Hematopoiesis in the Embryonic Mouse Spleen: An Electron Microscopic Study

By Meir Djaldetti, Hanna Bessler, and Richard A. Rifkind

The events of hematopoiesis in the C57/B16J embryonic mouse spleen are described, beginning with the appearance of the splenic rudiment (12th gestational day). Immature erythroid precursors appear on day 12 of embryonic development, but foci of mature red cell precursors were not seen until day 15. Red cell precursors were observed in the spleen up to day 19 after birth. Mature polymorphonuclear leukocytes were present by day 12, whereas megakaryocytic precursors appear on day 12. Although young cells of the lymphocytic series were identified on the 16th gestational day, a marked proliferation of lymphatic cells was noted at the end of the gestational period and immediately after birth.

There are four main sites of hematopoiesis in mammalian species during embryonic life, namely, yolk sac, liver, spleen, and bone marrow. As was shown in previous studies, C57B1/6J mice provide a useful system for studying the events of embryonic hematopoiesis. Erythropoiesis in embryonic livers of this strain was extensively investigated by Rifkind et al.1

The reports on hematopoietic events in the embryonic mammalian spleen are quite scarce and somewhat controversial. A few reports deal with different aspects of the embryonic splenic structure and hematopoiesis.2-5

The aim of the present study was to detect the appearance of the splenic rudiment in the embryonic C57/B16J mouse, to follow the morphogenetic events of hematopoiesis in the spleen from its very beginning, to detect the appearance of the precursor cells of each of the hematopoietic series, and, finally, to try to clarify the relationship between hepatic and splenic hematopoiesis.

MATERIALS AND METHODS

Animals

Four-week-old female C57B1/6J inbred mice, obtained from the Weizmann Institute of Science, Rehovot, Israel, and Jackson Laboratory, Bar Harbor, Maine, were hormonally primed6 and mated with 3–6-mo-old males of the same strain. The morning after mating was designated as 0 day of gestation.7

From the Department of Medicine B, Hasharon Hospital, Petah Tiqva, Tel-Aviv University Medical School, Tel-Aviv, Israel, and Department of Medicine, Department of Human Genetics and Development, Columbia University College of Physicians and Surgeons, New York.

Submitted August 9, 1971; revised November 15, 1971; accepted November 30, 1971.

Meir Djaldetti, M.D.: Head, Department of Medicine B, Hasharon Hospital, Petah Tiqva, and Senior Lecturer, Tel-Aviv University Medical School, Tel-Aviv, Israel. Hanna Bessler, M.Sc.: Department of Medicine B, Hasharon Hospital, Petah Tiqva, Israel. Richard A. Rifkind, M.D.: Professor, Department of Medicine and Department of Human Genetics and Development, Columbia University College of Physicians and Surgeons, New York.
Spleens were removed under a dissecting stereo microscope on sequential days of fetal development, beginning on gestational day 12 and on various days after birth as indicated below. On day 12 of the fetal development, the spleens were removed, occasionally attached to the stomach in order to avoid distortion of the splenic structure.

Microscopy

On the earlier days of gestation, whole embryonic spleens were immediately transferred to cold 1% phosphate-buffered glutaraldehyde (pH 7.2), whereas spleens of fetuses at later gestational days, as well as spleens of newborn and adult mice, were cut into small pieces and fixed promptly in glutaraldehyde. All spleens were postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812. For light microscopy, 1 μ sections were prepared and stained with methylene blue. Light microscopic studies of the 12th- and 13th-day embryonic spleens were performed on thick serial sections obtained at intervals of 10 μ. For electron microscopy, thin (500–800 Å) sections were prepared on an LKB Ultratome III, stained with uranyl acetate and lead citrate, and examined in an Hitachi HS-7S or Philips-300 electron microscope. At least five spleens of each developmental stage were studied.

Touch preparations obtained from embryonic spleens were stained for hemoglobin according to the method of Pearse. Hemoglobin staining of epon-embedded embryonic spleens was performed by the method described by Breton-Gorius. This method was useful also for demonstration of the granules in the granulocytic precursors.
RESULTS

Macroscopic Characteristics of the Fetal Spleen

By the 12th day of gestation the spleen was seen as a small, pale, triangular thickening in the dorsal mesogastrium (Fig. 1). It was firmly connected with the greater curvature of the stomach, as well as with the pancreas. On subsequent days, it assumes a sickle shape, and the color changes from yellowish-pink to red.

Cell Lines

Mesenchymal Cells: Mesenchymal cells represent the majority of the cell population in the earlier embryonic spleens (12–15 gestational days). These cells (Fig. 2) are characterized by their oval or irregular shape, junctional complexes composed of adherent zones with adjacent mesenchymal cells, large nuclei almost devoid of heterochromatin, and round nucleoli, scanty cytoplasm, a few mitochondria, and a small amount of endoplasmic reticulum. This cell type closely resembles the mesenchymal cells described in the early stages of development of mammalian fetal spleens. By day 16, with the increase of the number of cells of the red and white blood series, the relative number of the mesenchymal cells begins to decline. By day 20, their number is relatively very low, but these cells are still found in the early postnatal (0–2 days) spleens.
Fig. 3. (A) E.M. micrograph of 12th-day embryonic spleen. Two red cell precursors, most probably hemocytoblasts (H), are shown between mesenchymal cells (M). $\times$ 6000. (B) Hemocytoblast is shown in 12th-day embryonic spleen. It is possible to see that the cell is not firmly connected with surrounding cells. $\times$ 9000.
Fig. 4. E.M. micrograph of 13th-day spleen. Proerythroblast with abundant number of ribosomes causing a darker appearance of cytoplasm, invaginated nucleus with large prominent nucleolus, well-developed Golgi apparatus (Go), and large round mitochondria are typical for this type of cell. C, centrioles. × 11,000.

Red Cell Series: Erythroid Cell Precursors: Concerning the morphology of the red cell precursors, previously established criteria\textsuperscript{1,10-13} were used. Accordingly, the earliest erythroid precursor was tentatively identified as hemo-cytoblast, followed by proerythroblast, basophilic, polychromatophilic and

Fig. 5. Light micrograph of 14th-day embryonic spleen. Three basophilic erythroblasts are shown in middle of mesenchymal cells. × 1200.
Fig. 6. (A) Light micrograph of section from 16th-day spleen. Cluster of erythroid precursors at different stages of maturation is seen in vicinity of the splenic capsule. × 1800. (B) Light micrograph of a sinus within 16th-day spleen containing many matured blood cells, expelled nucleus (N), polychromatophilic erythroblast (P), and orthochromatic erythroblast (O). Nucleated cell is in process of entering into sinus (arrow). × 800.
Fig. 7. (A) E.M. micrograph of 20th-day embryonic spleen demonstrating three polychromatophilic erythroblasts under splenic capsule. $\times$ 7650. (B) E.M. micrograph of 0 day newborn mouse spleen. Cell polymorphism is evident. The two adjacent cells in the middle are very young erythroid precursors. O, orthochromatic erythroblast, P, polymorphonuclear, M, portion of macrophage with remnants of phagocytized nuclei. $\times$ 6000.
HEMATOPOIESIS IN SPLEEN

Fig. 8. Light micrograph of 12th-day embryonic spleen. Promyelocyte, characterized by its high nucleocytoplasmic ratio, prominent nucleolus, and a few cytoplasmic granules particularly emphasized by the peroxidase reaction, is seen between the mesenchymal cells. Portion of another granulocyte is marked with arrow. × 1600.

orthochromatic erythroblast, the latest being the last nucleated stage of the erythroid progenitors.

In the 12th-day embryonic spleen, only a few putative red cell precursors were seen (Fig. 3A). According to the above-mentioned criteria, these cells are designated as hemocytoblasts. A typical cell is shown in Fig. 3B. Proerythroblasts were first seen in the 13th-day spleen (Fig. 4). Staining for hemoglobin did not reveal hemoglobin-containing red cell precursors in the 12th- and 13th-day spleens. Small clusters of more mature erythroblasts (baso- and polychromatophilic) could be found on day 14 (Fig. 5). By day 15, orthochromatic erythroblasts in the process of expelling their nuclei were observed, indicating the production of reticulocytes. Erythropoiesis becomes very prominent beginning on day 16. At this time, large nests of erythroblasts at all stages of development are observed, principally under the marginal mesothelial monolayer (Fig. 6A). Figure 6B shows a large sinusoid from a 16-day spleen filled principally with nonnucleated cells. Some nucleated precursors of the red blood series are found in these blood vessels at this stage.

On the later gestational days, the predominant type of red cell precursor observed in the spleen was the polychromatophilic erythroblast (Fig. 7A), some of these containing ferritin. The same type of cells predominates in spleens immediately after birth (postnatal days 0–6), although in the day 0 postnatal spleen, a few proerythroblasts were still detected (Fig. 7B). Single orthochromatic erythroblasts were found up to the 19th day after birth.

Concerning the site of erythropoiesis, it is noteworthy that erythropoiesis appears to be developing outside of clearly defined sinusoids or blood vessels. Sinusoids, however, may be found as early as the 12th gestational day.

Yolk sac erythroid cells: Cells of yolk sac origin were easily identified by their pyknotic nuclei and abundant cytoplasm, containing hemoglobin as revealed by the hemoglobin staining methods. They were seen in the embryonic mouse spleen from the earliest day studied (day 12), most of them dispersed between the mesenchymal cells and fewer located in the splenic sinuses. With advancement of embryonic development their nuclei undergo
Fig. 9. E.M. micrograph of 12th-day embryonic spleen. Three cells of granulocytic series, characterized by their typical granules, are visible between the mesenchymal cells. × 4200.

progressive nuclear condensation, similar to that observed in the yolk sac erythroid cells in the peripheral blood, as described previously. These cells disappear from the peripheral blood on about day 16 and were no longer seen in the spleen after this gestational day.

White Blood Series: Granulocytic Series: The morphologic characteristics of the cells of mammalian granulocytic series are reviewed by Wetzel.

Cells of the granulocytic series were already identified in the 12th-day spleen, interspersed among the mesenchymal cells. The earliest granulocytic precursor that we were able to recognize in the 12th-day embryonic spleen was the promyelocyte, displaying a large nucleocytoplasmic ratio, large nucleolus, and a few cytoplasmic granules (Fig. 8). Granulocytic precursors were seen also with the electron microscope (Fig. 9). Their relative number progressively increased with the advance of embryonic development, and they were found mostly in clusters. It is to be noted that in the 18th-day spleen it was possible to detect, for the first time, eosinophilic granulocytes, easily recognized by the crystalloid inclusions in their granules. Mature granulocytes were detected in the postnatal spleens, although in relatively decreasing number.
HEMATOPOIESIS IN SPLEEN

Fig. 10. (A) 16th-day spleen. Two lymphoblasts are shown between mesenchymal cells. × 9800. (B) E.M. micrograph of 20th-day embryonic spleen. Cells from lymphocytic series of different stages of maturation (L), and polymorphonuclears (P) are seen between mesenchymal cells (M). × 4200.
Fig. 11. E.M. micrograph of adult mouse spleen. Polymorphonuclears (P) and macrophage (Ma), as well as cell in mitosis, are seen between mature lymphocytes. × 5000. (B) 12th-day spleen. Megakaryocytic precursor characterized by its granules and cysternal appearance of its endoplasmic reticulum and Golgi system. × 10,300.
Fig. 12. (A) 13th-day spleen. Sinus containing nucleated yolk sac cells is visible. Flattened endothelial cells (arrows) face sinus lumen. \( \times 1200 \). (B) Light micrograph of 12th-day embryonic spleen. Sinus displaying few nucleated cells that are most probably granulocytic precursors and two yolk sac cells. \( \times 1200 \).

Lymphocytic series: Ultrastructural criteria for cells from the lymphatic series are based on previously described features.\(^{16-18}\)

Applying these criteria, the first lymphoblasts were identified in the 16th-day embryonic spleen (Fig. 10A). They were seen throughout spleens of 17th- and 18th-day embryos, whereas on days 19 and 20 mature lymphocytes were detected (Fig. 10B). After birth the number of lymphoblasts and lymphocytes progressively increased, the lymphocytes being the predominant type of cell in the 6th-day postnatal spleen. In this sense, the splenic structure was fairly similar to that observed in the adult spleen (Fig. 11A).

Megakaryocytic Precursors and Megakaryocytes: The megakaryocytic precursors\(^1\) were observed in the 12th day embryonic spleen (Fig. 11B). Megakaryocytes\(^9\) were seen in the spleens from day 15 on, as well as in the of newborn and adult mice.

Erythrophagocytosis

Phagocytic activity was observed mostly in the peripheral areas of the embryonic spleens. Evidence of phagocytosis of yolk sac cells was recognized as early as the 12th-day spleen. Macrophages, containing the recognizable remains of one or more yolk sac erythroid cells, was observed. In the more
mature spleens, i.e., 18–20th gestational days as well as after birth, macrophages contain nuclei expelled from orthochromatic erythroblasts and mature red blood cells.

**Splenic Sinuses**

Splenic sinuses were already seen in the 12th-day spleen. They appeared as small spaces, their walls built up by flattened or spindle-shaped endothelial cells. Very often, cytoplasmic processes of these cells overlap each other, giving the impression of a membranelike structure, but we were not able to detect a real basement membrane. Most of the sinuses in the early spleens contained nucleated yolk sac cells (Fig. 12A), although in a few of them early precursors, most probably of the granulocytic series, could be observed (Fig. 12B).

**Collagen Fibers**

The first collagen fibers were noticed in the 17th-day embryonic spleen. They were detected in bundles principally located in the central areas of the spleen, thus forming splenic trabecula. Beginning on day 18 of gestation, collagen fibers were detected also at the periphery of the spleen, beneath the cells that most probably form the splenic capsule (Fig. 13).
DISCUSSION

To follow the events of hematopoiesis in the embryonic spleen, it was necessary to find out at which day of gestation this organ appears. According to Rugh, the spleen is first seen in the mouse embryo on day 13. On the other hand, Flamand was able to explant the spleen rudiment together with the pancreas already in the 12th-day Swiss albino mouse embryo. In most of the 12th-day embryos examined in the present study, we were able to detect the spleen in the dorsal mesogastrium firmly attached to the stomach and the pancreas.

Regarding erythropoiesis, a major question concerns the origin of hematopoietic precursors in the spleen: namely, are these stem cells indigenous to the organ or are they produced in the embryonic yolk sac or liver and later colonize the spleen? The literature concerning the subject is quite controversial. Although cells capable of colonizing the spleens of irradiated adult host mice are found in fetal yolk sac, blood, liver, and spleen, there is no convincing evidence for or against colonization in the establishment of hepatic and splenic hematopoiesis in the fetus.

Although circulating stem cells able to produce spleen colonies were demonstrated not only in embryonic blood but also in the blood of adult mice, it has not been excluded that stem cells may originate intrinsically in each hematopoietic organ.

Our findings do not exclude this possibility. In the present study, early erythroid precursors were observed in the 12th- and 13th-day spleens, surrounded by mesenchymal cells. By day 16 of gestation, a few erythroid precursors and expelled nuclei are found in the splenic sinuses. It seems likely that these intravascular precursors were “released” into the circulation through discontinuities in the sinusoidal endothelium. The existence of communications between the extravascular and intravascular compartments was postulated by Grasso et al. and demonstrated by Rifkind et al. in the mammalian fetal livers. It is not excluded that the final stages of maturation of these cells may take place, at least in part, in the splenic sinuses, as suggested by Thiel and Downey.

It appears that, from the stage of the very early erythroid cell precursor (hemocytoblast) first found in the 12th-day spleen, until the first appearance of the orthochromatic erythroblast in the 15th-day spleen, the process of erythroid cell maturation takes about 72 hr. Orlic et al. have shown that erythropoietin activation of stem cells in hypoxia-induced polycythemic adult mouse spleen induces an erythropoietic wave giving rise to mature erythroblasts and reticulocytes in about 72 hr.

The onset of splenic erythropoiesis in the fetus coincides in time with the increase of the erythropoietic activity in the spleen of the pregnant mouse. It was shown by Fowler and Nash that in pregnant C57Bl/6J mice the spleen weight reaches a peak at the 12th day of gestation. On the same day, simultaneously with the peak in splenic weight, a peak of erythropoietic activity in the spleen was recorded. It is possible that the erythropoietic stimulus that promotes the increase of erythropoietic activity in the maternal spleen is also responsible for induction of erythropoiesis in the embryonic spleen.
The origin of the polymorphonuclear leukocyte precursors in the embryonic spleen remains unclear. The earliest granulocytic precursor identified in the 12th-day spleen was the promyelocyte. Taking into consideration that the myeloblasts may exist in very low numbers, it is possible that they were simply missed. Alternatively, lack of reliable fine structural criteria that distinguish myeloblasts from erythroblasts\textsuperscript{18} introduces the possibility of misidentification.

Lymphatic cell precursors were first noted in the 16th-day embryonic spleen. Due to the close resemblance of lymphoblasts to other immature precursors seen in the spleen, this dating of lymphoblast appearance is not altogether sure. It is of interest that the number of lymphocytes, which are the main cellular element in the adult spleen, increases just before and immediately after birth.

The megakaryocytic precursors were already observed in the 12th day embryonic spleen. More mature cells of the megakaryocytic series were found in spleens from the 15th gestational day. It appears that it takes about 3 days for the megakaryocytic precursor to mature to a megakaryocyte. This observation is compatible with the turnover time of 2 or 3 days estimated by Ebbe and Stohlman\textsuperscript{29} for the rat megakaryocyte.

Macrophages were found in the spleens of all gestational days. We were not able to see plasma cells in the embryonic spleens but we did see them in the adult spleen.

The present study suggests that hematopoiesis in the embryonic spleen is principally an extravascular process. It is likely that cells of the extravascular compartment are released into the circulation through discontinuities in the sinusoidal lining. In this sense, fetal splenic hematopoiesis does not differ from the extravascular erythropoiesis described in the fetal livers of various mammals.\textsuperscript{10,30}

One of the most prominent features that distinguishes between erythropoiesis in the liver and the spleen in mouse embryos is the lack of evidence of epitheliomesenchymal interaction in the spleen. The existence of this interaction was identified by Rifkind et al.\textsuperscript{1} in the embryonic mouse liver, and the significance of such interaction in yolk sac erythropoiesis has been shown in cultures of isolated mesenchyme from other species.\textsuperscript{31,32} It may be speculated that in embryos at advanced gestational stages, the putative inductive functions of endodermal cells are replaced by humoral factors, including the hormone erythropoietin.

REFERENCES

HEMATOPOIESIS IN SPLEEN

841

Hematopoiesis in the Embryonic Mouse Spleen: An Electron Microscopic Study

Meir Djaldetti, Hanna Bessler and Richard A. Rifkind