Human Embryonic Hemopoiesis

Kinetics of Progenitors and Precursors Underlying the Yolk Sac → Liver Transition

G. Migliaccio, A. R. Migliaccio, S. Petti, F. Mavilio, G. Russo*, D. Lazzaro‡, U. Testa, M. Marinucci, and C. Peschle

*Department of Hematology, Istituto Superiore di Sanità, Rome; *Division of Obstetrics and Gynecology, Ospedale Civile di Avellino, Avellino, Italy; and ‡Istituto Patologia Generale, University of Rome "La Sapienza", Rome, Italy*

Abstract

Human embryonic development involves transition from yolk sac (YS) to liver (L) hemopoiesis. We report the identification of pluripotent, erythroid, and granulo-macrophage progenitors in YS, L, and blood from human embryos. Furthermore, comprehensive studies are presented on the number of hemopoietic progenitors and precursors, as well as of other cell types, in YS, L, and blood at precisely sequential stages in embryos and early fetuses (i.e., at 4.5–8 wk and 9–10 wk postconception, respectively). Our results provide circumstantial support to a monoclonal hypothesis for human embryonic hemopoiesis, based on migration of stem and early progenitor cells from a generation site (YS) to a colonization site (L) via circulating blood. The YS → L transition is associated with development of the differentiation program in proliferating stem cells: their erythroid progeny shows, therefore, parallel switches of multiple parameters, e.g., morphology (megaloblasts → macrocytes) and globin expression ($\zeta \rightarrow \alpha$, $\epsilon \rightarrow \gamma$).

Introduction

Human hemopoiesis is sustained by a pool of pluripotent stem cells, which are capable of both extensive self-renewal and differentiation into progenitors committed to the various hemopoietic lineages (1–3). In semisolid cultures, pluripotent progenitors from human adult marrow (4) and fetal liver (FL)[1] (5) give rise to mixed colonies, and are hence termed CFU-GEMM (colony-forming unit(s), granulocytic, erythrocytic, macrophage-monocytic, megakaryocytic). At least two erythroid progenitors have been identified in adult (6–8) and fetal (9–10) age, the erythroid burst-forming unit(s) (BFU-E), and the colony-forming unit(s) (CFU-E), which generate, respectively, large "bursts" and small clusters of erythroblasts. BFU-E represent early progenitors, closely deriving from the pluripotent compartment (11). They differentiate into late progenitors (CFU-E), which in turn feed into the erythroblastic pool. Granulo-macrophage progenitors (CFU-GM), identified in both adult (12) and fetal mammals (5), may be similarly subclassified as early and late progenitors (13).

The complex mechanisms controlling proliferation and differentiation of stem and progenitor cells in mammals are still debated. In both humans and mice a variety of glycoprotein positive regulators, produced by the T lymphocyte-macrophage complex, have recently been purified and the corresponding genes have been cloned (14, 15). In adult humans, in vitro formation of mixed colonies is induced by the "pluripotent" colony stimulating factor (CSF) (16), while BFU-E proliferation is apparently modulated by erythroid-potentiating factor or activity (EPA) (17–18). Erythropoietin (Ep) (19, 20) mainly modulates differentiation of CFU-E in vitro and in vivo (3, 21). In both human and murine cultures, granulocyte-macrophage (GM) proliferation is controlled by three types of CSF, termed GM-CSF, G-CSF, and M-CSF, respectively (14–15).

Ontogenic development of the human hemopoietic system involves a series of coordinated changes in embryonic and early fetal life (i.e., at 3–8 wk and 9–10 wk postconception, respectively) (22, 23). At 3–4 wk the first generation of hemopoietic cells proliferates in yolk sac (YS) and extraembryonic mesenchyme. These elements consist largely of "primitive" erythroblasts (megaloblasts), which are present in circulating blood from 4 wk onward. At 5–6 wk the YS is replaced by the liver (L) as the main hemopoietic site. In L parenchyma, "definitive" erythropoietic cells massively proliferate, thus giving rise to enucleated macrocytes. The definitive series enter into the bloodstream from 8 wk onward, thus gradually replacing circulating megaloblasts.

Although the general outline of these developmental phenomena has been established, their systematic analysis at precisely sequential stages has not been possible so far (see below). In particular, the kinetics of embryonic hemopoietic precursors have not been described in quantitative terms. More important, data on human embryonic stem and progenitor cells are virtually nonexistent so far.

We report the identification and the characterization of pluripotent, erythroid, and GM progenitors in human embryos. Detailed studies are presented on the time course of hemopoietic progenitors and differentiated precursors in YS, L, and blood at precisely sequential stages in the 5–10-wk postconception period.

Methods

Hemopoietic organs in embryos and fetuses. Our understanding of human embryonic hemopoiesis has been severely hampered so far by a variety of technical limitations. Human embryos have been scarce, particularly in the crucial 4–6-wk postconception period. Furthermore, it was difficult to obtain and process the embryos within a few hours of the abortion.

Address reprint requests and correspondence to Dr. Peschle, Dept. of Hematology, Istituto Superiore di Sanita, V. le Regina Elena, 299-00161 Rome, Italy.

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1. *Abbreviations used in this paper:* BFU-E, erythroid burst-forming unit(s); CFU-E, erythroid colony-forming unit(s); CFU-GEMM, colony-forming unit(s), granulocytic, erythrocytic, macrophagemonocytic, megakaryocytic; CFU-GM, colony-forming unit(s), granulo-macrophage; CRL, crown-rump length; CSF, colony stimulating factor; Ep, erythropoietin; EPA, erythroid-potentiating factor; FL, fetal liver; GM, granulocyte-macrophage; IMDM, Iscove's modified Dulbecco's medium; L, liver; NASDCA, napthtol AS-D chloroacetate esterase; YS, yolk sac.

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Finally, the embryos were rarely intact: hence, they could not be precisely dated, while the hemopoietic tissues were often contaminated by bacteria.

In an attempt to overcome these limitations, we have analyzed hemopoiesis in YS, L, and blood from 41 embryos and eight fetuses, obtained virtually intact by legal curettage abortions at 4.5–8 wk and 9–10 wk, respectively (24, 25, and Table I). After the abortion, all specimens were immediately washed, maintained in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Grand Island, NY) at +4°C, and finally processed within 3–6 h after the abortion. Hemopoietic organs (YS, L) were unequivocally recognized and dissected under the microscope in sterile conditions. Circulating blood was obtained by aspiration from heart and/or umbilical cord veins, free from any maternal blood contamination.

YS was obtained in 4.5-wk, 5-wk, and rarely 6-wk specimens, peripheral blood free of maternal contamination only from 5 wk onward. In the smallest embryos (end of the fourth week, stage 13, crown-rump length [CRL] 5–6 mm, see Table I) the L is constituted by a mere rudiment.

"Normal" bone marrow was obtained from 19–40-yr-old hematology-oncology patients without apparent marrow involvement, with seemingly normal hemopoiesis, and was obtained prior to initiation of treatment.

Fully informed consent had been obtained in advance from the mothers and the adult volunteers.

Cell preparation. A monocellular suspension from YS and L in IMDM, supplemented with bovine serum albumin (1.5%) (wt/vol) (Fraction V; Sigma Chemical Co., St. Louis, MO), was obtained by gently passing cells through progressively smaller needles down to No. 25. In the final suspension, viable cells (trypan blue staining) were always >95%, while cellular debris were not present. Progenitor cells were not purified, in order to avoid their partial loss.

Morphology studies. Cellular suspensions obtained from embryonic L were cytocentrifuged and stained by the conventional May-Grünwald Giemsa method. Cytochemical reactions included periodic acid Schiff, napthtol AS-D chloroacetate esterase (NASDCA), and Graham-Knoll peroxidase.

Histologic preparations of freshly dissected L were embedded in Epon Resin 812 (Fisher Scientific Co., Pittsburgh, PA); semithin sections (1.5 μm) were used for both light and transmission electron microscopy (TEM) according to standard procedures. In the time course studies by light microscopy, of each section, >500 cells were identified and counted in both peripheral-marginal and central area (at least five sections were examined for each specimen).

Table I.

	Age	CRL	Developmental stage	Analyzed tissues	Number of specimens
	wk	*mm*			
	Embryos 4–5	5–6	13	Yolk sac, liver rudiment	5
	5	7–9	14, 15	Yolk sac, liver, blood	6
	6	10–12	16	Liver, blood	11
	7	13–17	17	Liver, blood	8
	8	18–30	19–22	Liver, blood	11
	Fetuses 9	30–50	—	Liver, blood	5
	10–12	>50	—	Liver, blood	3

Cell culture. Each 1-ml plate contained the following components in IMDM (26): methylcellulose (0.8%, final concentration), α-thioglycerol (10^{-4} M), a selected heat-inactivated fetal calf serum (FCS; batch No. 2919130; Flow Laboratories, Glasgow, Great Britain) (40%), Ep (specific activity, 1,140 IU/mg of protein, generously provided by National Institutes of Health, Bethesda, MD), and nucleated cells ($0.5–3 \times 10^5$, in order to avoid overcrowded cultures). Each point was assayed at least in duplicate. The plates, incubated in a humidified 5% CO_2 in air atmosphere at 37°C, were removed from the incubator and examined up to three different times.

The culture conditions allowed optimal growth of erythroid and GM progenitors from FL, adult marrow, or blood. In particular, semipurified exogenous Ep was added at plateau levels (2–3 IU/plate). In this regard, the Ep dose-response curve of embryonic-fetal erythroid progenitors (results not shown here) is virtually superimposable to that of BFU-E and CFU-E from older fetuses (10). EPA and CSFs were present in the selected FCS batch at levels allowing peak growth of corresponding adult and fetal progenitors (results not presented here). The culture conditions also allowed growth of pluripotent progenitors.

Erythroid colonies were recognized in situ on the basis of their orange-red color. This was confirmed by control studies, whereby colonies were picked up, cytocentrifuged, and stained with May-Grünwald + Giemsa. Pure GM and mixed colonies were identified in situ (see 4), and the scoring criteria confirmed by control experiments on cytocentrifuged cells (not presented here).

Results

Total cell number in hemopoietic organs. In the 5–10-wk period, the L size undergoes a dramatic increase, associated with an

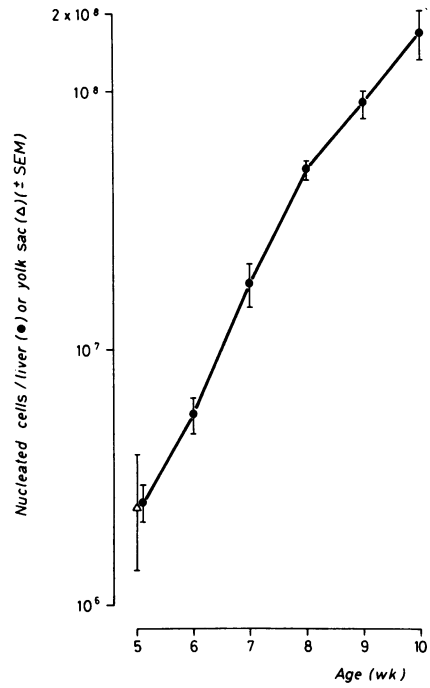

Figure 1. Time course of nucleated cells in L from human embryos (5–8 wk) and fetuses (9–10 wk) (mean±SEM values from 3–6 specimens per point). Corresponding values in YS from 5-wk embryos are also included. YS and L were dissected under the microscope and resuspended in IMDM supplemented with 5% FCS. Monocellular suspensions were obtained by carefully flushing the cells through progressively smaller needles down to No. 25. For further details see experimental procedures.

exponential rise of the total nucleated cell number from mean values of 2.3×10^6 to 1.7×10^8 (Fig. 1). Corresponding values in 5-wk YS are also shown (Fig. 1).

Identification and characterization of colonies generated by embryonic progenitors. In semisolid cultures of human FL, pluripotent (CFU-GEMM), early (BFU-E), or late (CFU-E) erythroid and GM (CFU-GM) progenitors generate specific types of colonies (5, 10). In the present studies, methylcellulose cultures of embryonic YS, L, and blood similarly gave rise to colonies of CFU-E, BFU-E, CFU-GM, and CFU-GEMM type. The colonies are assumed to be clonal, as demonstrated for corresponding adult aggregates, provided the plates are not overcrowded (27, 28). This postulate is in line with observations on cell dose-response regression curves (see below).

The morphology of embryonic CFU-E clusters, as well as BFU-E, CFU-GM, and CFU-GEMM colonies (not shown), was equivalent to that of corresponding fetal and adult aggregates. In regard to their size, CFU-E clusters comprise 20–150 cells, BFU-E and CFU-GEMM colonies comprise $6–8 \times 10^2$ to $4–6 \times 10^3$ elements, and CFU-GM aggregates include $1–2 \times 10^2$ to $2–3 \times 10^3$ cells.

The time-growth curve (not shown here) of embryonic colonies is virtually identical to that of corresponding fetal ones: CFU-E, BFU-E, CFU-GM, and CFU-GEMM derived clones reach peak values at 4, 10, 9–11, and 12 days, respectively. Their growth is therefore accelerated as compared to that of corresponding adult colonies (10).

Cell dose-response curves for embryonic progenitors from YS, L, and blood. The cell dose-response regression curves of CFU-E, BFU-E, and CFU-GM in YS and L from 5-wk embryos are shown in Fig. 2 (results on blood progenitors not included). All curves are linear through the origin, which indicates a lack of significant cell/cell cooperation in the explored cell concentrations (2.5×10^4 to 4×10^5 cells/plate). Similar results were obtained at later ontogenic stages (not shown, and 10). This pattern suggests clonality of these colonies, and confirms presence of plateau levels of hemopoietins in the culture medium. Therefore, in vitro assay of hemopoietic progenitors apparently allows reliable evaluation of their kinetics in vivo.

The low number of CFU-GEMM did not permit us to evaluate their cell dose/response curve, thus also rendering uncertain analysis of their in vivo dynamics.

Time course of the number of hemopoietic progenitors in 4.5–12-wk embryos and fetuses. At the end of the fourth week (i.e., 4.5-wk embryos) BFU-E are abundant in YS, but almost undetectable in L cell suspensions. Since hepatic sinusoids are prominent at this stage, the BFU-E number is presumably very low in circulating blood. CFU-E are numerous in YS but totally absent in L, and hence admittedly also in blood. The dynamics of CFU-GM are similar to that of BFU-E.

5-wk embryos. BFU-E are present in YS, blood, and L. At the YS level, their number becomes dramatically lower than at

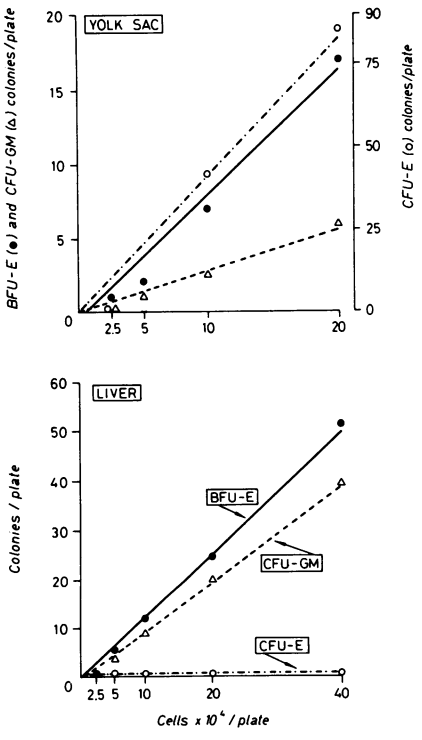

Figure 2. Cell dose-response regression curves of hemopoietic progenitors (BFU-E, CFU-E, and CFU-GM) from YS and L of 5–6-wk-old embryos (mean values from three different specimens: SEM were always <10% of corresponding mean values). Regression analysis: $P < 0.01$ for all curves. See also legend of Fig. 1 and text.

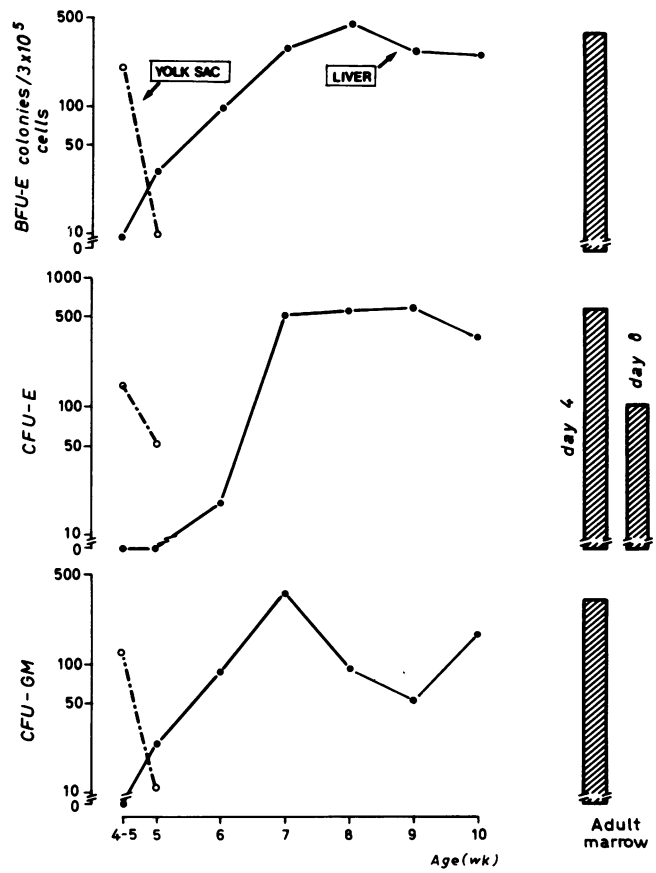

Figure 3. Time course of the number of hemopoietic progenitors in 5–12-wk embryos and fetuses, and corresponding values for progenitors in adult marrow, grown in the same culture conditions (mean values from 4–6 different specimens: SEM were always <10% of corresponding mean values). Day 4 CFU-E from adult marrow have been described by Ogawa et al. (8) and recently termed high Ep sensitive cells (HESC) (42). In 5-wk blood the number of BFU-E and CFU-GM progenitors was equivalent to that observed in YS, while CFU-E could not be detected. See also legend of Fig. 1 and text.

4.5 wk. Conversely, at the L level the BFU-E concentration becomes sharply more elevated than at 4.5 wk. Also note that the BFU-E concentration is distinctly more elevated in L than in blood: this difference indicates that BFU-E are largely located in the hepatic parenchyma rather than in sinusoids. Here again, the kinetics of CFU-GM are similar to those of BFU-E. As for CFU-E, they are still relatively abundant in YS, as compared with YS BFU-E. Conversely, they are virtually absent in L and blood. In this last regard, embryonic CFU-E apparently do not circulate in blood (results not shown), as also observed in fetal (29), neonatal (30), and adult life (8). It may be added that differentiated erythroid precursors are present in embryonic (23), fetal, and neonatal blood (31).

Later stages of development (Fig. 3). The concentration of BFU-E in L undergoes a rapid rise, up to plateau values from 8–9 wk onward. Interestingly, the saturation levels are similar to those observed in adult marrow under the same culture conditions. CFU-E are first detected in L at 6 wk. Their hepatic concentration shows a dramatic rise at 7 wk, followed by a plateau from 8 wk onward. Once again, saturation values are similar to those observed for day 4 CFU-E in adult marrow (also see legend of Fig. 3). CFU-GM dynamics are comparable to those of BFU-E, except for a possible decline in their relative number at 8–9 wk.

Cell dynamics in 5–10-wk L: a comprehensive analysis. As previously mentioned, 5-wk L essentially comprises megaloblasts in sinusoids, as well as blasts, mesenchymal, and hepatocytic cells in parenchyma (Fig. 4 *A*). In particular, 5-wk L is histologically characterized by cordonal formations of epithelial cells developing into mesenchymal tissue deriving from the septum transversum: in the contact areas the mesenchyme gives rise to the primitive system of L sinusoids, while the epithelial cords rapidly develop around them in an interdigitating pattern (Fig. 4 *A*). Numerous macrophages are observed at 5–6 wk, partic-

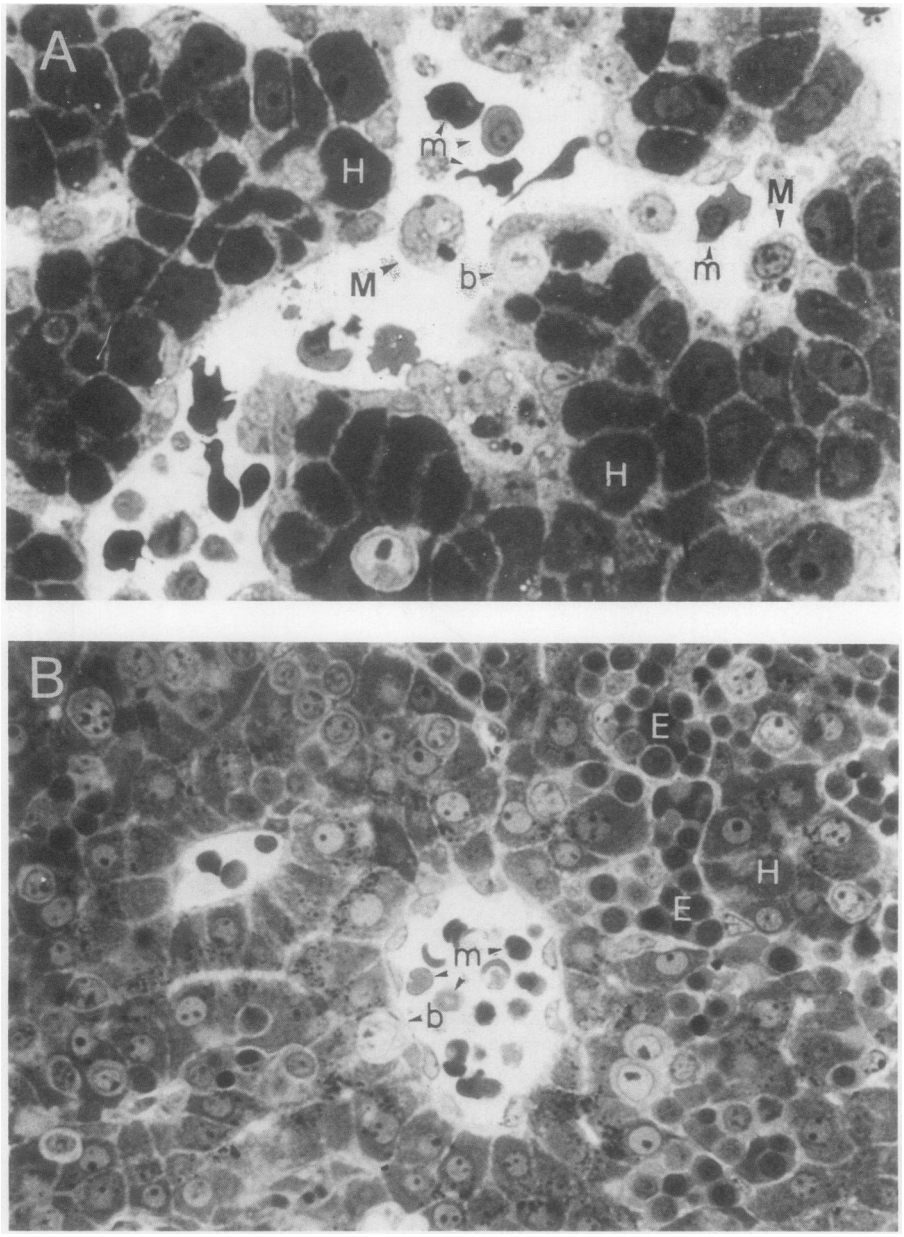

Figure 4. (*A*) Section of a 5-wk L stained with toluidine blue (× 600). (*B*) Section of a 7-wk L stained as in (*A*) (× 300). Symbols: *b*, blast cell; *E*, definitive erythroblasts organized in nests; *H*, hepatocyte; *M*, circulating or adherent Kupffer-like macrophages; *m*, megaloblasts.

ularly within the sinusoidal wall (Fig. 4 *A*). At 6–8 wk, discrete nests of definitive erythroblasts appear and then rapidly expand in parenchyma, while megaloblasts are still observed in sinusoids (Fig. 4 *B*).

The time course of L blasts and hemopoietic cells has been first evaluated in cytocentrifuged smears (Fig. 5 *A*) (this technique does not allow recognition of cytocentrifuged and hence clumped-damaged hepatocytes and mesenchymal elements). It is thereby apparent that the wave of definitive erythroid differentiation, initiating at the end of the fifth week, is associated with an inverse decline of blastlike cells. Some blasts may represent pro-erythroblasts at an early stage of differentiation, and thus, may be difficult to recognize (see below). As expected, immature erythroblasts peak earlier than mature ones (Fig. 5 *B*). In the final steady state condition, the relative ratio of pro-erythroblasts, immature and mature erythroid cells is similar to corresponding values in adult marrow (Fig. 5 *B*). A discrete number of differentiated granulo-monocytic cells is clearly observed at the end of 4 wk and at 5 wk, but becomes negligible at later stages, due to the overwhelming erythroid proliferation (Fig. 5 *A*). Identification of granulocytic elements was confirmed by NASDCA and peroxidase reactions (see Methods, and results not shown here), as well as electron microscopy (not shown). Rare megakaryocytes are observed throughout the 5–10-wk period (not shown here).

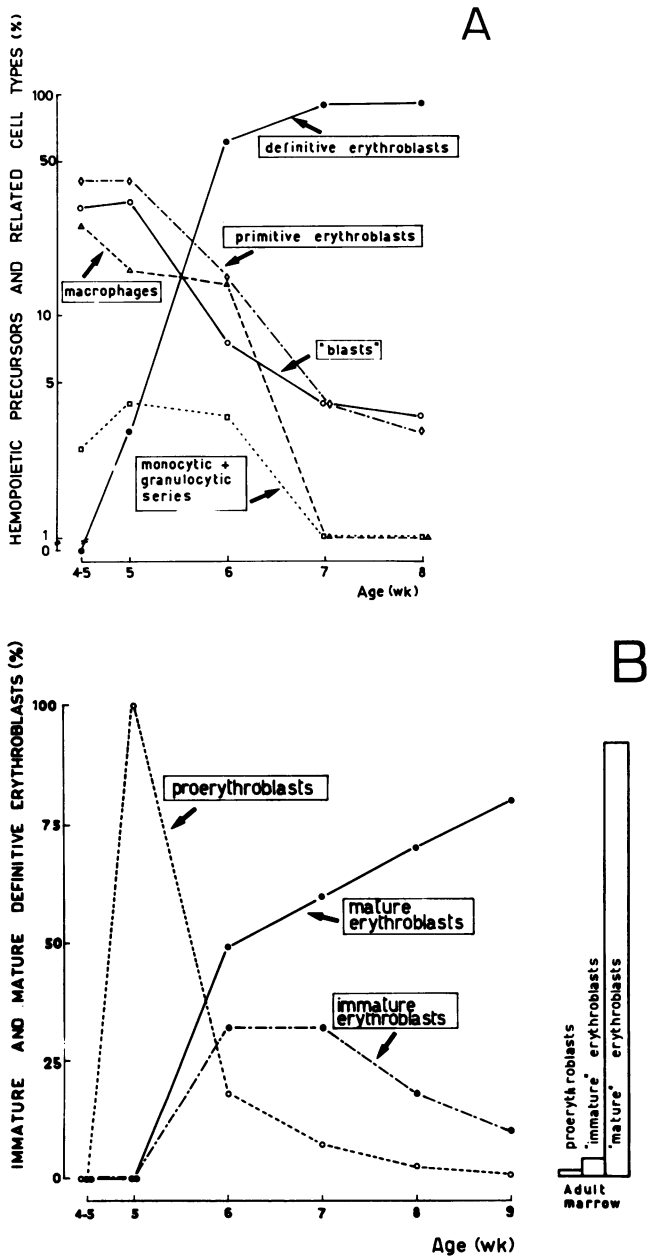

Figure 5. Time course of hemopoietic precursors (*A*) and definitive erythroblasts (*B*) in embryonic-fetal L (percent values from a minimum of three specimens per point). Cellular suspensions, obtained as described in the legend of Fig. 1, were cytocentrifuged and stained by the conventional May-Grünwald + Giemsa method.

We then attempted to provide a comprehensive analysis of cell dynamics in 4.5–10-wk developing L. Results on progenitors, derived from clonogenic assays (see above), were plotted together with those on hemopoietic precursors and other cell types, obtained by analysis of tissue sections (see Methods).

All values were expressed in terms of the total number of each type of cell in L: this was calculated on the basis of both the relative frequency of each element (in L cultures [Fig. 3] or tissue sections [data not shown]) and the total number of L nucleated cells (Fig. 1).

The identity of granulo-monocytic precursors cells could hardly be established in sections. Since the mean relative frequency of other precursors in the overall hemopoietic population was virtually identical in both sections and smears, the number of GM elements was extrapolated from smear values. Furthermore, the L histology is not homogenous at the end of 4 wk and at 5 wk. In the latter stage the central part is highly vascularized, while in peripheral portions mesenchymal cells are distinctly prevalent; the data reported below refer to mean values from sections through both central and peripheral structures.

Although different L specimens yielded homogenous results, the calculated mean values are admittedly only indicative. Nevertheless, the marked fluctuations observed for the majority of cell types render these values of considerable significance, and clearly identify time-course patterns specific for each cell type.

The impressive wave of definitive erythropoiesis is clearly depicted in Fig. 6 *A*. Its timing is obviously in line with that observed in smears. As expected, the rise of the number of CFU-E parallels that of erythroblasts. BFU-E kinetics is characterized by an early 5–6-wk phase of rapid increase without detectable differentiation, and a secondary 7–10-wk period of slower rise, associated with massive erythroid differentiation.

The number of intrasinusoidal megaloblasts shows a considerable increase in the 5–6-wk period, presumably as a consequence of their production in YS. Later, the size of their intrahepatic pool is virtually at steady state level.

The number of blasts shows a gradual rise, slower than that of the total nucleated L cells. The physiological significance of these elements is uncertain. A small part thereof admittedly represents hemopoietic stem and early progenitor cells. The ultrastructural features of some blast elements (not shown here) are virtually identical with those described for purified murine stem cells (32). Other blasts may constitute early proerythroblasts, which are difficult to identify by conventional microscopy. In this regard, electron microscopy observations indicate that some blast cells are apparently more differentiated than other ones (not presented here). Those blast cells that are more differentiated are characterized by more prominent ribosomes and endoplasmic reticulum and a lower number of mitochondria, as

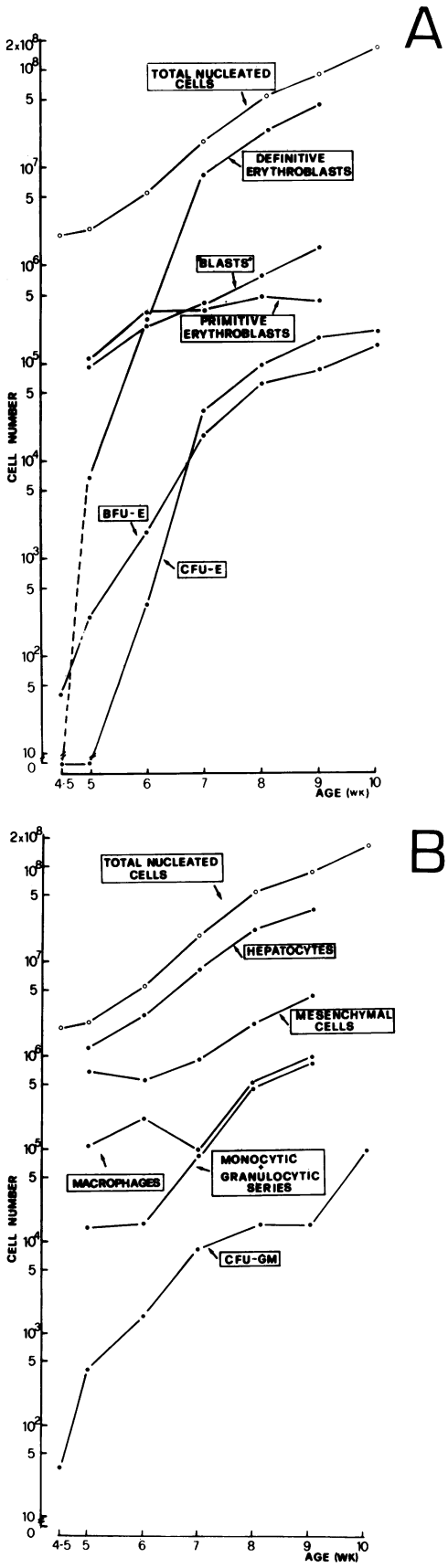

Figure 6. Time course of the number of: (*A*) erythroid progenitors (BFU-E, CFU-E) and precursors ("primitive" and "definitive" erythroblasts) in embryonic-fetal L; (*B*) granulo-monocytic progenitors (CFU-GM) and precursors (macrophages and granulo-monocytes), as well as hepatocytes and mesenchymal cells, in embryonic-fetal L.

The total number of progenitors has been calculated from their frequency values (Fig. 3) and the overall number of nucleated cells in L (Fig. 1). The total number of morphologically recognizable cells has been calculated from the corresponding percent values, as evaluated on hepatic sections (see Methods), and the total number of nucleated cells in L (Fig. 1). The identity of granulo-monocytic cells was difficult to establish in sections, and was thus extrapolated from corresponding smears. In this regard, the mean relative frequency of other precursors in the overall hemopoietic population was virtually identical in both sections and smears. For further details see text.

compared with the less differentiated blast cells. In view of the impressive wave of erythropoiesis in embryonic L, we suggest that the more differentiated blasts represent at least in part early pro-erythroblasts. In this regard, note that in steady state conditions (i.e., at 7–9 wk) the number of blasts falls neatly between the number of erythroblasts and the number of erythroid progenitors.

As mentioned above, some L blast cells presumably represent pluripotent and early progenitors. Hence, it is of interest that we observed all the sequential stages of the crossing of the L endothelial barrier by blast cells (i.e., adherence of blasts to the intraluminal side of the endothelial wall, gradual passage of them through this barrier, and location of blasts on the other side of the sinusoidal wall and within the epithelial cords) (Fig. 7 *A–D*). More important, 5-wk L sections often show preferential presence of blast cells around the lumen of developing sinusoids (Fig. 8). In later stages of development, blasts are randomly interspersed within the L parenchyma, without a preferential location around the sinusoids (not shown). In spite of its inherent limitations, this morphological analysis is compatible with active crossing of the sinusoidal wall by a large number of blast cells, prevailingly from blood into L parenchyma (but also vice versa, see Discussion), perhaps via chemotactic mechanisms (33).

The kinetics of other cell types are presented in Fig. 6 *B*. A discrete wave of granulo-monocytic differentiation is apparent (see also Fig. 5 *A*). It initiates at 5 wk, earlier than its erythroid counterpart. Later, it undergoes a relatively mild increase, which parallels that of the total L cell number. The amplification of the differentiated granulo-monocytic pool is thus markedly less prominent than that of erythroblasts, although the kinetics of BFU-E and CFU-GM are similar.

At 7–9 wk, the number of macrophages is comparable to that of granulo-monocytic elements. At 5–6 wk, however, the former parameter greatly exceeds the latter. This might suggest that, in this ontogenic stage, macrophages may be generated at least in part by mesenchymal cells, and/or CFU-GM undergo preferential differentiation in the macrophage pathway.

Finally, it is not surprising that the kinetics of hepatocytic and mesenchymal cells parallel those of total nucleated L elements.

Discussion

We report the identification and the partial characterization of pluripotent, erythroid, and GM progenitors in human embryos, as well as the fluctuations of the number of hemopoietic progenitors and precursors in hemopoietic organs at 4.5–10 wk after conception.

BFU-E, CFU-E, and CFU-GM have been identified in YS,

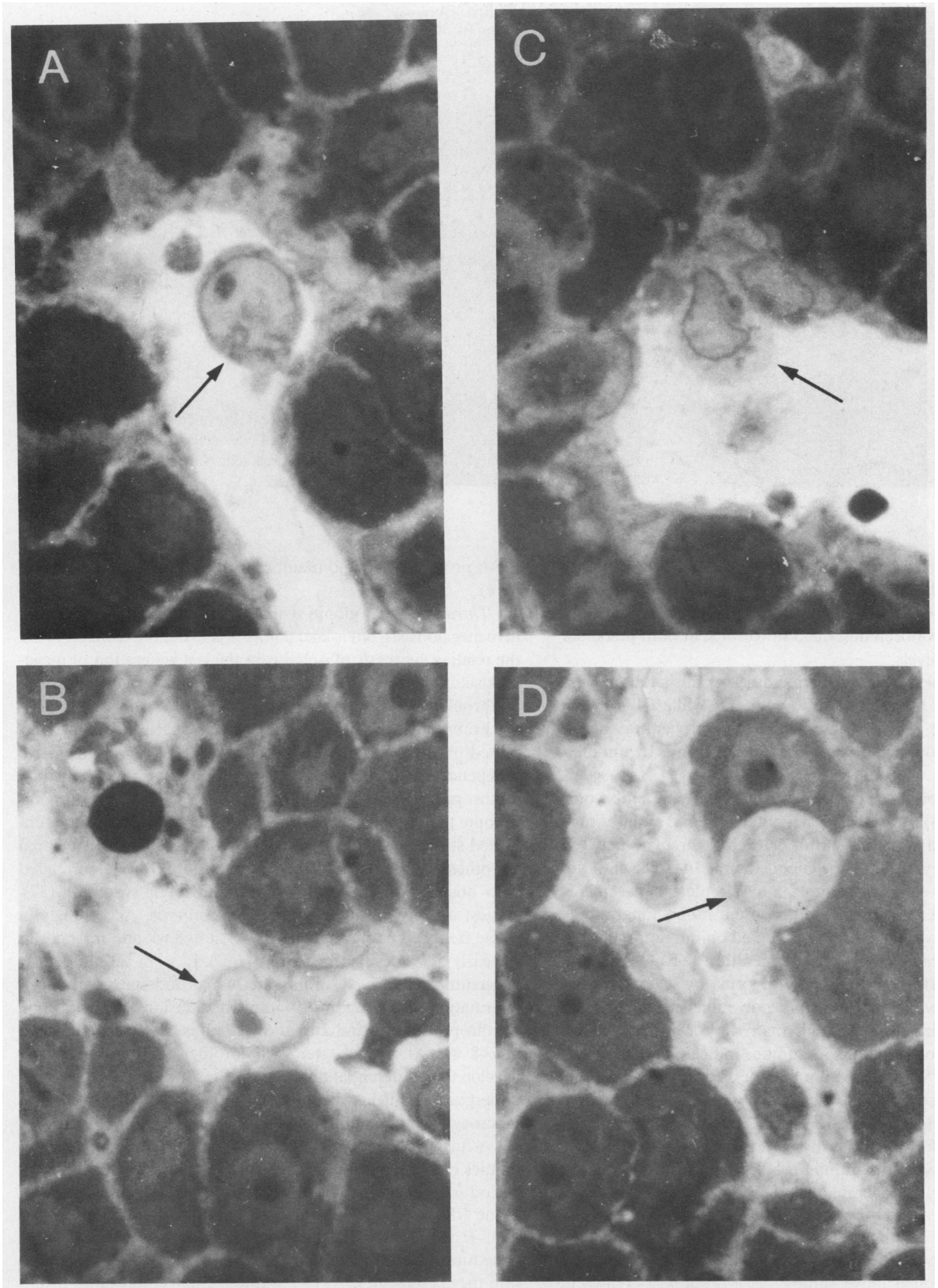

Figure 7. (*A–D*) Sequential phases of the crossing of a sinusoidal wall by a blast cell (indicated by the arrow) in 5-wk L (× 1,000). For further details see text.

L, and circulating blood by standard clonogenic cultures in semisolid medium. We have also noted mixed colonies generated by pluripotent progenitors (CFU-GEMM); however, it was difficult to analyze them because of their relatively low number. The clonogenic characteristics of the progenitors have been described above.

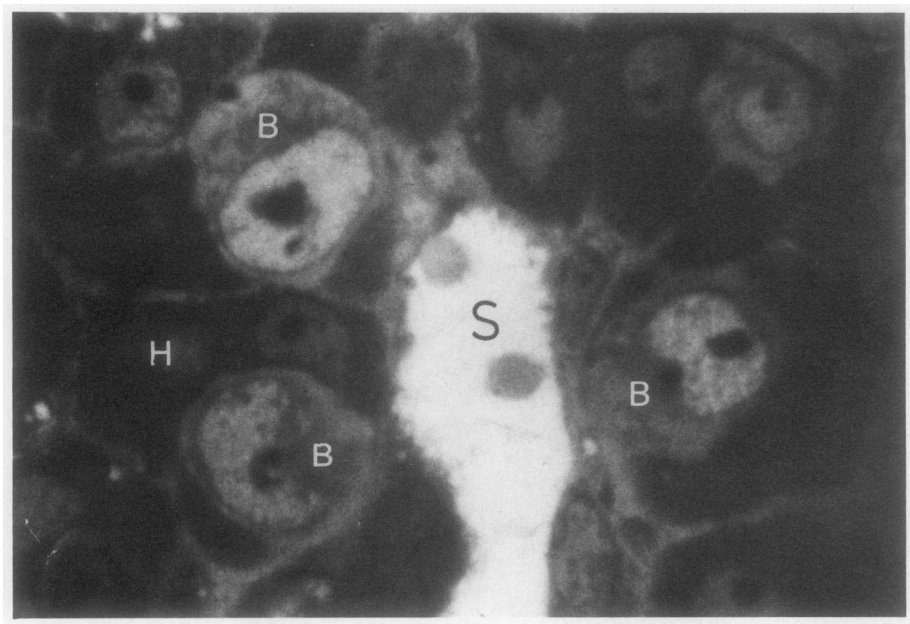

Figure 8. Prevalent perisinusoidal location of blast cells in 5-wk L (× 12,000). *B*, blast cell; *H*, hepatocyte; *S*, sinusoidal space.

Results reported here allow us to delineate the dynamics of hemopoietic cells in 4.5–8-wk embryos and 9–10-wk fetuses. The primary "megaloblastic" phase and the secondary "macrocytic" period have been described in Results, at both precursor and progenitor levels.

The early "megaloblastic" period deserves further discussion. In 5-wk L early progenitors are abundant, while CFU-E and erythroblasts are virtually absent. Do early progenitors derive from YS via blood, and/or are they generated in L by undifferentiated cells?

Cell migration is a key mechanism underlying embryonic development (33). In fetal (29), neonatal (30), and adult human life (8), stem and early progenitor cells cross-circulate between the hemopoietic sites and the bloodstream. In 5-wk embryos pluripotent and early progenitors may circulate between YS, blood, and L. In this regard, we propose a monoclonal model for human embryonic hemopoiesis, based on migration of stem and early progenitor cells from a generation (YS) to a colonization site (L). This hypothesis is supported by several lines of circumstantial evidence: *first,* studies in mice suggest that YS stem cells colonize the L parenchyma (34–36).

Second, our observations in humans indicate that: (*a*) in the fifth week, the YS BFU-E pool undergoes a dramatic depletion, while BFU-E become readily detectable in the bloodstream and their number undergoes a striking rise in L parenchyma. Depletion of YS BFU-E can hardly be attributed to their massive differentiation, since the number of YS CFU-E does not show a rise, but rather, a moderate decline. By implication, these observations are best interpreted in terms of massive YS → L migration of BFU-E (migration of YS CFU-E is seemingly excluded, since these progenitors are absent in 5-wk blood and L).

(*b*) As mentioned above, some L blasts presumably represent pluripotent and early progenitors. Morphological observations are compatible with active crossing of the sinusoidal wall by a large number of blast cells, prevailingly from blood into L parenchyma.

(*c*) Early progenitors in 5-wk YS, L, and blood exhibit identical features, in terms of clonogenetic characteristics, Hb synthesis program (26), and membrane antigen pattern (not shown here).

Third, the $\epsilon \rightarrow \gamma$ globin switch is gradually initiated in primitive megaloblasts, but is later completed in definitive macrocytes: the relative synthesis of ϵ-chains in the last generation of megaloblasts is slightly more elevated than in the earliest definitive erythroblasts (37).

Embryonic hemopoiesis in the avian species is often interpreted in terms of a biclonal model (38–39): accordingly, two independent differentiation events would generate two distinct erythropoietic lineages, deriving from YS or other intraembryonic tissues, particularly the L. In mammals, the aforementioned studies provide circumstantial support to a monoclonal hypothesis: particularly, the findings reported here, correlated with observations on embryonic hemoglobin switching (37), suggest a unifying model in human embryos. Accordingly, a single stem cell pool originates in YS at 3–5 wk and migrates in the early 5-wk developing L. In 5–6-wk L, stem cells and early progenitors intensively proliferate, while undergoing little differentiation, thus leading to rapid expansion of their pool. Erythropoiesis in L initiates at 6 wk and is massively enhanced at 7–8 wk. At 5–6 wk, stem cells proliferating in L gradually develop their differentiation program, i.e., their erythroid progeny undergoes parallel switches of morphology (megaloblasts → macrocytes) and globin synthesis ($\zeta \rightarrow \alpha$, $\epsilon \rightarrow \gamma$).

A further aspect worth discussion is represented by the dynamics of erythroid vs. GM cells in 5–8-wk L. The initial 5-wk period is characterized by a very rapid expansion of both BFU-E and CFU-GM pool. This is associated with virtual absence of CFU-E and erythroblasts, in contrast with the presence of a discrete number of differentiated granulo-monocytic elements and rare megakaryocytes. At 6–7 wk, appearance and rapid proliferation of CFU-E and erythroblasts are associated with an inverse relative decline of granulo-monocytic cells. The GM elements are apparently of hepatic origin. Indeed, it seems unlikely that they derive from YS, wherein they are virtually absent (23; and authors' observations). Furthermore, they are not observed in blood, while granulocytic elements at all stages of maturation

are present within L parenchyma at a distance from sinusoids (data not shown).

These observations clearly indicate that stem cells in L undergo a "differentiation switch". At 5 wk their intensive proliferation is associated with discrete GM differentiation. From 6–7 wk onward they are massively channeled into the erythroblastic pathway. The switch obviously may be attributed to intrinsic and/or extrinsic mechanism(s). In the avian model, switching from primitive to definitive erythropoiesis is possibly mediated by initiation of Ep release in the hemopoietic microenvironment (38). In mammals, fetal L cells produce Ep in vitro (40) and in vivo (20). In 5-wk human L, Ep production has not been observed so far in culture (results not shown here), which is in line with virtual absence of CFU-E in vivo (Fig. 3). Hence, it is suggested that in 6–7-wk L, initiation of Ep production may underlie the above-mentioned switch: studies are currently in progress in an attempt to verify this hypothesis. On the other hand, the relatively low GM differentiation in embryonic-fetal L may be related to the relatively low number of CSF receptors on CFU-GM therein, as observed in fetal mice (41).

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References

1. McCulloch, E. A. 1983. Stem cells in normal and leukemic hemopoiesis. *Blood.* 62:1–13.

2. Ogawa, M., P. N. Porter, and P. Nakahata. 1983. Renewal and commitment to differentiation of hemopoietic stem cells. *Blood.* 61:823–829.

3. Peschle, C., G. Migliaccio, A. R. Migliaccio, A. Covelli, A. Giuliani, F. Mavilio, and G. Mastroberardino. 1983. Hemoglobin switching in humans. *In* Current Concepts in Erythropoiesis. D. R. Dunn, editor. Wiley/London. 339–387.

4. Fauser, A. A., and H. A. Messner. 1979. Identification of megakaryocytes, macrophages and eosinophils in colonies of human bone marrow containing neutrophilic, granulocytes and erythroblasts. *Blood.* 53:1023–1027.

5. Hann, I. M., M. P. Bodger, and A. V. Hoffbrand. 1983. Development of pluripotent hematopoietic progenitor cells in the human fetus. *Blood.* 62:118–123.

6. Axelrad, A. A., D. L. Mcleod, M. M. Shreeve, and D. S. Heath. 1974. Properties of cells that produce erythrocytic colonies in vitro. *In* Hemopoiesis in Culture. W. A. Robinson, editor. Government Printing Office, Washington, DC. 226–234.

7. Gregory, C. J., A. D. Tepperman, E. A. McCulloch, and J. E. Till. 1974. Erythropoietic progenitors capable of colony formation in culture: response of normal and genetically anemic W/W^v mice to manipulations of the erythron. *J. Cell. Physiol.* 84:1–12.

8. Ogawa, M., O. C. Grush, R. F. O'Dell, H. Hara, and M. D. MacEachern. 1977. Circulating erythropoietic precursors assessed in culture: characterization in normal men and patients with hemoglobinopathies. *Blood.* 50:1081–1092.

9. Rowley, P. T., W. B. M. Ohlsson, and B. A. Farely. 1978. Erythroid colony formation from human fetal liver. *Proc. Natl. Acad. Sci. USA.* 75:984–988.

10. Peschle, C., A. R. Migliaccio, G. Migliaccio, R. Ciccariello, F. Lettieri, G. Quattrin, G. Russo, and G. Mastroberardino. 1981. Identification and characterization of three classes of erythroid progenitors in human fetal liver. *Blood.* 58:565–572.

11. Ferrero, D., M. Gabbianelli, C. Peschle, B. Lange, and G. Rovera. 1985. Surphace phenotypes of human progenitor cells defined by monoclonal antibodies. *Blood.* 66:496–502.

12. Metcalf, D. 1977. Hemopoietic colonies. In vitro cloning of normal and leukemic cells. Springer Verlag, Berlin.

13. Ferrero, D., H. E. Broxmeyer, G. L. Pagliardi, S. Venuta, B. Lange, S. Pesano, and G. Rovera. 1983. Antigenically distinct subpopulations of myeloid progenitor cells (CFU-GM) in human peripheral blood and marrow. *Proc. Natl. Acad. Sci. USA.* 80:4114–4118.

14. Dexter, T. M. 1984. The message in the medium. *Nature (Lond.).* 309:746–747.

15. Metcalf, D. 1985. The granulocyte-macrophage colony stimulating factors. *Science (Wash. DC).* 229:16–18.

16. Welte, K., E. Platzer, Li Lu, J. L. Gabrilove, E. Levi, R. Mertelsmann, and M. A. S. Moore. 1985. Purification and biochemical characterization of human pluripotent hematopoietic colony-stimulating factor. *Proc. Natl. Acad. Sci. USA.* 82:1526–1530.

17. Westbrook, C. A., J. C. Gasson, S. E. Gerber, M. E. Selsted, and D. W. Golde. 1984. Purification and characterization of human T-lymphocyte-derived erythroid-potentiating activity. *J. Biol. Chem.* 259:9992–9996.

18. Gasson, J. C., D. W. Golde, S. E. Kaufman, C. A. Westerbrook, R. M. Hewick, R. J. Kaufman, G. G. Wong, P. A. Temple, A. C. Leary, E. L. Brown, E. C. Orr, and S. C. Clark. 1985. Molecular characterization and expression of the gene encoding human erythroid-potentiating activity. *Nature (Lond.).* 315:768–771.

19. Miyake, T., C. K. Kung, and E. Goldwasser. 1977. Purification of human erythropoietin. *J. Biol. Chem.* 253:5558–5564.

20. Jacobs, K., C. Shoemaker, R. Rudersdorf, S. D. Neill, R. J. Kaufman, A. Mufson, J. Sechra, S. S. Jones, R. Hewick, F. E. Fritsch, M. Kawakita, T. Shimizu, and T. Miyake. 1985. Isolation and characterization of genomic and cDNA clones of human erythropoietin. *Nature (Lond.).* 313:806–810.

21. Eaves, C. J., R. K. Humphries, and A. C. Eaves. 1979. In vitro characterization of erythroid precursor cells and the erythropoietic differentiation process. *In* Cellular and Molecular Regulation of Hemoglobin Switching. G. Stamatoyannopoulos and A. W. Nienhuis, editors. Grune and Stratton, New York. 251–278.

22. Wood, W. G., J. B. Clegg, and D. J. Weatherall. 1977. Developmental biology of human hemoglobin. *In* Progress in Hematology, X. E. D. Brown, editor. Grune and Stratton, New York. 43–90.

23. Kelemen, E., W. Calvo, and T. M. Fliedner. 1979. Atlas of human hemopoietic development. Springer Verlag, Berlin.

24. Moore, K. L. 1982. The developing humans. Saunders, Philadelphia.

25. England, M. A. 1983. "Color atlas of life before birth". Yearbook Medical Publishers, Inc., Chicago.

26. Peschle, C., A. R. Migliaccio, G. Migliaccio, M. Petrini, M. Calandrini, G. Russo, G. Mastroberardino, M. Presta, A. M. Gianni, P. Comi, B. Giglioni, and S. Ottolenghi. 1984. Embryonic → fetal Hb switch in humans: studies on erythroid bursts generated by embryonic progenitors from yolk sac and liver. *Proc. Natl. Acad. Sci. USA.* 81:2416–2420.

27. Prchal, J. F., J. W. Adamson, L. Steinman, and P. J. Fialkow. 1976. The single cell origin of human erythroid colonies. *J. Cell. Physiol.* 89:489–492.

28. Lim, B., N. Jamal, D. Tritchler, and H. A. Messner. 1984. G6PD isoenzyme analysis of myeloid and lymphoid cells in human multilineage colonies. *Blood.* 63:1481–1487.

29. Linch, D. C., L. J. Knott, C. H. Rodeck, and E. R. Huehns. 1982. Studies of circulating hemopoietic progenitor cells in human fetal blood. *Blood.* 59:976–979.

30. Tchernia, G., F. Miclot, L. Coulomber, and M. Mohandas. 1981. Characterization of circulating erythroid progenitors in human newborn blood. *J. Lab. Clin. Med.* 97:322–331.

31. Williams, W. J., E. Beutler, A. J. Erslev, and M. A. Lichtman. 1983. *Hematology.* McGraw-Hill, Inc., New York. 365–376.

32. Van Bekkum, D. W., G. J. van den Engh, G. Wagemaker,

S. J. L. Bol, and J. W. M. Visser. 1979. Structural identity of the pluripotent hemopoietic stem cell. *Blood cells.* 5:143–159.

33. Le Douarin, N. M. 1984. Cell migration in embryos. *Cell.* 38: 353–360.

34. Moore, M. A. S., and D. Metcalf. 1970. Ontogeny of the haemopoietic system: yolk sac origin on *in vivo* and *in vitro* colony forming cells in the developing mouse embryos. *Br. J. Haematol.* 18:279–296.

35. Johnson, G. R., and M. A. S. Moore. 1975. Role of stem cell migration in initiation of mouse fetal liver haemopoiesis. *Nature (Lond.).* 258:726–728.

36. Johnson, G. R., and D. C. Barker. 1985. Erythroid progenitor cells and stimulating factors during murine embryonic and fetal development. *Exp. Hematol.* 13:200–208.

37. Peschle, C., F. Mavilio, A. Carè, G. Migliaccio, A. R. Migliaccio, G. Salvo, P. Samoggia, S. Petti, R. Guerriero, M. Marinucci, D. Lazzaro, G. Russo, and G. Mastroberardino. 1985. Hemoglobin switching in humans embryos: asynchrony of $\zeta \rightarrow \alpha$ and $\epsilon \rightarrow \gamma$ globin switches in primitive and definitive erythropoietic lineage. *Nature (Lond.).* 313:235–238.

38. Labastie, M. C., J. P. Thiery, and N. M. Le Douarin. 1984. Mouse yolk sac and intraembryonic tissues produce factors able to elicit differentiation of erythroid burst-forming units and colony-forming units, respectively. *Proc. Natl. Acad. Sci. USA.* 81:1453–1456.

39. Beaupain, D., C. Martin, and F. Dieterlein-Liévre. 1981. Origin and evolution of hemopoietic stem cells in the avian embryos. *In* Hemoglobins in development and differentiation. G. Stamatoyannopoulos and A. W. Nienhuis, editors. Alan R. Liss, Inc., New York. 161–170.

40. Zucali, J. R., V. Stevens, and E. A. Mirand. 1975. In vitro production of erythropoietin by mouse fetal liver. *Blood.* 46:85–90.

41. Metcalf, D., and N. A. Nicola. 1985. Role of the colony stimulating factors in the emergence and suppression of myeloid leukemia populations. *In* Molecular Biology of Tumor Cells. B. Wahren, G. Homl, S. Hammarstrom, and P. Perlman, editors. Raven Press, Inc., New York. 215–232.

42. Alter, B. P., and H. S. Gilbert. 1985. Hydroxyurea and hemoglobin F in patients with myeloproliferative syndromes. *In* Experimental Approaches for the Study of Hemoglobin Switching. G. Stamatoyannopoulos and A. W. Nienhuis, editors. Alan R. Liss, Inc., New York. 455–462.