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# Properties of the earliest clonogenic hemopoietic precursors to appear in the developing murine yolk sac

(fetal liver/stem cell/hemoglobin switching)

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**ABSTRACT** **We have found that a variety of clonogenic hemopoietic cells can be obtained in a viable state from mouse conceptuses as early as day 7 of gestation when tissues are disaggregated in a crude collagenase solution containing fetal bovine serum. Examination of the time course of colony formation, and the ultimate size and lineages represented in colonies produced in semisolid medium containing methylcellulose, together with analysis of individual erythroid colonies stained with rabbit antisera specific for adult (HbA) and embryonic (HbE) mouse hemoglobins, revealed the presence on days 7 and 8 of gestation (but not later) of erythropoietic progenitors that give rise to mature erythroid colonies containing up to 100 HbE-containing erythroblasts after 4–6 days of growth in culture. These progenitors are highly sensitive to the disaggregation conditions used. Clonogenic progenitors of exclusively HbA-positive erythroblasts can also be detected in the day-7 conceptus. Assays of progenitors from separately disaggregated yolk sacs and embryos from day-8 conceptuses yielded colonies only from yolk sac suspensions, and again these contained either HbE- and HbA-positive erythroblasts or only HbA-positive erythroblasts. These findings demonstrate the very early appearance in the yolk sac of a population of erythroid progenitors with a number of unique properties. Although most of these yield HbE-positive erythroblasts *in vitro*, some produce erythroblasts containing HbA only. Such a developmental pattern is consistent with the hypothesis that definitive erythropoiesis in the mammalian fetal liver is derived from stem cells that originate in the yolk sac blood islands.**

In the developing mouse embryo, hemopoiesis begins with the formation of the yolk sac blood islands on the seventh day of gestation (1). Two days later the circulatory system is complete, and "primitive" erythroblasts are the predominant cell type found in the embryonic blood at that time (1–4). Unlike adult erythroid cells, these circulating primitive erythroid cells remain nucleated until they disappear around the 15th day of gestation (1–5). In addition, they are unique in their ability to synthesize embryonic hemoglobin (HbE), although with time they also produce some adult hemoglobin (HbA) (6). Erythrocytes containing exclusively HbA first appear in the fetal liver, which is formed shortly after day 10 of gestation (7, 8).

The restriction of HbE production to a gestationally early cohort of erythroid cells that show other unique differentiation features has led to the concept of a developmentally separate lineage of primitive hemopoietic cells. In previous studies, we showed that progenitors of exclusively HbA-positive erythroid cells were already present in the 9-day-old conceptus, at a time when the fetal liver is not yet formed (9). In addition these studies revealed an unusual sensitivity of gestationally early precursors to conventional methods for obtaining single-cell suspensions, although organ cultures of day-8 conceptuses did suggest the existence of two populations of erythroid progenitor cells (10). To characterize these progenitor populations further and to investigate their ontogeny, we therefore sought a procedure that might allow their assessment by colony formation in semisolid medium. Recently we discovered a suitable enzymatic disaggregating procedure (11) that allowed the presence of clonogenic progenitors of primitive erythroblasts to be detected (10). In this report we present the results of more detailed studies, in which progenitor cell types present during the initial phases of hemopoietic cell development in mammalian embryogenesis have been quantitated and characterized.

## MATERIALS AND METHODS

**Animals and Tissues.** Normal adult BALB/c mice were obtained from Cumberland View Farms (Clinton, TN). One or two females were caged late in the afternoon with a male and examined for vaginal plugs the next morning. The morning on which the plug was found was designated day 0 of gestation. On days 7–10 of gestation, pregnant mice were killed by cervical dislocation. Conceptuses were removed and placed in alpha minimal essential medium with 2% fetal bovine serum (FBS; Flow Labs, Inglewood, CA).

**Disaggregation of Tissues with Collagenase.** Just prior to use, a solution of 0.1% collagenase (Sigma, cat. no. 0130) in $Ca^{2+}$- and $Mg^{2+}$-free phosphate-buffered saline supplemented with 20% FBS was prepared (11). Usually, four 7-day or three 8-day conceptuses, or three 9-day or 10-day yolk sacs, were placed in 5 ml of the collagenase solution in a 50-ml tube. These were then incubated at 37°C for 3 hr with occasional shaking. Forty milliliters of alpha medium plus 2% FBS was then added, clumps were allowed to settle (5 min), and the cells remaining in suspension were harvested. These were washed once again before final resuspension in alpha medium with 2% FBS. The majority of the cells obtained in this way were present as single isolated cells. For collagenase treatment without FBS, the procedure was the same except that FBS was omitted from the collagenase solution and the incubation period was shortened to 20–30 min.

**Methylcellulose Cultures.** Cells were mixed in a culture medium consisting of alpha medium plus 0.8% (wt/vol) methylcellulose, 1% (wt/vol) deionized bovine serum albu-

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Abbreviations: Epo, erythropoietin; FBS, fetal bovine serum; PWM-SCCM, pokeweed mitogen-stimulated spleen cell conditioned medium; CFU-E, erythroid colony-forming unit; BFU-E, erythroid burst-forming unit; HbE, embryonic hemoglobin; HbA, adult hemoglobin.

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min, 30% (vol/vol) FBS, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 2–3 units of partially purified erythropoietin (Epo) (12) per ml, and 1.3% (vol/vol) pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCCM) (13). The Epo preparations used contained no detectable interleukin 3 at the concentrations tested (12). Aliquots (0.7 ml) of the final methylcellulose mixture were then plated in 35-mm petri dishes and incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Usually all colonies were scored at a single time point on the sixth day of incubation. Clusters of from 8 to 100 well-"hemoglobinized" cells were operationally defined as originating from a progenitor referred to as a day-5 erythroid colony-forming unit (CFU-E). Colonies containing 100–300 well-hemoglobinized cells were classified as small bursts (from small-burst-forming units, SBFU-E). Those containing more than 300 erythroid cells were classified as large bursts (from LBFU-E). All colonies that clearly consisted of erythroid cells and cells of at least one other hemopoietic lineage were classified as mixed (from E-mix). Giemsa–Wright staining of many of these mixed colonies indicated that macrophages were the most common cell type present, other than erythroid cells, in the mixed colonies produced in these cultures. All other colonies containing >50 cells, mainly granulocytes and macrophages, but no detectable erythroid cells were classified as non-E.

**Immunocytologic Studies.** The preparation and characterization of specific fluorescein isothiocyanate- and rhodamine-labeled rabbit anti-mouse HbA and HbE antisera have been described (8). To assess erythroblast hemoglobin phenotypes, individual colonies were plucked with a micropipette and smeared on a glass slide. These were then air-dried, fixed in acetone/methanol (1:9, vol/vol), and stained with both anti-HbA antibodies at 1:8 dilution and anti-HbE antibodies at 1:10 dilution for 1 hr at room temperature. After extensive washing, the slides were examined under a Zeiss microscope equipped with a 200-watt mercury-vapor lamp and appropriate filters for fluorescein and rhodamine fluorescence.

## RESULTS

**Differential Sensitivity to Collagenase Treatment.** A preliminary survey of various procedures for disaggregating day-7 embryonic tissues revealed that incubation for 3 hr in a 0.1% solution of a crude collagenase preparation containing a FBS supplement yielded a single-cell suspension from which hemopoietic colonies could be obtained. Physical methods or enzymatic treatment with trypsin, dispase (neutral proteinase, EC 3.4.24.4), or collagenase alone did not produce significant growth. Table 1 shows the effect of the FBS supplement in the collagenase solution on all types of hemopoietic progenitors detectable in day-7 embryonic tissue and the disappearance of this effect with increasing gestational age. This protective FBS effect was demonstrable with two of three different batches of collagenase.

**Colony Kinetics.** Repeated assessment of the same cultures after various periods of incubation revealed differences in the rates at which different types of colonies matured and thus became identifiable (Table 2). In assays of 7- and 8-day-old tissues, the majority of colonies obtained were erythroid. In contrast to assays of fetal liver or adult hemopoietic tissue, the first colony type to mature consisted of a cluster of up to 100 erythroblasts in which hemoglobin production was not obvious until the third day in culture. Maximum numbers of such colonies were detectable after 4–5 days of incubation; later, their numbers declined due to colony lysis. They are therefore referred to here as day-5 CFU-E, although they were usually counted on the sixth day of incubation to facilitate simultaneous assessment and scoring of later-maturing colony types. Larger colonies with a burst-like

Table 1. Colony formation by various hemopoietic progenitor cell types after collagenase treatment of 7- to 9-day-old embryonic tissues in the presence or absence of FBS

| | No. of colonies per dish | | | | | |
|---|---|---|---|---|---|---|
| | Day 7 | | Day 8 | | Day 9 | |
| Progenitor | + FBS | − FBS | + FBS | − FBS | + FBS | − FBS |
| Day-5 CFU-E | 15 ± 4 | 0 | 29 ± 11 | 3 ± 2 | 0 | 0 |
| SBFU-E | 4 ± 1 | 0 | 19 ± 3 | 1 ± 1 | 8 | 3 ± 1 |
| LBFU-E | 2 ± 1 | 0 | 16 ± 5 | 0 | 37 ± 1 | 19 ± 1 |
| E-mix | 0 | 0 | 1 ± 1 | 1 ± 1 | 16 ± 1 | 15 ± 1 |
| Non-E | 1 | 0 | 8 ± 1 | 3 ± 2 | 38 ± 5 | 24 ± 2 |

The mean egg cylindrical length of day 7 and 8 embryonic tissues was 0.6 mm and 1.4 mm, respectively. Separately removed yolk sacs were used to obtain single cells for cultures of day-8 and -9 tissues in these experiments, but whole conceptuses were used for the day-7 study. No. of cells plated per dish was $2 \times 10^3$, for cultures of day-7 tissues; $9 \times 10^3$, for day-8 tissues; and $2 \times 10^4$, for day-9 tissues. Data are mean (± SEM) number of colonies per dish based on counts from two to four replicates in one set of experiments. These experiments were repeated at least twice and similar results were obtained.

morphology were first seen to contain mature erythroblasts after 5 days of incubation, and the same association between ultimate colony size and maturation time typical of adult progenitors was a feature of the small and large bursts obtained in assays of cells from embryonic tissues. Qualitatively, most of the erythroid colonies generated by the progenitors classified as LBFU-E (>300 cells) in assays of day-7 and day-8 conceptuses were not as large as those produced in assays of cells from 9- to 10-day-old yolk sacs. The average size of LBFU-E colonies in assays of the former was less than 1000 cells per colony, whereas day-10 yolk sac cultures set up using the same culture reagents yielded LBFU-E colonies that contained on average more than 3000 cells. The size of the E-mix colonies observed in these cultures also varied markedly, ranging from approximately 500 to more than 5000 cells per colony. From the data in Table 2, it can also be seen that the plating efficiency of day-5 CFU-E, in contrast to progenitors of all other colony types, was not dependent on the addition of PWM-SCCM to the culture medium.

**Changes in Progenitor Numbers in the Yolk Sac with Increasing Gestational Age.** Fig. 1 shows the results of experiments in which 7- and 8-day-old conceptuses and 9- and 10-day-old isolated yolk sacs were assayed for their content of various types of hemopoietic progenitor cells. As

Table 2. Time course of colony formation in methylcellulose assays of day-8 embryonic tissues

| Days in culture | PWM-SCCM | No. of colonies per plate | | | | |
|---|---|---|---|---|---|---|
| | | Day-5 CFU-E | SBFU-E | LBFU-E | E-mix | Non-E |
| 3 | − | 55 ± 5* | 0 | 0 | 0 | 0 |
| | + | 46 ± 7* | 0 | 0 | 0 | 0 |
| 5 | − | 73 ± 5 | 2 ± 1 | 0 | 0 | 0 |
| | + | 47 ± 2 | 8 ± 2 | 8 ± 1 | 3 ± 1 | 9 ± 2 |
| 7 | − | 37 ± 2 | 2 ± 1 | 0 | 0 | 4 ± 1 |
| | + | 29 ± 2 | 10 ± 1 | 10 ± 3 | 5 ± 1 | 6 ± 2 |

Day-8 embryonic tissues (i.e., both embryo and yolk sac cells; mean egg cylindrical length = 1.6 mm) were digested with collagenase in the presence of FBS. Plating concentration was $1.1 \times 10^4$ cells per ml. Data are the mean (± SEM) number of colonies in three replicate plates of a single experiment. Similar results were obtained in two other experiments.

*Colonies did not contain hemoglobin, as judged by microscopic examination.

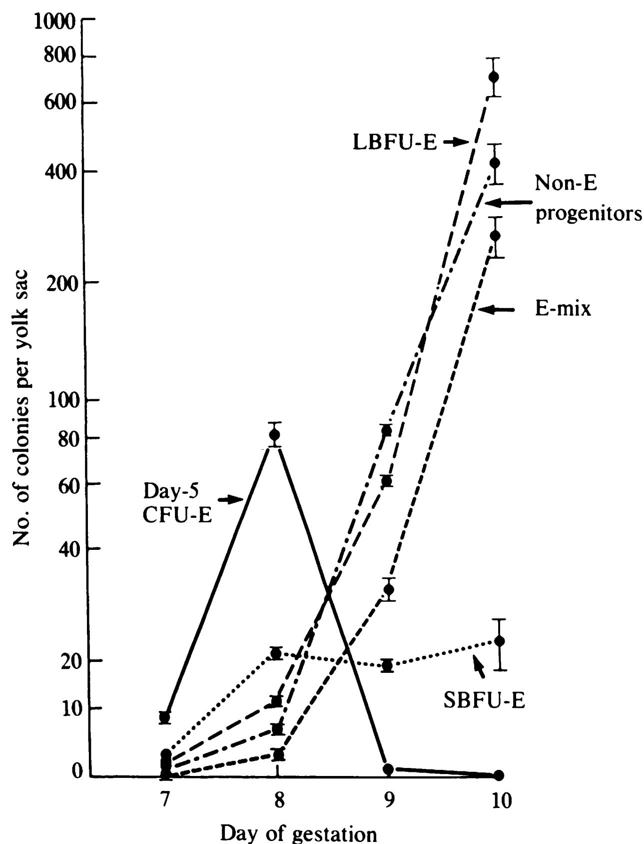


FIG. 1. Changes in various progenitor populations in the yolk sac as a function of gestational age. Values shown are means ± SEM of data from at least two experiments, assayed in two to four replicates per time point. Data for days 7 and 8 are from assays of the whole conceptus, at which time all progenitors are still localized in the yolk sac (see Table 3). For days 9 and 10, yolk sacs were dissected and assayed separately.

suggested by the data shown in Table 1, day-5 CFU-E was initially the predominant progenitor cell type present, although the numbers of all progenitor types increased during the first day of hemopoietic cell development. During this same interval, the total number of cells recovered per disaggregated conceptus increased from $1–2 \times 10^3$ to $3–5 \times 10^4$. Thus the initial increase in day-5 CFU-E between the seventh and eighth day of gestation was roughly proportional to the increase in total cell mass. By the ninth day of gestation, day-5 CFU-E had almost disappeared, whereas the numbers of other types of erythroid, granulopoietic, and pluripotent progenitors were increasing rapidly, again roughly in proportion to the increase in the total cell mass of the conceptus. Progenitors of small bursts showed kinetics intermediate between these two patterns.

To determine the distribution of progenitors between the yolk sac and the embryo proper at different times of gestation, a series of experiments was carried out in which the extraembryonic yolk sacs were dissected away from the embryo proper before disaggregation of tissues for methylcellulose culture. The earliest time that this could be done was at the 7–10 somite stage (egg cylindrical length = 1.2–1.5 mm). As shown in Table 3, cultures of 8- and 9-day-old disaggregated yolk sac cells yielded various types of hemopoietic colonies, whereas parallel cultures of cells from the 8-day-old embryo proper did not yield any colonies and relatively few colonies were obtained in assays of 9-day-old embryos.

**Immunofluorescence Determination of the Type of Hemoglobin Present in Individual Colonies.** Table 4 shows the types of hemoglobin detectable in individual colonies produced by cells present in 7- to 9-day-old embryonic tissues. These analyses show that initially (colonies from day-7 embryonic tissues) the majority of erythroid colonies (9 of 10) contained erythroblasts in which HbE as well as HbA could be readily detected. Based on the intensity of fluorescence, the amount of HbA in these erythroblasts was considerably lower than that seen in identical 6-day-old cultures initiated with cells from slightly older (day 8) embryonic tissues. In addition, only half of all the erythroid colonies detected when day-8 tissues were disaggregated in the FBS-supplemented collagenase solution contained HbE-positive erythroblasts. Disaggregation of day-8 embryonic tissues in collagenase without added FBS selectively eliminated HbE-positive colonies. By day 9 of gestation, very few progenitors of HbE-positive erythroblasts could be detected under any conditions.

Although most of the colonies analyzed were homogeneous in terms of the type of erythroblast present, a small number of colonies appeared to include both types of erythroblasts (Table 4). In these, most of the erythroblasts

Table 3. Distribution of progenitors between the yolk sac and the embryo at days 8 and 9 of gestation

| | No. of progenitors per yolk sac or per embryo | | | |
|---|---|---|---|---|
| | Day 8 | | Day 9 | |
| Progenitor | Yolk sac | Embryo | Yolk sac | Embryo |
| Day-5 CFU-E | 113 ± 26 | 0 | 1 ± 1 | 0 |
| SBFU-E | 32 ± 8 | 0 | 20 | 2 ± 1 |
| LBFU-E | 22 ± 6 | 0 | 62 ± 1 | 3 ± 2 |
| E-mix | 1 ± 1 | 0 | 32 ± 4 | 0 |
| Non-E | 10 ± 3 | 0 | 86 ± 2 | 1 ± 1 |

Cells were obtained by enzymatic treatment using collagenase and FBS. The mean egg cylindrical length of day-8 embryonic tissues was 1.4 mm. Day-9 tissues were obtained from 18-somite embryos. Decapitated day-9 embryos were bled for 5 min before the enzymatic treatment. Data shown are the calculated numbers (mean ± SEM) of progenitors per yolk sac or per embryo, based on the number of colonies scored (after 6 days of incubation) in at least two experiments. Total numbers of cells obtained per yolk sac or per embryo were as follows: day-8 yolk sac, $2 \times 10^4$; day-8 embryo, $3 \times 10^4$; day-9 yolk sac, $8 \times 10^4$; day-9 embryo, $3 \times 10^5$. Cell plating concentrations used in assays of 8-day-old yolk sacs and embryos were equivalent to one yolk sac or embryo per ml, and those used in assays of day-9 tissues were $6 \times 10^4$ cells per ml and $2 \times 10^5$ cells per ml, respectively, for yolk sacs and embryos.

Table 4. Immunofluorescence of individual erythroid colonies with rabbit anti-mouse HbA and HbE

| Day of gestation | FBS in collagenase | Number of colonies: EA | A + some EA | A |
|---|---|---|---|---|
| 7 | + | 9[a] | 0 | 1 |
| 8 | +* | 11[b] | 2 | 9[c] |
| | − | 0 | 1 | 4 |
| 9 | + | 0 | 1 | 5 |
| | − | 0 | 1 | 9 |

Cells from BFU-E and/or E-mix colonies in several cultures containing 2–3 units of Epo per ml and 1.3% PWM-SCCM were examined after 6 days of incubation except where noted (see superscript letters). When all erythroid cells in individual colonies stained positive with both anti-HbE and anti-HbA, these colonies were classified as EA. When most erythroid cells stained with anti-HbA only but some with both anti-HbA and anti-HbE, they were classified as A + some EA. When all the erythroid cells were stained with anti-HbA only, they were classified as A.

*Some of the data in this group have been reported elsewhere (10).

[a–c]In these groups, some day-5 CFU-E were included in the colonies examined: [a]6 out of 9, [b]8 out of 11, [c]2 out of 9.

contained HbA only, but a number contained both HbE and HbA.

## DISCUSSION

Attempts to study the relationship between progenitors of primitive and definitive hemopoiesis during the initial development of these cells in mammals have been hampered by the difficulty of obtaining viable single-cell suspensions from very early embryonic tissues (10, 14). In this study we have shown that this may be due to a unique sensitivity of these cells to enzymatic treatment with crude collagenase solutions, but that this sensitivity may sometimes be overcome if FBS is added, presumably because the FBS neutralizes contaminating toxic proteases present in crude collagenase preparations.

Cell suspensions prepared in this way from 7- to 10-day-old embryonic tissues yielded erythroid, granulopoietic, and multilineage colonies in standard methylcellulose cultures containing Epo and PWM-SCCM. Erythroid progenitors were initially the predominant hemopoietic cell detected, and during this period (days 7 and 8 of gestation) the majority of the erythroid colonies obtained displayed unique growth and maturation characteristics. Because of the time taken for these colonies to mature and their ultimate CFU-E-like single-cluster morphology, we scored them as a separate entity referred to here as day-5 CFU-E. Subsequent studies indicated that colonies derived from day-5 CFU-E achieved a maximum size of approximately 100 cells and produced almost exclusively HbE-positive erythroblasts.

In another report (10), we showed that omission of exogenous Epo from assay cultures of 8-day-old conceptuses resulted in a moderate reduction of all erythroid colony types, indicating that embryonic progenitors of HbE-positive erythroblasts, like adult erythroid progenitors, are Epo-responsive. In contrast, factor(s) present in PWM-SCCM were not required to support the production of (HbE-positive) day-5 CFU-E-derived colonies (Table 2), although higher-proliferative-potential progenitors in the early (day-8) yolk sac that yield mainly pure HbA-positive colonies were dependent on the presence of such factor(s), as are similar high-proliferative-potential progenitors found later in the fetal liver and adult. Studies with purified reagents will be necessary to establish whether this factor is interleukin 3 (15).

The immunofluorescence data support the concept that separate populations of erythroblast progenitors can be distinguished and that these appear at different times during development. It is of interest that a small number of colonies were found in which most of the erythroid cells stained positively with anti-HbA only, but some stained positively with both anti-HbA and anti-HbE antisera. One possibility is that these latter colonies did not arise from single cells, although the plating cell concentration was very low in the cultures of 8-day-old embryonic tissues where they were detected. Alternatively, such colonies may indicate the persisting presence of an earlier cell type capable of giving rise to both HbE-containing and pure HbA-positive erythroblasts. The assumption that these erythroblasts generated *in vitro* exhibited the same pattern of globin-gene expression as would have been obtained *in vivo* might also be questioned. Although the present findings do not suggest such a difference, the concept invites further investigation.

A number of studies have indicated that the yolk sac contains cells that can give rise to a variety of hemopoietic cells, and it has therefore been suggested that pluripotent stem cells arising in the yolk sac are later responsible for populating other hemopoietic organs (16, 17). This hypothesis has, however, been challenged by quail–chick "yolk sac chimera" experiments (18, 19). The results of these experiments indicate that the hemopoietic organs in the embryo of birds may be populated by intraembryonic stem cells rather than by cells of yolk sac origin. Our previous studies have shown that large numbers of progenitors capable of giving rise to HbA-positive cells are present in the fetal circulation before the establishment of the fetal liver in the embryo (9). In addition, murine fetal hepatic erythropoiesis *in vitro* has been shown to be dependent on an influx of exogenous stem cells (20). The present studies show that on day 8 of gestation, progenitors capable of giving rise to pure HbA-positive erythroblasts *in vitro* can be readily demonstrated in the yolk sac but not in the embryo proper. Taken together, these data are consistent with the hypothesis that fetal hepatic hemopoiesis is seeded by circulating hemopoietic stem cells derived from the yolk sac.

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