the presence of erythroid progenitor cells at serial gestational ages in the yolk sac (1, 2), liver (3–6), bone marrow (3, 7), and blood (3, 8, 9) of fetuses, as well as in the bone marrow and blood of newborn infants (3, 6, 10) and adults (11, 12).

We have previously demonstrated that colonies derived from newborn and adult blood BFU-E have different globin synthesis phenotypes (13, 14). The studies presented here were designed to further characterize fetal, newborn, and adult blood erythropoiesis with regard to Ep sensitivity, kinetics of colony growth, number of progenitor cells, and proportion of γ-globin synthesis. We present evidence that erythropoiesis is distinct at each stage of ontogeny.

MATERIALS AND METHODS

Blood studies. All procedures were approved by the Institutional Review Board of the Mount Sinai School of Medicine. Fetal blood was obtained at the time of elective termination of pregnancy from the umbilical veins of normal 18- to 23-wk gestational age fetuses. Newborn blood was obtained from the umbilical cords of term newborn infants immediately after delivery. Adult blood was from the antecubital vein of normal donors, 20–45 y of age. Blood samples were collected into syringes containing 50 U heparin (Liquaemin; Organon Inc., West Orange, NJ)/mL blood or into heparinized Vacutainer blood collection tubes (Becton-Dickenson, Rutherford, NJ). To prepare MNC, blood was diluted 1:1 with alpha medium (GIBCO Laboratories, Grand Island, NY), layered onto an equal volume of Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), and centrifuged at 450 × *g* for 30 min at 18°C (13, 15).

Fetal liver studies. Fetal livers were obtained from normal 18- to 23-wk gestational age fetuses after elective termination of pregnancy. The livers were cut into small pieces with sterile scissors and then incubated in alpha medium containing 5 mg collagenase (Sigma Chemical Co., St. Louis, MO)/mL for 30 min at 37°C. After incubation, liver cell suspensions were passed through a sterile fine-wire mesh, then vigorously pipetted, and finally passed through successively smaller bore syringe needles to prepare single-cell suspensions. Fetal liver MNC were prepared by layering fetal liver cell suspensions onto equal volumes of Ficoll-Paque, followed by centrifugation at 450 × *g* for 30 min at 18°C.

Erythroid cultures. MNC were cultured in 0.8% methyl cellulose according to methods previously described (13, 15). Each mL of culture contained: 2.5–5.0 × 10^4 fetal, 1 × 10^5 newborn, or 3 × 10^5 adult MNC, 0.8% methyl cellulose (Fisher Scientific Co., Pittsburgh, PA) in alpha medium without nucleosides (GIBCO L Laboratories), 30% fetal bovine serum (Armour Pharmaceutic Intergen Company, Purchase, NY), 1% BSA (Cohn faction IV; Sigma Chemical Co.), 10^{-4} M 2-mercaptoethanol (Sigma Chemical Co.), 0.1 U penicillin, and 0.1 μg streptomycin/mL (GIBCO Laboratories). Ep was kindly provided by Ortho Pharmaceutical Corporation (Raritan, NJ). All cultures (except for Ep dose-response studies) contained 2 U Ep/mL culture. Cultures were done in triplicate in 0.3-mL volumes in NUNC four-well dishes (Intermed, Rosklide, Denmark) and incubated

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at 37°C in 4% CO_2. Colonies derived from CFU-E were counted on culture d 7 (seen only in fetal liver cultures), and those derived from BFU-E were counted on d 9, 13, 17, and 20 after plating using a Bausch & Lomb Stereozoom dissecting microscope (Bausch & Lomb, Rochester, NY). BFU-E/mL = BFU-E/100 000 cells plated × number of MNC/mL of blood.

Globin synthesis. Globin synthesis by colonies was examined by labeling with ^{3}H-leucine, electrophoresis of culture lysates on polyacrylamide gels containing acetic acid, urea, and Triton X-100, and fluorography of gels, followed by densitometric analyses. BFU-E-derived colonies were labeled from culture d 9–10, 13–14, 16–17, and 20–21. Fetal Hb (Hb F) is composed of two α- and two γ-globin chains. There are two γ-chains, $^G\gamma$ and $^A\gamma$, with glycine or alanine in amino acid position 136. Adult Hb (Hb A) is composed of two α- and two β-chains. The percentage of γ-globin (or Hb F) = $100 \times \gamma/(\gamma + \beta)$. The percentage of $^G\gamma$-globin = $100 \times {}^G\gamma/({}^G\gamma + {}^A\gamma)$. Detailed procedures have been reported elsewhere (13, 15). All data shown are the means of triplicate analyses.

RESULTS

Ep dose-response curves for fetal, newborn, and adult blood BFU-E-derived colonies on d 13 of culture are shown in Figure 1. No colonies were observed without the addition of erythropoietin. When average growth was compared at a low dose of Ep (0.1 U Ep/mL), the highest proportion of total growth was observed in fetal blood cultures (31% of maximal growth), newborn colony growth was intermediate (23%), and adult colony growth was the lowest (13%) (Table 1). The most significant observation was that some of the fetal and newborn cultures grew well at <0.1 U Ep/mL, whereas none of the adult cultures did so. The Ep ½ max (Fig. 2 and Table 1) and the Ep plateau (Fig. 3 and Table 1) indicate the sensitivity of erythroid progenitor cells to Ep. The range of Ep ½ max was lower for fetal cultures than for newborn or adult cultures. All fetal cultures reached half-maximal growth with doses of Ep ≤0.52 U/mL, whereas one newborn and two adult cultures required higher doses of Ep to achieve half-maximal growth. The Ep plateau was ≤2 U/mL in four of five fetal cultures and seven of seven newborn cultures. In contrast, only four of nine adult cultures reached plateau growth with doses ≤2 Ep/mL, and the remaining five cultures required 4 U Ep/mL for colony growth to plateau.

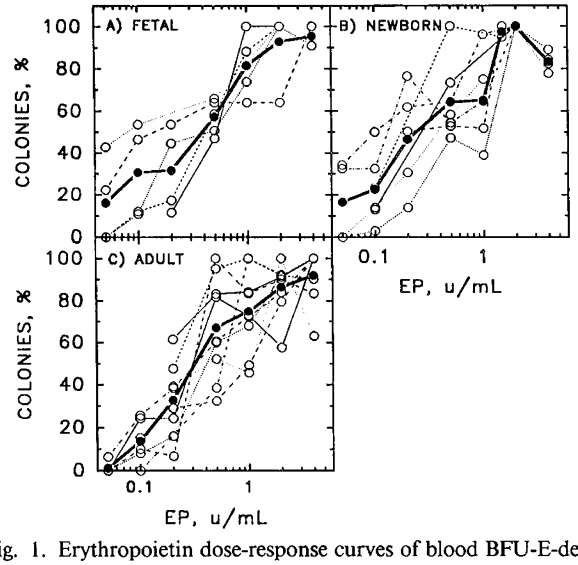

Fig. 1. Erythropoietin dose-response curves of blood BFU-E-derived colonies from fetuses, newborn infants, and adults. Data were normalized as the percentage of maximal growth. Each *curve* with *open symbols* represents a different donor, and each *point* is the mean of triplicate cultures. The curve with *solid symbols* represents the mean of all curves. Fetal (*A*), newborn (*B*), and adult (*C*) blood.

Thus, fetal and newborn BFU-E tended to be more sensitive to Ep than adult BFU-E, requiring lower doses to achieve maximal growth.

Figures 4 and 5 demonstrate the growth time course of blood BFU-E-derived colonies, and the data are summarized in Table 1. In two of 13 fetal cultures, maximal growth occurred by d 10 of culture. In five more, peak growth was by d 13. Four more peaked by d 16, and only two more by d 20. Peak growth of newborn colonies was slightly later, with no peak growth before d 13, at which time only three of 13 achieved maximal growth; eight more reached peak growth by d 16, and two more, by d 20. Growth of normal adult colonies peaked at the same time as newborn colonies. In adult cultures, the earliest peak growth was by culture d 13 in only eight of 20 cultures; seven more peaked by d 16, and the remaining five, by d 20.

Figure 6 compares blood BFU-E-derived colony growth on d 13 and on the peak day, which is often later than d 13 as shown above. In *panels A* and *B*, the data are expressed as colonies/100 000 MNC plated. On both d 13 and the day of peak growth, fetal colonies were the most numerous, followed by newborn colonies, whereas adult colonies were the fewest in number. Because the number of MNC/mL of blood obtained by Ficoll-Hypaque centrifugation is often higher from newborn than fetal or adult blood (mean × 10^5 cells/mL blood ± 1 SD: fetal = 19 ± 11, newborn = 40 ± 25, adult = 17 ± 6), data are expressed as BFU-E/mL blood in *panels C* and *D* to provide a more physiologic comparison. This comparison demonstrated that the concentration of fetal and newborn BFU-E were similar to each other and both were significantly higher than adult BFU-E on d 13 and the peak day. The means, ranges, and *p* values for these experiments are shown in Table 1.

Fetal liver erythroid progenitor cell growth is shown in Figures 5 and 7. In contrast with fetal blood, fetal liver MNC contained both CFU-E and BFU-E. Fetal liver BFU-E-derived colonies were more numerous than fetal blood BFU-E-derived colonies on both culture d 13 and the day of peak growth ($p = 0.023$ and 0.017, respectively). Fetal liver BFU-E-derived colonies peaked slightly later than fetal blood colonies (mean day of peak growth = 16.4 *versus* 14.5, respectively). No fetal liver cultures achieved peak growth by d 10, two of five peaked by d 13, one more peaked by d 16, and maximal growth occurred by d 20 in the remaining two cultures.

Figure 8 demonstrates the time course of globin synthesis. A temporal decline in γ-globin synthesis was observed at all stages of ontogeny. At all times, fetal was highest and newborn was next, followed by adult γ-globin synthesis. $^G\gamma$-globin did not decline with time and was highest in the fetal cultures. Figure 9 shows globin synthesis in individual cultures of fetal, newborn, and adult blood BFU-E-derived colonies on d 13. Means, ranges, and *p* values are shown in Table 1.

DISCUSSION

We have compared the growth characteristics of peripheral blood erythropoiesis from fetuses, newborn infants, and adults. For the parameters that were examined, each ontogenic stage has a unique "phenotype."

Ep sensitivity. The Ep sensitivities of fetal, newborn, and adult blood progenitor cells were compared by examining the proportion of colony growth at a low dose of Ep (0.1 U Ep/mL), Ep ½ max, and Ep plateau. Fetal cultures had the highest proportion of growth with low Ep (31%) and the lowest Ep ½ max (≤0.5 U Ep/mL). Newborn cultures had intermediate growth at low Ep (23%), and the Ep ½ max was slightly higher than in fetal cultures and similar to that in adult cultures. The Ep plateau was similar in all cultures. The Ep sensitivities in our studies were similar to those previously reported for fetal (8, 9, 16), newborn (3, 6, 10, 17), and adult (9, 17–19) blood progenitors. The apparent bimodality of the Ep dose-response curves in some fetal and newborn studies is consistent with the possibility that blood at

Table 1. *Characteristics of blood BFU-E-derived colonies*

								p values*		
		Fetal	*n*	Newborn	*n*	Adult	*n*	F *vs* N	N *vs* A	F *vs* A
Colonies/100 000 cells, on d 13	Mean†	141 ± 109	13	59 ± 22	13	17 ± 11	20	0.01	<0.001	<0.001
	Range	24–383		26–107		2–40				
Colonies/100 000 cells, on peak day	Mean	190 ± 120	13	95 ± 42	13	21 ± 11	20	0.01	<0.001	<0.001
	Range	24–383		40–176		4–40				
BFU-E/mL, on d 13	Mean	2886 ± 2921	13	2256 ± 1509	13	277 ± 204	20	0.49	<0.001	<0.001
	Range	323–8966		575–5959		27–758				
BFU-E/mL, on peak day	Mean	4299 ± 3878	13	3517 ± 2302	13	365 ± 225	20	0.54	<0.001	<0.001
	Range	323–11730		732–9535		45–1007				
Day of peak growth	Mean	14.5 ± 3.2	13	15.9 ± 2.2	13	15.5 ± 2.8	20	0.21	0.60	0.38
	Range	10–20		13–20		13–20				
0.1 U Ep/mL, % maximal growth	Mean	31 ± 23	4	23 ± 17	6	13 ± 9	9	0.5	0.1	0.05
	Range	11–54		3–50		0–26				
Ep ½ maximal (U/mL)	Mean	0.3 ± 0.2	5	0.4 ± 0.3	7	0.4 ± 0.3	9	0.77	0.84	0.56
	Range	0.1–0.5		0.1–1.1		0.1–1.0				
Ep plateau (U/mL)	Mean	2.2 ± 1.1	5	1.7 ± 0.6	7	2.7 ± 1.6	9	0.33	0.13	0.52
	Range	1.0–4.0		0.5–2		0.5–4.0				
% γ-globin on d 13	Mean	82 ± 5	11	44 ± 12	13	13 ± 9	25	<0.01	<0.01	<0.01
	Range	70–88		30–70		5–40				
% $^{G}\gamma$-globin on d 13	Mean	66 ± 6	11	55 ± 7	11	53 ± 14	22	<0.01	0.62	<0.01
	Range	58–78		42–70		26–76				

* F, fetal; N, newborn; and A, adult.
† ±1 SD.

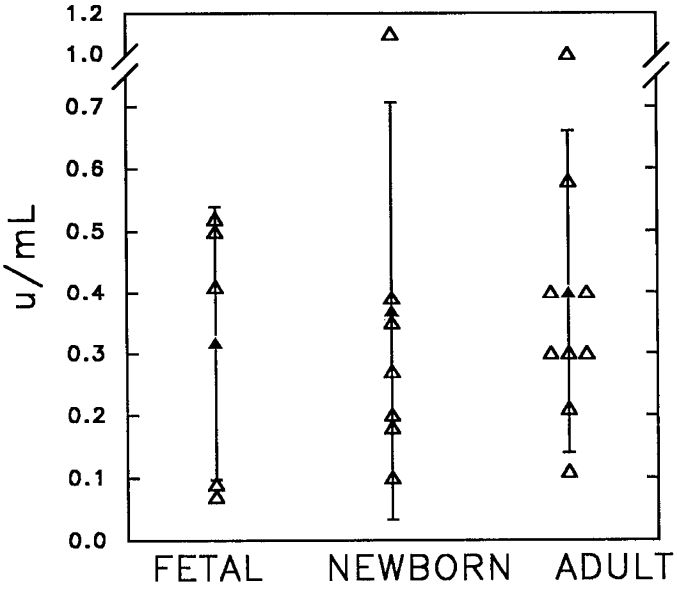

Fig. 2. Ep ½ max for growth of blood BFU-E-derived colonies from fetuses, newborn infants, and adults. The Ep ½ max was obtained from corresponding Ep dose-response curves. *Open symbols* represent individual experiments; *solid symbols* represent the mean ± 1 SD.

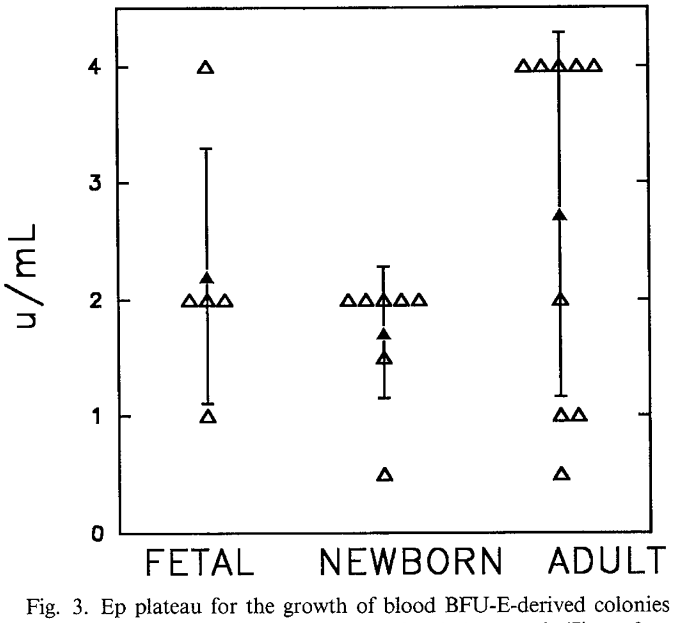

Fig. 3. Ep plateau for the growth of blood BFU-E-derived colonies from fetuses, newborn infants, and adults. Symbols are as in Figure 2.

those stages contains fetal and adult progenitor cells (13); the fetal progenitors grew at Ep concentrations ≤0.1 U/mL.

Time course. Many laboratories assay blood BFU-E at a single time point, d 13 or 14 of culture (5, 7, 12). We examined cultures at 3- to 4-d intervals from d 9 to d 20, and we noted that less than half of all cultures reached their maximal growth potential by d 13. We therefore present our data on d 13 for comparison with other laboratories and also our data on the day of peak growth to demonstrate the maximum growth potential. The day of peak growth was earliest for fetal blood cultures and later for newborn and adult cultures, which were similar to each other.

Growth potential. The number of colonies/100 000 cells was greatest (2.5 times newborn and 10 times adult) in fetal, intermediate in newborn (three times adult), and lowest in adult cultures. Although the total number of each type of colony/100 000 cells increased by the peak day, the relative frequency between groups remained similar. The number of colonies/100 000 cells previously reported for fetal (7–9), newborn (3, 5, 6, 8, 13, 17, 20–23), and adult (5, 6, 8, 12, 18, 20, 24–27) blood cultures were 40–500, 9–220, and 2–256, respectively. Our values fall within the ranges reported by others. However, careful inspection of the literature fails to adequately demonstrate the dramatic differences that actually exist between ontogenic classes of progenitor cells. These differences become more apparent when all ontogenic stages are compared in the same laboratory with the same techniques and reagents as in our studies. Our data are also presented as the number of BFU-E/mL in the circulation to provide a more physiologic comparison. Fetal and newborn BFU-E/mL were similar to each other, and both were significantly more numerous than adult BFU-E/mL on d 13 and on the peak day (>3000/mL *vs* approximately 300/mL). The relatively high numbers of BFU-E/mL in fetuses and newborn

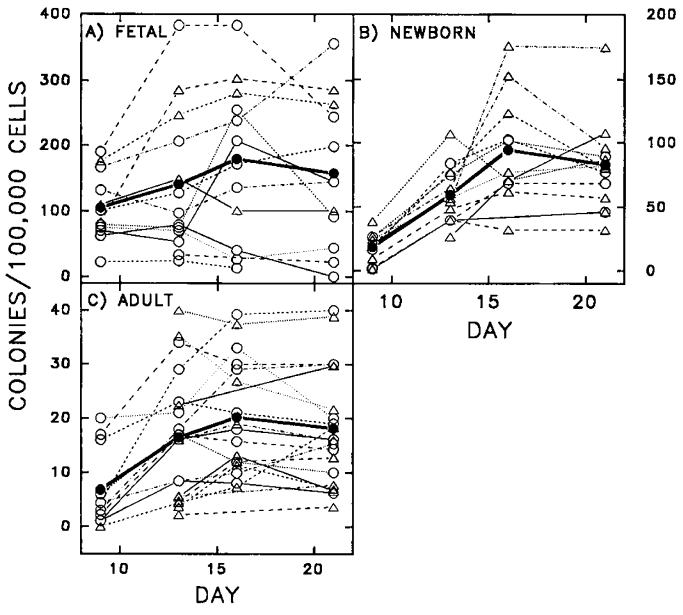

Fig. 4. Time courses of blood BFU-E-derived colony growth from fetuses, newborn infants, and adults, showing numbers of colonies/100 000 MNC plated. Data were normalized as the percentage of maximal growth. Each *curve* with *open symbols* represents a different donor, and each *point* is the mean of triplicate cultures. The curve with *solid symbols* represents the mean of all curves.

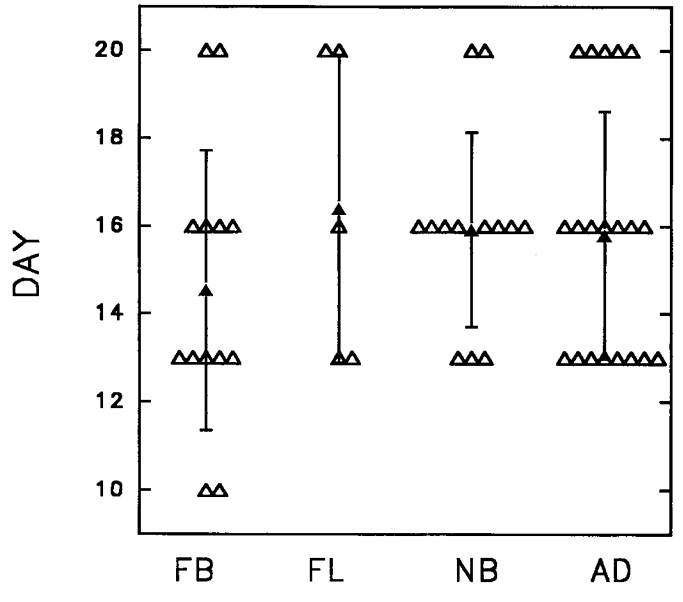

Fig. 5. Day of maximal BFU-E-derived colony growth from fetal liver (*FL*) and blood from fetuses (*FB*), newborn infants (*NB*), and adults (*AD*). Symbols are as in Figure 2.

infants may reflect the continuous expansion of the erythropoietic pool in rapidly growing fetuses and newborn infants. It may also be a compensatory response to the physiologic stresses associated with delivery. In contrast, normal adults are generally in an erythropoietic steady state.

Globin synthesis. Relative γ-globin synthesis on d 13 was 2-fold greater in fetal (82%) than newborn cultures (44%), and 3-fold greater in newborn than adult cultures (13%). In cultures from all three ontogenic classes of progenitors, a temporal decline in the proportion of γ-globin synthesis was observed, as reported previously (8, 28, 29). The proportion of γ-globin that was $^{G}\gamma$ also decreased with ontogenic stage.

In summary, erythropoiesis in cultures of fetal blood BFU-E are the most sensitive to Ep, and develop into colonies earliest.

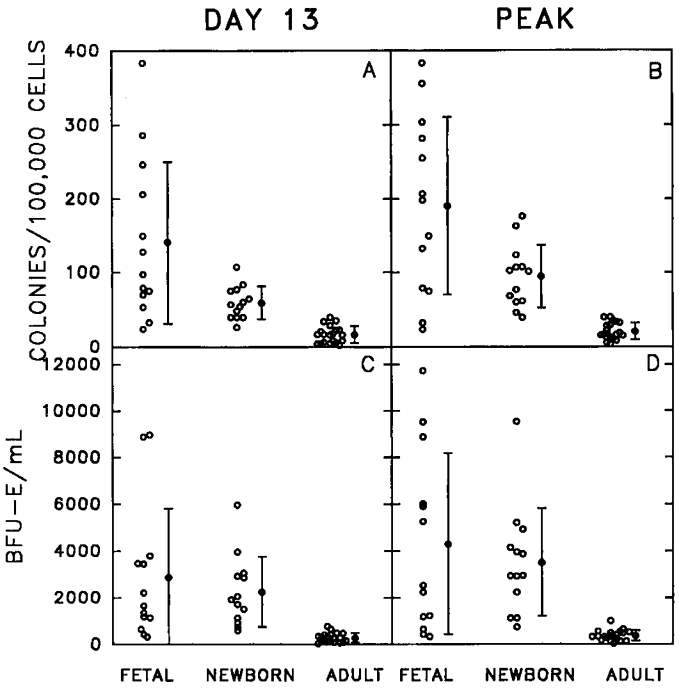

Fig. 6. Growth potential of blood erythroid progenitors from fetuses, newborn infants, and adults. *A* and *C* are data from d 13; *B* and *D* are data from the day of peak growth. In *A* and *B*, data are expressed as colonies/100 000 cells plated. In *C* and *D*, data are expressed as BFU-E/mL of blood. Symbols are as in Figure 2.

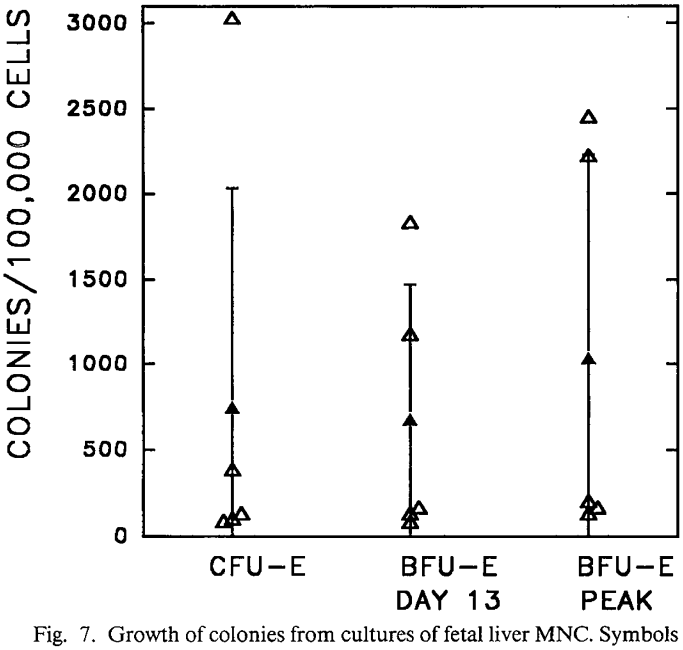

Fig. 7. Growth of colonies from cultures of fetal liver MNC. Symbols are as in Figure 2.

The fetal colonies are the most numerous and synthesize the highest proportion of γ-globin. Cultures from newborn infants are less sensitive to Ep, contain an intermediate quantity of colonies, and their colonies synthesize an intermediate proportion of γ-globin. Adult cultures exhibit Ep sensitivity that is similar to newborn cultures; their colonies are fewest in number and synthesize the lowest proportion of γ-globin. We conclude that erythropoiesis is unique at each stage of ontogeny.

Our cultures used total MNC, in media-containing sera. We thus cannot discriminate between ontogenic uniqueness of BFU-E per se and differences in accessory cells (monocytes and lymphocytes) in the MNC fraction. The composition of the

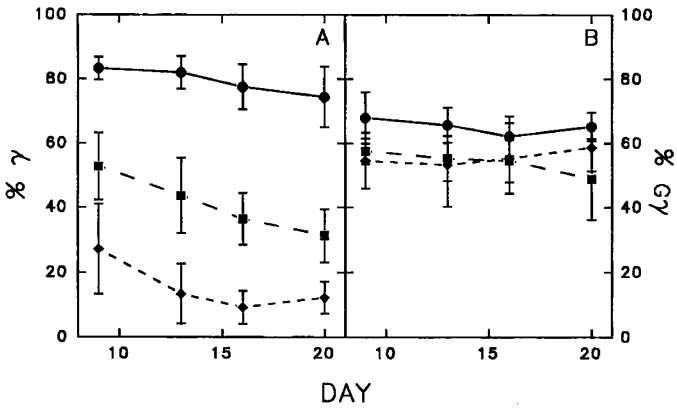

Fig. 8. Time courses of globin synthesis by blood BFU-E-derived colonies from 11 fetuses (●), 10 newborn infants (■), and 25 adults (♦). Data shown are the mean ± 1 SD. *A*, % $\gamma = 100 \times \gamma/(\gamma + \beta)$; *B*, $\%^{G}\gamma = 100 \times {}^{G}\gamma + {}^{A}\gamma)$.

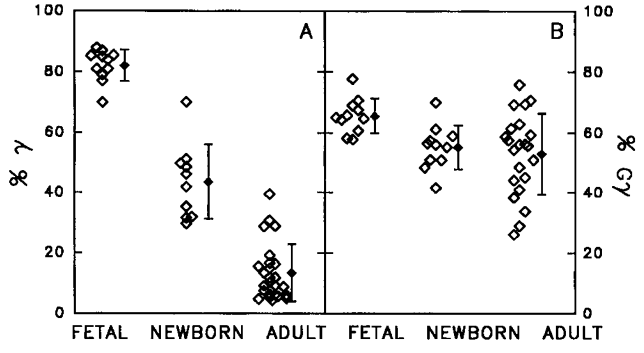

Fig. 9. Globin synthesis by d 13 blood BFU-E-derived colonies from individual fetuses, newborn infants, and adults. Symbols as in Figure 2. *A*, % γ; *B*, % $^{G}\gamma$.

accessory cells certainly changes during ontogeny, and the production of hematopoietic growth factors by those accessory cells may also change. Our previous results suggested that the BFU-E themselves were different during ontogeny [with regard to the relation between the percentage of $^{G}\gamma$ of $^{A}\gamma + {}^{G}\gamma$ and the percentage of γ of $\gamma + \beta$ synthesis (13)], but those conclusions were also inferential. In fact, we know from blood differential counts that fetuses have fewer monocytes and granulocytes than newborns or adults (30, 31). We have also found that removal of adherent cells (*i.e.* monocytes) removes only 20% of fetal MNC compared with 50% of newborn and adult MNC (13, and unpublished data).

The results reported in this paper indicate that the phenotype of erythropoiesis changes during ontogeny. The present data do not distinguish between ontogenic changes in BFU-E and in their milieu. The next phase will require removal of accessory cells, purification of erythroid progenitors, and addition of specific defined hematopoietic growth factors to define more specifically the components responsible for the ontogenic differences. Definition of those components may permit identification of manipulations that might enhance erythropoiesis in anemia or reactivate fetal Hb production for hemoglobinopathies.

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