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ERYTHROPOIESIS IN THE BONE MARROW
OF THE FETAL RABBIT: A MORPHOLOGICAL
STUDY.

The Ohio State University, Ph.D., 1965
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ERYTHROPOIESIS IN THE BONE MARROW OF THE FETAL RABBIT:
A MORPHOLOGICAL STUDY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

John Edward King, B.A.

* * * * * * *

The Ohio State University
1965

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INTRODUCTION

Since the discovery by Neumann in 1868 that the red bone marrow gives rise to a number of the cells of the circulating blood, the question of the exact mode of origin of these blood cells and their genetic interrelationships has been one of the most fascinating and perplexing problems in cell biology.

Traditionally, the problem of red blood cell development and differentiation has been approached from a morphological standpoint. Accepting that cell development and differentiation are for the most part revealed by visible structural changes, hematologists have proposed sequential series of cells whose architectural similarities are interpreted as evidence of genetic relationship. Earlier views have been reinforced through the use of newer techniques: phase contrast microscopy, electron microscopy and tritiated thymidine studies.

Hence, there has accumulated a body of information concerning the lineage and potentialities of the free cells of the erythrocytic series which is now generally accepted. However, the relative position of these cells within the bone marrow and their genetic relationship to the fixed precursor cells have not been conclusively demonstrated.

Because of the great complexity of the bone marrow organ and because of difficulties encountered in achieving perfect visualization of bone marrow morphology, disagreements concerning the cell
of origin of the erythrocytic series and the precise localization of
developing erythrocytic elements within the bone marrow parenchyma
have not been completely resolved in our opinion. Various theories
of erythropoiesis have been proposed and can be conveniently divided
into three distinct views: (1) Erythrocytes arise in the extra-
vascular space of the bone marrow parenchyma from undifferentiated
mesenchymal cells which have invaded the marrow cavity from the
periosteum (Maximow '07; Schridde '09; Drinker, Drinker and Lund '22;
Maximow '24; Maximow '27; Bargmann '30; Weinbeck '38; Gilmour '41;
Schleicher '46a; Schleicher '46b; Hamre '47; Dacie and White '49;
Lennert '52; Knoll '57; Rohr '60; Burkhardt '64). After differentia-
tion and development, the mature erythrocytes gain entrance into the
general circulation through discontinuities in the sinusoidal wall.
According to this view, both erythrocytic and myelocytic precursors
develop at the expense of the same multipotential stem cell under
similar environmental conditions. It is implicit that the stem cell
is irrevocably determined along the erythrocytic or myelocytic line
at a very early stage of development. (2) The second view suggests
that differential factors controlling the development of erythrocytes
are largely extrinsic, i.e., environmental, and consist in conditions
present within the blood vessels. Undifferentiated stem cells
develop into myelocytic elements when located in the extravascular
spaces; and into erythrocytes, in an intravascular environment
(Danchakoff '18; Sabin '23; Jordan '35; Jordan '39; McDonald '39).
Erythrocytes arise, differentiate, and mature either in patent
vessels (Danchakoff '18) or within endothelium-lined spaces not con-
tinuous with patent vessels but representing, nonetheless, intra-
vascular conditions (Jordan '35). (3) A modified view of intravascular
erythropoiesis has been proposed, suggesting not only an intravascular
localization for the origin and differentiation of erythrocytes in
birds and mammals but also the origin of erythrocytic precursors from
endothelial cells of collapsed bone marrow vessels — the so-called
intersinusoidal capillaries (Cunningham and Doan '23; Doan '22;
Sabin '23; Doan et al. '25; Muller '26; Peabody '26; Muller '27;
Doan '31; Doan '38). According to this view, the vascular system
of the bone marrow is completely closed; mature erythrocytes gain
entrance into the general circulation by the dilation of the col-
lapsed capillaries in which they have developed.

Of these three theories of erythropoiesis, the extravascular
view appears to be the most widely accepted at the present time.
The concept of intravascular erythropoiesis and/or endothelial cell
origin of erythrocytes in the mammalian bone marrow has not been
generally accepted; and the results of recent light and electron
microscopic studies (Pease '56; Zamboni and Pease '61; Weiss '61;
Burkhardt '64; Weiss '65) have failed to indicate the presence of
intersinusoidal capillaries or of erythropoiesis occurring within
bone marrow vessels. In spite of the general acceptance of extra-
vascular erythropoiesis in the mammal, we do not believe that the
problem of the site of erythropoiesis in the mammalian bone marrow
has been completely resolved. We are not convinced that the
morphological evidence presented up to this time has conclusively ruled out the possibility of endothelial contributions in erythropoiesis or of the preferential localization of erythropoiesis in endothelium-lined spaces. Conventional methods of tissue preparation have produced ruptures in the delicate sinusoidal walls and shrinkage of the extravascular parenchyma which obscure the fine details of bone marrow architecture and preclude, in our opinion, a critical study necessary to establish unequivocally the site of erythropoiesis and the cell of origin of the erythrocyte. Although electron microscopy has shown the fine structural detail of the cells of the bone marrow and of isolated segments of the sinusoidal walls, due to the size of the tissue examined and the methods employed to obtain such small pieces, this technique has been unable to show, in our opinion, a large enough area of intact bone marrow suitable for the study of cellular relationships.

In order to determine more precisely the exact mode of origin of the erythrocytic elements in the mammalian bone marrow and to resolve the controversy over the intra- or extravascular site of erythropoiesis, it is imperative that the following considerations be made:

1. The bone marrow must be perfectly preserved in order to eliminate ruptures in the sinusoidal walls and shrinkage of the stromal elements.

2. The development of erythrocytes should be observed and studied when the bone marrow is in its simplest form (the fetal
marrow) without the super-imposed complexities of large myelocytic and fat cell populations.

3. The sinusoidal walls must be carefully examined for discontinuities and for evidence of cell migrations.

4. The bone marrow parenchyma and vessels must be closely surveyed for the presence of lymphocytes and for the possible invasion of lymphocytes into the extravascular marrow spaces.

5. The pattern and location of erythrocytic colonies must be closely examined to determine whether or not the colonies are enclosed in endothelial cell lined spaces.

6. Endothelial cells must be closely examined for evidence of further differentiation into hemopoietic precursor cells.

It is the purpose of this investigation to describe the origin of the first erythrocytes from the intact bone marrow of the fetal rabbit employing new methods of tissue fixation and preparation; and in the light of improved visualization, to present critical morphological evidence which indicates conclusively to us the extravascular origin of the erythrocyte in the bone marrow of the fetal rabbit.
MATERIALS AND METHODS

The intact rib cages and long bones of the appendages were removed from sixty-five rabbits ranging from eighteen days of gestation to two days after birth. The bones were treated according to the following procedures:

1. One group of bones was fixed in 10 percent aqueous acrolein for two and one-half hours, rinsed in running water and decalcified in 5 percent aqueous nitric acid for three to twelve hours. The decalcified bones were dehydrated in absolute acetone and embedded in plexiglas-methacrylate according to the method of Cathey (Cathey '63). Two micron sections of the plastic embedded material were cut with a rotary microtome, fixed to glass slides with albumin and stained with 0.5 percent aqueous toluidine blue and examined with the light microscope.

2. One group of bones was fixed for twenty-four hours in formal-Zenker fluid, washed in running water, decalcified in 5 percent aqueous nitric acid, dehydrated in ethanol, cleared in benzene and embedded in paraffin. Serial paraffin sections were stained with hematoxylin and eosin or toluidine blue and examined with the light microscope.

Wright's stained preparations of peripheral blood and bone marrow films were examined for all the animals used in this study.
The ribs and long bones of the fetal rabbit follow nearly identical developmental patterns; the developing rib, however, was chosen as a model for this study because its small diameter permitted faster penetration of the fixative into the bone marrow cavity, thus giving the best preservation of morphological detail.
OBSERVATIONS

Development of the Primary Marrow Space

Beginning with the eighteenth day of gestation, the proximal portions of the cartilage models of each of the upper four or five ribs became surrounded by a thin bony collar; at the same time the chondrocytes of these areas became hypertrophic. Near the periphery of the cartilage model the cytoplasm of the chondrocytes appeared to fragment and the lacunae were occupied only by degenerating naked nuclei. On the nineteenth day of gestation, mesenchymal cells from the periosteum of the bony collar invaded the periphery of the cartilage model of the rib at a point in the proximal one third of the shaft and occupied the degenerating cartilage lacunae. This invasion of mesenchymal cells into the cartilage model marked the first appearance of the primary marrow space; it appeared as an irregular, perforated cylinder bounded at the circumference by the progressively thickening and proliferating bony collar and at the end by the receding epiphyseal plates. The contents of the primary marrow space -- the primary bone marrow or the bone marrow before the appearance of hemopoiesis -- underwent a series of changes with respect to cellular organization and vascular pattern before the first appearance of erythrocytic elements.

The most primitive organization of the primary bone marrow was observed immediately after the first invasion of the cartilage
model by the mesenchymal cells. The primary bone marrow at this stage was populated by degenerating chondrocytes and various morphological forms of mesenchymal cells which ranged from spindle shaped cells with moderately dense, flattened nuclei similar to the cells of the osteogenic layer of the periosteum to typical ameboid mesenchymal cells having abundant, pale cytoplasms and a large irregular nuclei with insignificant nucleoli. It appeared that the mesenchymal cells and the degenerating chondrocytes were lying free in an unorganized mass of semi-fluid ground substance and calcified cartilage debris. Although most of the chondrocytes observed at this stage showed clear morphological signs of degeneration, a few chondrocytes in the periphery of the primary marrow space, occupying ruptured lacunae, showed evidence of viability. No indications of the fate or potentialities of these cells were observed. This initial unorganized stage of the primary bone marrow persisted only for a short time; for soon after the establishment of the mesenchymal cells in the primary marrow space, numerous typical osteoblasts lined up along the calcified cartilage spicules. These cells were clearly related by transitional forms to the mesenchymal cell population of the cavity (Figs. 1-3; 17-19). With the appearance of osteoblasts and with the continuous enlargement of the cavity, the primary bone marrow took on a certain degree of organization. In the semi-fluid ground substance of the extravascular spaces there appeared a delicate syncytium-like network of fine anastomosing fibers (Figs. 1-6; 18-20); associated with the fibers was a modulated form to the mesenchymal
cell -- the reticulum cell. This cell had a small, dense oval nucleus with one or more small nucleoli and lightly basophilic cytoplasm with long delicate processes apparently continuous with the fibers of the ground substance.

Striking changes in the vascular pattern of the primary marrow space accompanied the changes in cell population and organization of the ground substance. The first vessels of the primary marrow space appeared with the initial mesenchymal cell invasion of the cartilage model; they appeared to be capillaries with slightly dilated lumina. Erythrocytes were observed free in the unorganized ground substance in the vicinity of these capillaries, thus suggesting that these vessels were damaged and allowed for the seepage of erythrocytes into the extravascular spaces. Subsequent stages in the development of the marrow space showed vessels with extremely thin walls and wide lumina. The size of the lumina of these vessels appeared to vary with their location in the marrow cavity. In the older mid-shaft region, the site of the initial invasion of the cartilage model, only one or two very large sac-like vessels were seen whose walls were composed of stretched out endothelial cells with thin dark nuclei. Near the epiphyses, however, a greater number of vessels was evident; the walls of these vessels appeared thicker since the endothelial cells comprising the vessel wall were not so stretched or extended. The sinusoid walls in all stages of development were carefully examined for evidence of endothelial cell contributions to the hemopoietic precursor cell population; although there appeared
considerable variation in their size and shape, no endothelial cells were observed which were suggestive of their undergoing further differentiation. Further, no endothelial cells differentiated in situ into erythroid precursors and no cords of endothelial cells consistent with the notion of collapsed capillaries were observed in the fetal rabbit bone marrow.

Initial Erythropoietic Activity

On the twenty-fifth day of gestation the first typical pro-erythroblasts were observed in the extravascular spaces in the older parts of the primary rib marrow (Figs. 3-10); they appeared as large round cells with scant basophilic cytoplasm and large round nuclei with coarsely clumped chromatin. These cells were related structurally to the surrounding mesenchymal cells by intermediate transitional forms. Mesenchymal cells in various stages of rounding up, increasing cytoplasmic basophilia and nuclear chromatin clumping were readily recognized in the thin plastic sections (Figs. 2-16). The relative position of the first proerythroblasts with respect to the sinusoidal walls was quite variable: some cells lay near the walls; others, among the reticulum cells and fibers, some distance from the vessels. Their relative position in the bone marrow cavity was also inconsistent: some proerythroblasts lay near the periphery of the cavity near the periosteal bone; others, near the center. In no instance were proerythroblasts seen in the sinusoids or surrounded by cell processes in such a way that they could be conceived to lie within the collapsed vessels.
Vessel walls and lumina were carefully examined throughout the development of the bone marrow cavity for evidence of invasion of the extravascular space by blood-borne lymphocytes; none were observed in the vessels of the bone marrow until the thirty-first day of gestation and in no instance were lymphocytes observed in the parenchyma of the bone marrow.

By the twenty-sixth to twenty-seventh days of gestation, erythroblasts and normoblasts appeared in large numbers in the fetal rib marrow, first in the oldest part of the marrow and then extending toward the epiphyses (Figs. 10-12).

Erythroblasts and normoblasts occurred first in colonies associated with proerythroblasts and rounded mesenchymal cells; as their number increased in the later stages of embryonic development, they were scattered diffusely in the extravascular spaces with or without associated precursor cells. The early cells of the erythrocytic series were never seen within vessel lumina or in any consistent relationship to the vessel walls; cells of the erythrocytic series never appeared in tightly packed colonies or cords consistent with the notion of their being enclosed within cell processes or in any way isolated from the surrounding cells and fibers of the bone marrow stroma -- rather they lay scattered at random among the elements of the marrow parenchyma. It is remarkable that only rarely were observed colonies of cells in the same stage of development; rather all stages of development from rounded mesenchymal cells to mature erythrocytes appeared mixed at random in the extravascular space.
At the same time there appeared megakaryocytes in various stages of
development; the mature cells of this series usually occupied a
position near the wall of the sinusoid but lay nonetheless in the
same extravascular space as the developing erythrocytes. At the
twenty-seventh day of gestation numerous mature erythrocytes appeared
among the immature cells of the erythroid series; normoblasts ex-
truding their nuclei were numerous in the extravascular spaces at
this time.

From the twenty-seventh to the twenty-eighth day of gestation
(Figs. 11-15) granulocytes made their first appearance in the older
mid-shaft region of the rib marrow after erythropoiesis had been
well established. Transitional forms structurally relating rounded
mesenchymal cells to myeloblasts occurred distributed at random among
the population of erythrocytic elements. The relative position of
the myeloblast and subsequent generations of more differentiated
myelocytic forms was variable with respect to the sinusoidal walls
and the endosteum. Cells of the myelocytic series never appeared
isolated or walled off from the surrounding erythrocytic cell popu-
lation; rather, the two populations were mixed at random in the same
extravascular space of the bone marrow.

There appeared to be a reciprocal relationship between the
populations of osteoblasts and hemopoietic elements. At the epi-
physeal plates the osteoblasts and their mesenchymal cell precursors
were the most numerous cellular elements; whereas, the hemopoietic
elements were scarce. In the old mid-shaft region of the bone,
hemopoetic elements made up the larger part of the cell population and osteoblasts were only sparsely represented along the bony spicules. Thus, a marrow area rich in osteoblasts was transformed within one to two days into an area showing only a few of these cells; it was remarkable that during this transformation few if any degenerating osteoblasts could be identified. Series of transitional forms structurally relating typical osteoblasts to spindle-shaped reticulum cells and to rounded mesenchymal cells were encountered in the primary marrow space and very frequently encountered in the later stages after hemopoiesis had begun (Figs. 17-20). Further, little evidence of migration of osteoblasts from an older part of the marrow space to the epiphyseal areas was observed, thus suggesting that at least some osteoblasts transform into cells morphologically indistinguishable from those having undiminished developmental potentialities.
DISCUSSION

Erythropoiesis in the mammalian bone marrow has been extensively studied in the past; at the present time it is generally accepted that erythrocytes arise from undifferentiated mesenchymal cells in the extravascular spaces of the bone marrow. The results of our observations, we believe, conclusively establish this site and mode of origin of erythrocytic elements in the fetal bone marrow. Many of the difficulties inherent in earlier investigations have been eliminated by improved methods of fixation and tissue preparation which have permitted us to study fetal erythropoiesis in nearly artifact free section.

It may be necessary to re-examine some of the problems and theoretical considerations related to erythropoiesis in the light of the results of this investigation as well as other recent studies of bone marrow morphology. The following problems related to erythropoiesis might well be considered: (1) the entrance of mature erythrocytes into the circulation, (2) the source of precursor cells of erythrocytes and (3) the factors determining the differentiation of multipotential stem cells along the pathway of erythrocyte development.

Most investigators (Drinker, Drinker and Lund '22; Maximow '27; Bargmann '30; Weinbeck '38; Hamre '47; Dacie and White '49;
Lennert '52; Knoll '57; Rohr '60; Weiss '61; Burkhardt '64) have agreed that mature red blood cells flow into the general circulation from the extravascular spaces through discontinuities in the sinusoidal walls. The fenestrations in the walls of the sinusoids permit erythrocytes and plasma of the circulating blood to flow freely into the extravascular spaces and wash mature erythrocytes free from the surrounding stroma and into the venous sinusoids (Maximow '27; Weiss '61; Zamboni and Pease '61; Burkhardt '64). Certain proponents of intravascular erythropoiesis (Cunningham and Doan '22; Doan '22; Sabin '23; Doan et al. '25; Muller '26; Peabody '26; Muller '27; Doan '31; Doan '38), however, have described a closed circulation in the mammalian bone marrow and have suggested that the red blood cells develop within collapsed intersinusoidal capillaries which can dilate to release the mature erythrocytes into the general circulation. This and other recent investigations (Pease '56; Zamboni and Pease '61; Weiss '61; Burkhardt '64; Weiss '65) have failed to recognize the existence of intersinusoidal capillaries in the mammalian bone marrow or of a closed bone marrow circulation.

Electron microscopy (Pease '56; Zamboni and Pease '61; Weiss '61; Weiss '65) has shown that the walls of the sinusoids of the bone marrow are very thin and represent an extremely labile system of fenestrated membranes which allow the circulating blood to communicate freely with the extravascular spaces. These membranes represent rather transient features which at one instant may form an effective barrier between the circulating blood and the
extravascular spaces; at the next instant portions may be absent, permitting areas of developing cells in the extravascular spaces to be exposed to the circulating blood of the sinusoids. So labile are the membranes and so inconsistent are the walls of the sinusoids that Weiss (Weiss '61) has suggested that the terms extra- and intravascular are meaningless when applied to the adult mammalian bone marrow. Our observations on fetal material, however, suggest that there is adequate morphological evidence to separate the intra- and extravascular spaces; even though the walls of the sinusoids are fenestrated there are very definite and well-defined channels through which the circulating blood flows. The number of fenestrations in the walls appear to increase with the age of the fetus; in the early stages of bone marrow development only a few discontinuities are evident in the sinusoids, while in the older fetuses and in the neonatal rabbit, fenestrations are more numerous. It has been shown (Burkhardt '64) that the number of discontinuities in the sinusoidal walls also varies in pathological conditions. In certain severe anemias where delivery of erythrocytes into the circulation is relatively low, only few fenestrations are seen; while in conditions of increased "washing out" of erythrocytes, discontinuities in the sinusoidal walls are extremely numerous. It appears that in situations where the rate of delivery of erythrocytes into the circulation is relatively low, e.g., early fetal bone marrow and in the bone marrow of certain severe anemias, the sinusoidal wall is a more effective barrier between the extravascular spaces and the venous
sinusoids than in situations of increased delivery of erythrocytes into the circulation. The possible correlation between the rate of delivery of erythrocytes from the extravascular spaces and the number of discontinuities in the sinusoidal walls offers further evidence that these discontinuities represent the site of entrance of erythrocytes into the circulating blood.

Four cell types have been proposed for the cell of origin of the erythrocyte: (1) the mesenchymal cells which have invaded the primary bone cavity from the periosteum (Maximow '07; Schridde '09; Maximow '24; Maximow '27; Bargmann '30; Weinbeck '38; Gilmour '41; Schleicher '46a; Schleicher '46b; Hamre '47; Dacie and White '49; Lennert '52; Knoll '57; Rohr '60; Burkhardt '64), (2) the endothelial cells of the bone marrow (Cunningham and Doan '22; Doan '22; Sabin '23; Doan et al. '25; Muller '26; Peabody '26; Muller '27; Doan '31; Doan '33), (3) the circulating lymphocyte (Jordan '39; Yoffey '54), and (4) the osteoblast which may represent an additional source of hemopoietic precursor cells (Heller et al. '50; Branemark and Breine '64). In order to establish morphologically an endothelial cell origin of erythrocytes in the bone marrow, the following would have to be observed: endothelial cells differentiating in situ into large round basophilic cells; mitoses of endothelial cells perpendicular to the sinusoidal wall; and cords of cells representing collapsed endothelial tubes containing developing erythrocytic elements.

The results of this investigation as well as other recent studies (Pease '56; Zamboni and Pease '61; Weiss '61; Burkhardt '64)
have failed to visualize any of these cellular relationships; and at the present time it appears that the possibility of an endothelial cell contribution to erythropoiesis in the mammalian bone marrow is extremely remote. The possibility that blood-borne lymphocytes may invade the extravascular spaces of the bone marrow and there undergo further differentiation into erythrocytes has been carefully considered in this study. Morphological evidence which would establish a lymphocytic contribution to erythropoiesis must demonstrate differentiation of lymphocytes within the extravascular spaces and sequential series of cells representing transitional forms from the circulating lymphocyte to developing erythrocytic elements. None of the sections examined in this study disclosed lymphocytes in the circulating blood of the bone marrow sinusoids until the thirty-first day of gestation; moreover, examination of peripheral blood smears of the fetuses showed very few lymphocytes until the twenty-seventh day of gestation -- a time when erythropoiesis was well under way in the bone marrow. No typical lymphocytes appeared in the bone marrow stroma of the sectioned material examined.

Thus, although the possibility of lymphocytic contribution to erythropoiesis exists, we have been unable to obtain morphological evidence in support of this concept in the rabbit fetus. We suggest that the main source of erythrocytic precursors in the fetal bone marrow of the rabbit is the mesenchymal cell which has invaded the primary bone marrow cavity from the periosteum. The flat, spindle-shaped cells of the inner or osteogenic layer of the periosteum
invade the open lacunae of the degenerating cartilage cells and there differentiate into stellate cells similar to those of undifferentiated embryonic mesenchyme. These undifferentiated mesenchymal cells then, through sequential stages of rounding up and increasing cytoplasmic basophilia, differentiate into osteoblasts, megakaryocytes, fat cells, precursor cells of the erythrocytic and myelocytic elements (hemocytoblasts) and the stromal cells of the bone marrow. Transitional forms morphologically relating typical osteoblasts to rounded up mesenchymal cells have been observed in the fetal bone marrow; these observations support the results of experimental investigations (Heller et al. 50; Branemark and Breine '64) in suggesting that osteoblasts may represent a possible additional source or hemopoietic precursor cells.

Certain investigators have suggested (Danchakoff '18; Sabin '23; Jordan '35; Jordan '39) that environmental factors determine the differentiation of a multipotential stem cell along the erythrocytic or myelocytic line of development and that these environmental factors consist in a set of conditions present in the circulating blood. According to this view, a stem cell exposed to the circulating blood is determined along the erythrocytic line of development; whereas, a stem cell isolated from the circulating blood is determined along the myelocytic line. In order to support this argument with morphological evidence, the following conditions must be observed in the bone marrow: (1) the isolation of one group of stem cells in such a way that one group is exposed to the circulating blood at the
exclusion of the other, (2) the isolation of developing erythrocytic and myelocytic elements into different compartments of the bone marrow. Differential localization of centers of hemopoietic activity has been described for the adult bone marrow (Weinbeck '38; Lennert '52; Rohr '60; Burkhardt '64); both erythropoietic and myelopoietic areas are clearly located in the extravascular spaces; however, according to these authors, the erythrocytic elements lie in closer association to the walls of the sinusoids than do the myelocytic elements.

Our observations of fetal bone marrow have not disclosed any separation either of stem cells or of developing erythrocytic elements into separate compartments within the bone marrow; nor have we observed any preferential localization of erythrocytes in any particular area of the bone marrow. Rather, this study has shown in the fetal marrow a random arrangement of both erythrocytic and myelocytic elements in the extravascular spaces. The results of electron microscopic studies of definitive bone marrow (Pease '56; Zamboni and Pease '61; Weiss '61) are in agreement with our observations; developing erythrocytic and myelocytic elements have been observed mixed at random in the extravascular spaces and exposed to the same tissue fluid. The objection may be raised here: How is it possible for a population of morphologically similar cells existing under identical environmental conditions to differentiate along more than one line of development? We suggest that even though the multipotential stem cells are morphologically similar, we can by no means
accept the theory that the cells have identical developmental potentialities. Ackerman (Ackerman '64) has suggested that blast cells exhibiting cytoplasmic basophilia indicative of protein synthesis are already differentiated; and that such differentiation must have taken place in an earlier stage of development, e.g., in the stellate reticulum or mesenchymal cell, as a result of differences in the sequential coding of RNA. Histochemical studies have demonstrated biochemical differentiation in the very early developmental stages (the mesenchymal cell) of the megakaryocyte in the fetal liver (Ackerman and Knouff '60); in this case morphologically similar mesenchymal cells showed chemical evidence of different developmental potentialities. It may be suggested, then, that stem cells of the bone marrow may be irrevocably determined along a specific line of development even before evidence of cytoplasmic basophilia and/or morphological differentiation can be observed. The actual factors involved in initiating and regulating the differentiation of erythrocytes are unknown; however, we suggest that these factors must exert their influence at a very early stage of erythrocytic development -- mesenchymal cells -- and that environmental factors such as the relative position of stem cells within the bone marrow are not involved in erythrocytic development in the fetus. Further investigations must be carried out to determine whether or not developmental potentialities of the mesenchymal cells of the bone marrow can be predicted through differences in biochemical and enzymatic activities.
SUMMARY

Erythropoiesis in the ribs and long bones of sixty-five rabbits ranging from eighteen days of gestation to the second day after birth was studied by improved methods of fixation and tissue preparation. Our observations indicated that --

1. Erythrocytes develop in the extravascular spaces of the bone marrow and enter the circulating blood through discontinuities in the sinusoidal walls.

2. Endothelial cells of the fetal bone marrow make no contributions to erythropoiesis.

3. Blood-borne lymphocytes make no contribution to erythropoiesis in the fetal marrow.

4. The precursor cells of both myelocytic and erythrocytic elements are morphologically indistinguishable; no evidence was observed suggesting that precursor cells were determined along one line of differentiation by their relative position within the bone marrow.

5. Developing erythrocytes and myelocytic elements appeared mixed at random in the extravascular spaces; no isolation of erythrocytic elements from the developing myelocytes or stromal elements was observed.

6. Osteoblasts may represent a possible additional source of hemopoietic precursor cells in the fetal marrow.
Figure 1

Cross section through the rib of the fetal rabbit of twenty days gestation. Note the osteoblasts (o) lined up along the calcified cartilage spicules (s). The extravascular space is filled with a heterogeneous population of mesenchymal cells (m) and fibers in a semi-fluid ground substance. The bone marrow vessels (ss) at this stage are represented by slightly dilated capillaries with very irregular walls. Toluidine blue staining.

Figure 2

Fetal rabbit rib at twenty-three days of gestation. The extravascular spaces show more organization than in Figure 1. The osteoblasts appear more regularly lined up along the calcified cartilage spicules. The vessels at this stage are represented by extremely thin-walled sinusoids (ss). The extravascular space between the sinusoidal walls and the osteoblasts is filled with mesenchymal cells in various stages of rounding up (m) and increasing cytoplasmic basophilia. Toluidine blue stain.
Figure 3

The bone marrow of the rib of the fetal rabbit of twenty-three days of gestation showing further examples of the rounding up of mesenchymal cells in the extravascular spaces. Toluidine blue stain.

Figure 4

The bone marrow of the fetal rabbit of twenty-five days of gestation. Note the proerythroblasts and erythroblasts (arrows) in the extravascular spaces among the rounded mesenchymal cells (m) and osteoblasts (o). Toluidine blue stain.
Figure 5

Cross section of the rib of the fetal rabbit of twenty-five days of gestation. Note the transitional forms (arrows), clearly located in the extravascular space, relating mesenchymal cells (m) to proerythroblasts (p). Toluidine blue stain.

Figure 6

Similar to Figure 5 showing intermediate stages (arrows) in the transformation of mesenchymal cells into proerythroblasts. A mitotic (x) figure is shown in an endothelial cell of the sinusoidal wall. Bone marrow of the fetal rabbit of twenty-five days of gestation. Toluidine blue stain.

Figure 7

Section of bone marrow of the fetal rabbit of twenty-five days of gestation showing numerous proerythroblasts and erythroblasts (arrows) lying in the extravascular space. An osteoclast (c) is also shown. Toluidine blue stain.

Figure 8

Bone marrow of the fetal rabbit of twenty-five days of gestation showing numerous proerythroblasts (p) and erythroblasts (e) in the extravascular space. Arrows indicate mitotic figures in erythroblasts. Note the extreme thinness of the sinusoidal walls.
Figure 9
Bone marrow of the fetal rabbit at twenty-six days of gestation. Shown between the osteoblasts (o) and the sinusoids (ss) are two reticulo-endothelial cells (r). One reticulo-endothelial cell is surrounded by erythroblasts (arrows); this arrangement is suggestive of the phenomenon of rhopheocytosis.

Figure 10
Similar to Figure 9 showing developing erythrocytic elements among the fibers of the extravascular spaces. Also shown are osteoblasts (o) and a reticulo-endothelial cell (r). Bone marrow of the fetal rabbit at twenty-six days of gestation. Toluidine blue stain.

Figure 11
Bone marrow of the fetal rabbit of twenty-seven days of gestation. Two developing myelocytes (arrows) are shown in the extravascular space among the population of developing erythrocytic elements. Also shown is a reticulo-endothelial cell (r) exhibiting evidence of active phagocytosis. Toluidine blue stain.

Figure 12
Similar to Figure 11 showing developing myelocytic elements in the extravascular space along with numerous developing erythrocytic elements. A reticulo-endothelial cell (r) is shown surrounded by erythroblasts. Bone marrow of the fetal rabbit at twenty-seven days of gestation. Toluidine blue stain.
Figure 13

Bone marrow of the fetal rabbit at twenty-eight days of gestation showing the extravascular space filled with developing erythrocytic and myelocytic elements as well as reticulo-endothelial and mesenchymal cells. Arrows indicate a discontinuity in the sinusoidal wall. Toluidine blue stain.

Figure 14

Similar to Figure 15 showing an increase in the bone marrow population and a crowding of the extravascular space. Bone marrow of the fetal rabbit at twenty-eight days of gestation. Toluidine blue stain.

Figure 15

Bone marrow of the fetal rabbit at twenty-eight days of gestation showing a transitional series of developing erythrocytic elements from the mesenchymal cell (m) to the normoblast (n). Also shown are reticulo-endothelial cells (r). Toluidine blue stain.

Figure 16

Bone marrow of the fetal rabbit at twenty-nine days of gestation showing developing erythrocytic and myelocytic elements in the extravascular spaces. Areas of discontinuities in the sinusoidal walls are indicated by arrows. A megakaryocyte (k) is shown lying near the sinusoidal wall. Toluidine blue stain.
Figure 17

Bone marrow of the fetal rabbit at twenty days of gestation showing numerous cells (arrows) representing transitional forms between osteoblasts (o) and mesenchymal cells (m). Toluidine blue stain.

Figure 18

Similar to Figure 17 showing numerous transitional forms (arrows) which suggest a relationship between osteoblasts (o) and mesenchymal cells (m). Bone marrow of the fetal rabbit at twenty days of gestation. Toluidine blue stain.

Figure 19

Bone marrow of the fetal rabbit of twenty days of gestation showing numerous examples of transitional cell forms (arrows) intermediate between osteoblasts (o) and mesenchymal cells (m). Toluidine blue stain.

Figure 20

Similar to Figure 19 showing further evidence of cells (arrows) which are intermediate in a transition from osteoblasts (o) to mesenchymal cells (m). Bone marrow of the fetal rabbit at twenty days of gestation. Toluidine blue stain.
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