STUDIES ON BLOOD

THE VITALLY STAINABLE GRANULES AS A SPECIFIC CRITERION FOR ERYTHROBLASTS AND THE DIFFERENTIATION OF THE THREE STRAINS OF THE WHITE BLOOD-CELLS AS SEEN IN THE LIVING CHICK'S YOLK-SAC

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In 1920 the writer [1] published an account of a study of the origin of the vascular sytem as it can be made out by watching the living chick blastoderm of the second day of incubation. The method of the origin of vessels can be made out in such specimens, in the area pellucida of the yolk-sac, in stages which range from the time just before the first somite through the stage of about twenty somites.

In the area pellucida there are three well-known layers, the ectoderm, the double layer of the mesoderm lining the extra-embryonal cœlom, and the endoderm. The blastoderms are mounted in a hanging drop preparation, in Locke-Lewis solution, with the endoderm against the coverslip. The area pellucida is so thin that the endoderm, the vascular zone between the endoderm and mesoderm, and the mesoderm can all be analyzed with an oil immersion lens. The technique was described in 1920.

Blood-vessels begin by the differentiation of a new type of cell from mesoderm. This cell moves out of the mesoderm, develops a dense, basophilic, azurophilic cytoplasm and becomes physically more refractive than mesoderm. As soon as this cell divides, it shows one of its essential characteristics, namely, the tendency of the cells to stay together to form syncitial masses. These masses of cells put out sprouts by which they join similar masses to make a plexus. Both, while such solid clumps are still isolated, and after the plexus is formed, the cells become transformed into vessels by a liquefaction of the central part of the mass to make blood-plasma, while the periphery differentiates into endothelium.

There is a progressive differentiation of angioblasts, beginning in the periphery of the area vasculosa at the stage of two somites; the cells gradually appearing nearer and nearer the embryo, until, at the stage of five or six somites, angioblasts differentiate in the axial line of the embryo as forerunners of the endothelium of the heart and aorta. The heart, aorta and main vessels of the embryo differentiate *in situ* from angioblasts and increase by the addition of newly differentiated cells as well as by the cell-division and the sprouting of their endothelial walls. The amount of the differentiation of new cells grows less but at what stage it ceases is not known. Thus there is established the fundamental morphology of the vascular system.

Blood-vessels arise by the development of a new type of cell, the vasoformative cell of Ranvier, or the angioblast of His. This cell produces the first fluid of the blood; thus, endothelium is primary and blood-plasma secondary. Since there is a tissue fluid before angioblasts arise, endothelium is from the start a membrane between two different fluids, tissue-fluid and plasma. Moreover, the process of liquefaction is intra-cellular, that is, it can be seen in chains of single angioblasts which become vessels and it can also be seen to take place in the sprouts which are processes of cells, hence the lumen of vessels is embryologically intracellular and thus not a tissue-space.

In the living chick of the second day, it can be seen that both angioblasts and the endothelial cells give rise to red blood-cells. Erythroblasts begin in the chick in the vessels of the outer margin of the area opaqua in the stages of from 7 to 11 somites. In the area pellucida, where they can be seen in the living specimen, angioblasts differentiate during the stages of from 5 to 11 somites, while the vessels form and erythroblasts begin during the stage of from 11 to 14 somites. The heart begins to beat at the stage of 10 somites and the circulation starts when the chick has 16 to 17 somites.

During the past year, I have been continuing these studies on the living chick, and have found that it is possible to mount the entire blastoderm on a large cover-slip, one measuring 42 by 50 mm., throughout the third and fourth days of incubation. The cover-slips must be entirely free from grease or the membrane will not flatten out on the glass. From the fifth day on, the chick is too heavy to mount in the hanging-drop form, but if the specimen be transferred to a dish of Locke-Lewis solution, the amnion can be opened, the allantois pushed aside and the yolk-sac cut off close to the embryo and then spread out and mounted. The circulation, of course, stops but the membrane can be mounted and kept alive certainly for from three to five hours. So far, I have studied these living membranes only through the first seven days of incubation. It is an advantage to mount the chick with the membranes because the preparations are all fixed after the vital studies have been made and many of them are stained and mounted *in toto*. If the embryo has been left attached, it can be cut off in the alcohol and then the entire ectoderm can be dissected off from the area vasculosa. The specimen is thus made thinner and much easier to analyze. When the embryo has been cut away at the start, the ectoderm clings too closely to the specimen to be taken off and makes one more layer of stained cells in the final specimen.

I have found it a great advantage to study the embryos with a vital dye and add 1 to 3 drops of a 1 per cent aqueous solution of neutral red to 10 c. c. of the Locke-Lewis solution, making a dilution of possibly 1 to 10,000 of the dye, or less. This may be termed the physiological dilution of the dye.

After the specimens have been studied, they are fixed by floating the cover-slip, embryo down, on Bouin's picro-formol and then keeping them in 70 per cent alcohol until all the picric acid is removed. They are then stained in hæmatoxylin and counterstained in eosin with a little orange G. This fixation is excellent for the granulocytes, but quite worthless for the erythrocytes after the primitive stage. After fixation in Bouin's solution the young erythrocytes show only a wide-meshed reticulation having no relation whatever to any of the substances that can be made out in the living cell. No fixation of the blastoderms is adequate to follow the changes in the red cells, but they can be identified best after fixation in the vapor of formalin if not applied too long. I use it for from ten minutes to half an hour. In this the hemoglobin is well preserved but not the basophilic cytoplasm and indeed the earliest traces of hemoglobin which can be seen in the living cell by a distinct yellow color cannot be detected in the fixed specimens. Helly's fluid, which preserves the basophilic substance better, cannot be used, because the blastoderms float off from the cover-slip almost immediately and wrinkle so that they can never be studied as a section with an oil immersion lens.

In connection with the study of these blastoderms, a drop of blood is drawn from the vessels when the egg is first opened and used for a film. These films of blood are studied vitally by Pappenheim's method. They are made as follows: A clean glass rod is dipped into a dye and drawn across a perfectly clean glass slide. The two dyes which I have used are neutral red and brilliant cresyl blue, made up either in a 1 per cent aqueous solution or in a saturated alcoholic solution. The even film of the stain dries quickly and its strength is estimated by the color. The film must not be dense enough to stain any nuclei. A drop of blood is then drawn out with a fine glass cannula, placed on a cover-slip which is then inverted on the film of stain, ringed at once with salvoline and placed in a warm box. The vital stains develop slowly, on an average, in ten minutes, and if permanent preparations are wanted, the specimen is watched under an oil immersion lens until the staining is best, then the cover is drawn off from the slide and the film of blood counterstained with one of the blood stains. I have used Wright's eosin-methylene blue. In blood from chicks on the second and third days of incubation the amount of the specific substance of the red cells, that is, the megaloblasts, stainable in the vital dye is so massive, that it is necessary to differentiate the specimens after staining by Wright's method. This can be done in absolute alcohol and the decolorizing stopped with xylol. The white cells are so very unevenly distributed in the vessels of the early blastoderms that no film of blood represents adequately the amount of differentiation for the stage. Hence, each stage must be studied by both methods, by a survey of the total area pellucida and by drops of blood with specific stains.

In these studies, it can be seen that there are three different strains of blood-cells, first, those that arise from endothelium, which include both the red cells and the monocyte strain of the white cells; secondly, the granulocytes, and thirdly, the lymphocytes. The red cells begin to differentiate on the second day of incubation. On the third day the endothelium gives rise to the monocytes, that is, to the large mononuclears and the transitional forms of Ehrlich. In two different specimens I have seen an occasional monocyte on the second day, but the process becomes active only on the third day. The group of the monocytes of the blood is especially well illustrated by Pappenheim on his Plate 1,[33] as the third row of cells, and as the fourth and fifth rows on Ferrata's Plate 12.[13]

At the same time that the endothelium gives rise to the monocytes, namely, beginning on the third day, it gives rise to a much more numerous extravascular group of cells, identical with the monocytes, which are the clasmatocytes of the connective tissues. On the third day also the granulocytes begin to differentiate as a new type of cell from the mesoderm. These cells develop a specific type of granulation and wander into the blood-vessels. The third strain is the lymphocytes. These I have never seen differentiating in the wall of the yolk-sac; they begin to appear in the blood stream on the fourth day but do not become marked until the fifth and sixth days. It may be that they arise only within the embryo itself.

ERYTHROBLASTS

In these studies it has been possible to establish a criterion for a primitive red cell. As was discovered by Pappenheim,[31] the primitive red cell has a basophilic, azurophilic cytoplasm so finely granular as to appear like ground glass. Fixed to show this basophilic cytoplasm, the cell looks like a single angioblast. In the living cell a droplet of yolk is occasionally to be seen. If, however, one stains the cell supravitally with either neutral red or with brilliant cresyl blue, there appears a very massive granulation which at first completely fills the cell. This granulation is completely soluble in alcohol and in all of the usual fixatives; it disappears also in the vapor of formalin. If, however, it be stained with neutral red or with brilliant cresyl blue, it becomes insoluble in methyl alcohol and hence can be seen in films stained with Wright's eosin-methylene blue. For these double stains brilliant cresyl blue is slightly better. This special granulation is then very easy to bring out in films of blood. It is not so easy to stain in the total blastoderm, because the dilution which stains the neutral red granules of endothelium, the yolk and all of the stainable substances of the clasmatocytes, that is, the dilution of 1 to 10,000, is too weak to stain the granulation of the reds, but it does, nevertheless, make it just visible. The primitive red cell in the living blastoderm can be stained, however, by injecting the dye directly into the blood-stream; or if a drop of 1 per cent solution of neutral red is put on the blastoderm for a few seconds and then washed off with clear Locke-Lewis solution, the red cells show the stain well.

The question must come up as to whether the stainable substance actually exists in the living cell in some state from which it is precipitated by the dye, just as Mott has shown that the Nissl substance, in its stainable form, develops only as the nerve cell dies. Indeed, when Israel and Pappenheim [11] first described the vital staining of substances in erythrocytes, they

saw that the staining commenced only as the cell began to die. This may be true. The criterion I have used for the actual death of the cell is whether the nucleus stains or not, and this specific substance does stain in dilutions which do not stain the chromatin of the nucleus at all. The exact dilution necessary to stain it must be worked out.

For the permanent films of blood on the second and third days, the granulation is so dense that, after counterstaining with Wright's stain, the cells must be differentiated in absolute alcohol, the decoloration being stopped with xylol.

On the second and third days, the red cells being megaloblasts, both the granulation and the basophilic cytoplasm completely fill the cell. On the fourth day a narrow rim of clear cytoplasm appears in many of the cells making erythroblasts, showing hemoglobin around the edge free from granules, while the granules make a very dense rosette or wreath around the nucleus. These rosettes are very characteristic and in stained preparations they greatly obscure the nucleus. By the fourth day the red cells have no longer the somewhat uniformly round shape of the earlier stage and there is no longer a comparative uniformity in size, but rather there are many much larger forms together with many small irregular or oval cells. This period of great variation in size is a stage of active division of the cells as well as of growth of individual cells. In the small oval cells, the rosettes are oval and in division the rosettes divide, so that each daughter nucleus is surrounded by a wreath of granules before the cells separate.

On the fifth day a few of the red cells begin to show a diminution of the granulation, some of the cells grow much larger and the granules and rods begin to spread out into the cytoplasm, the cells showing the polychromasia and the reticulation which is known to be characteristic of the so-called reticular forms occuring in anæmias in human blood. By the seventh day there are large numbers of the cells in the reticular stage, while there are still many of the primitive cells and the wreath forms. Gradually the amount of the basophilic cytoplasm and of the vitally stainable granulation decreases while the hemoglobin increases. Finally, the basophilic cytoplasm disappears, but a small amount of the specific granulation remains in each erythrocyte. At the time of hatching and for three days afterward, all of the red cells in the circulating blood show from one to eight or ten vitally stainable granules. I have not carried the studies farther nor studied the stages between the seventh day of incubation and the time of hatching. It is clear, however, that one can work out the types of cells that are characteristic for each stage of the developing chick. Of course, at any stage there are some cells characteristic of preceding stages. On the second day only the primitive stage, the megaloblast, is present, and then, with a given increment of time, the different stages in the development of this specific granulation are added. When such a study has been made for a mammalian form and especially for the human embryos, as is now feasible, we shall be in a position to estimate just how primitive are the cells that appear in the circulation in anæmias.

The first account of the staining of the specific granulation of the red cells, which I have been able to find is in a paper by Israel and Pappenheim[17] in 1896, in which they say that if a few dry grains of neutral red are placed on a slide and used for a film of fresh blood, there will appear a granulation in some of the red cells just as the cell begins to die. In 1901, Bettmann[2] described the use of vital neutral red in the staining of red cells in pathological conditions, but did not discriminate between a staining of the nucleus and the specific granulation around it. Three years later, Rosin and Bigergeil[35-36] described the methods of vital staining and the dyes to be used, but it was not until 1907 that we have a clear account of the vitally stainable granulation of the red cells. In 1907 there appeared three papers in the Folia Hæmatologica, by Caesaris-Demel,[4] by Pappenheim[24] and by Ferrata,[13] in which the vitally stained granules are described and illustrated. Caesaris-Demel shows the stage of the wreath around the nucleus, not, however, in as primitive a stage as on the fourth day of incubation in the chick. He shows also the reticular stages and the final stage of a few granules. He distinguished between the deeply staining granules and the more faintly staining filaments.

The primitive basophilic cell, which is the first red blood-cell, was first differentiated by Pappenheim and called the megaloblast. It has a basophilic cytoplasm and a large nucleus, poor in chromatin, and a conspicuous nucleolus. It becomes an erythroblast and all the stages of the development of this cell, as far as concerns the decreasing of its basophilic cytoplasm, the increasing of its content of hemoglobin and the changes of its nucleus, have been worked out with the eosin-azur technique in final perfection by Pappenheim,[21-24] Ferrata,[12] Danchakoff,[5,6] Maximow[22-24] and Weidenreich.[39-42] The development of this cell with reference to its specific granulation is now necessary to complete its life history.

In the chick, it has been shown that all of the primitive blood-cells on the second day of incubation are megaloblasts which become erythroblasts as soon as a trace of hemoglobin can be made out. These cells are derived from angioblasts and from endothelium. As far as the specific granulation is concerned, the first stage, on the second and third days of incubation, has the granulation throughout the cell. From the fourth to the sixth day, there are the rosette or wreath forms in which the granulation is around the nucleus. On the seventh day most of the cells show the reticular form of the granulation. All of these stages show diffuse basophilia. With further studies it will be possible to tell just when basophilia and the reticular forms disappear in the majority of the red cells. At the time of hatching all of the cells in the circulation have acidophilic, hemoglobin-bearing cytoplasm with a few vitally stainable granules. It is, of course, clear that at any stage in development, while the majority of the cells are in a specific phase, a few of the earlier types may be found. In normal human blood about one per cent or less of the erythrocytes show a few vitally stainable granules.

The question which must come up first in connection with this granulation is its relation to the so-called basophilic punctation, and both Pappenheim and Ferrata agree that these two substances are entirely different. The basophilic punctation stains in azur after fixation and Ferrata [13] thinks that it is an abnormal clumping "conglobation" of the azurophilic cytoplasm, and he shows the gradual production of the punctate forms in red cells after experimental lead poisoning on his Plate VIII. It is thus easy to see why basophilic punctation does not occur in embryonic blood, whereas the vitally stainable granulation is specifically characteristic of development.

Another point in which this specific granulation may prove of interest is that it offers a chance to study the development of hemoglobin in the cell by testing the granulation for the presence or the absence of iron. Both the azurophilic cytoplasm and the granulation disappear as hemoglobin develops, but the granulation alone is characteristic of the red cell as distinct from all other blood cells.

In the developing blood there are always a few cells containing the Howell-Jolly bodies. These are fragments of nuclei staining just like chromatin, which were discovered by Howell,[16] in 1890, in a study of the blood of the cat after hemorrhage. Of course the corpuscles of the chick are all nucleated, so that the question of the extrusion of nuclei does not come up, but an occasional cell, from the very beginning of the formation of blood on the second day, shows a fragmented nucleus. I interpret such cells as dead. They are to be found in the early blood islands before the cells become free and are very interesting as showing that cell death occurs in the early stages of marked cell-division and growth.

ORIGIN OF MONOCYTES AND CLASMATOCYTES FROM ENDOTHELIUM

The separation of the clasmatocyte as a distinct type of cell of the connective tissues is due to Maximow.[20] He showed that by introducing two sterile cover-slips under the skin in rabbits, one could separate three types of cells by the speed with which they passed between the covers, leucocytes appearing first; a special cell, the clasmatocyte, wandered in during the first nineteen hours and the fibroblast in from two to four days. Then he showed that the clasmatocyte was specifically sensitive to neutral red,[21] while Bouffard,[5] Goldman [14,15] Evans,[7-11] Schulemann [7-9] and a large group of workers have demonstrated that it is the cell of the connective tissues most specifically differentiated to phagocytize and store particulate matter. The specific reaction of this cell to vital, neutral red is that the dye stains certain granules of the cell and certain large fluid spheres which are called vacuoles, the so-called "neutral red granules and vacuoles" of Lewis and Lewis.[12] These vacuoles are organs into which the cell passes phagocytized particulate matter. In the vacuoles the fine particles which the cell has taken up become clumped and, as Evans and Scott [11] have shown, may even be re-crystallized.

Aschoff and Kiyono [1] then showed that an identical reaction to a vital dye could be obtained by certain cells of the blood, namely the group Naegeli [30] has called the monocytes, which are the large mononuclear and transitional forms of the Ehrlich school. Thus they distinguished and related histiocytes of the blood and histiocytes of the connective tissues. Moreover, they regarded the histiocytes of the blood as of endothelial origin.

Pappenheim and Ferrata have illustrated the separation of the monocytes, the leucocytes, and the lymphocytes on purely morphological grounds, believing that there is a common stem-cell, a hypothetical hæmatoblast for them all. Aschoff and Kiyono separate the monocytes, calling them histiocytes of the blood, on a physiological basis, and I think that I can demonstrate on a fundamental embryological basis that the monocyte and the clasmatocyte are identical cells, derived from endothelium, thereby making one of the three great groups of connective-tissue cells that contribute to the blood.

If a blastoderm of the third day of incubation be stained in vital neutral red, the endothelium stands out with numerous granules staining in the dye which are both around the nuclei and scattered in the thin periphery of the cytoplasm. The endothelium of the capillaries and the veins often becomes reduplicated. Endothelium is more refractive than mesoderm, and this property, as well as the granules stainable with neutral red, characterizes both of these layers of endothelium. One of the cells of the inner row can then be seen to enlarge, protrude into the lumen and develop the vacuoles which are characteristic of clasmatocytes. The periphery of the cell then puts out a film of cytoplasm in which there is a central process more refractive than the rest, simulating a flagellum, and these films are in constant motion. In fact the eye is attracted to these cells both by the stained vacuoles and by the motion of the peripheral films of cytoplasm. Such a cell then gradually becomes free. The characteristic motion of the peripheral films continues, keeping the surrounding fluid moving, though the cell itself shows very little locomotion. In the study of the origin of the red blood-cells on the second day of incubation (Sabin)[27] it was noted that the erythroblasts formed great clumps of cells attached to the inner surface of a complete endothelium. The monocytes, on the other hand, differentiate and drop off as single cells, leaving the original endothelial cell from which they came as the wall of the vessel.

An endothelial cell may become phagocytic while it is yet in place, for I have seen them with red cells engulfed just as Maximow [22] shows for a mammal in his Fig. 4 on Plate XVIII. This means that an endothelial cell which is actually a part of the wall of a vessel, not one of the reduplicated forms already on the road toward becoming free, may be phagocytic. That is to say, endothelium is itself phagocytic as well as having the power to give off free cells which are phagocytic. In the same figure quoted above, Maximow shows three free monocytes, very characteristic, labeled Edph. He recognized them as desquamated endothelium but did not identify them as monocytes. In fact all of the early stages of the develop-

ment of blood are beautifully illustrated by the two plates of Maximow in this same article.

While these few cells are getting free in the lumen of the vessel to make the monocytes of the blood, the outer row of the reduplicated endothelium divides rapidly in irregular patches, making the outlines of the vessels, both capillaries and veins, have an exceedingly irregular contour, very different from the smooth contour of the earlier capillaries and from the wall of the omphalomesenteric arteries which now have a single layer of smooth muscle. The clumps of cells along the outer wall of the vessels develop the vacuoles characteristic of clasmatocytes and become free as clasmatocytes. Many hundreds of the extra-vascular cells are formed from the endothelium to one intra-vascular. The extra-vascular forms tend to be larger and have larger vacuoles, but I have seen one of the large cells wander into a vessel. The original endothelium has granules that stain in neutral red; it may also have vacuoles. The free cells all have both vacuoles and granules and a differentiation of the periphery of the cell into motile films. Studied with vital neutral red, the monocytes and the clasmatocytes are conspicuous because they are stained.

Thus, in the early chick, endothelium gives rise to two groups of cells, the megaloblasts which develop hemoglobin and become erythroblasts and a strain of cells termed histiocytes by Aschoff. The extra-vascular histiocytes have been termed clasmatocytes, the intra-vascular ones monocytes. They are identical and are specifically differentiated along the line of phagocytosis. They take up particulate matter and debris in solid form which they segregate and store in certain preformed vacuoles filled with fluid. They do not store this insoluble material permanently because it is gradually returned to the circulation and excreted by the kidney. So they represent a mechanism for taking care of foreign matter in excess of the amount that the body can excrete at the time. In the blastoderms from the third to the seventh day there is comparatively little differentiation of new angioblasts in the area pellucida. In fact, in about fifty specimens, I have found only three masses of solid angioblasts. The hollow isolated vesicles made from these solid masses are, however, more numerous, indicating that this stage lasts longer than the solid stage. In one specimen of the third day of incubation there was a long mass of solid angioblasts which started to liquefy to form a vessel, and while the center of the mass was liquefying to form a vessel, two cells wandered off from the periphery as clasmatocytes. Thus angioblasts can also give rise to clasmatocytes.

If one takes the group of monocytes as they are shown in the third row of Pappenheim's Plate I,[23] or the fourth and fifth rows of Ferrata's Plate XII,[12] it will be seen that they include all of the large mononuclear forms and the transitionals of the circulating blood. Both of these types can be seen early in the chick coming from endothelium; an endothelial derivative which is larger and less vacuolated is the mononuclear cell, a smaller and more vacuolated type, the transitional. The transitional is thus shown to be a finished type of cell like the cell of the adult form, for which the term transitional is therefore a misnomer. The large mononuclear type always has an excentric nucleus; it is distinguished most easily in the films of blood, stained with brilliant cresyl blue and counterstained with eosin-azur. It lacks the specific granulation of the erythroblast and has a very clear distinctive blue cytoplasm in Wright's blood-stain. With the group of the clasmatocyte in the connective tissues, Maximow divided the cells into resting and active cells. With the group of endothelial derivatives in the blood, it is not wholly clear whether the larger or mononuclear forms are resting or are old forms. In the embryo, the large mononuclear forms appear less specifically differentiated. Both forms can be seen in the living chick to come from endothelium, the transitional cell being developed specifically along the line of phagocytosis and storing of particulate, solid matter and possessing a certain type of motion of the cell *in situ* and very slow locomotion like the clasmatocyte of the connective tissue.

The Granulocytes or Leucocytes

On the third day, also, granulocytes begin, represented by the cells which are analogous to the neutrophilic myelocyte of mammals. In the chick the granulocyte with fine granules is pseudo-eosiniphilic. The first sign of the beginning of the granulocytes is that a cell appears close to a vessel which cannot be distinguished from a single angioblast. I have not found in these cells any substance stainable in neutral red except the specific granulation which stains paler than the granules of the endothelium, but am not yet entirely sure that this will be a sufficient distinguishing mark between this cell and a single angioblast. When, however, such a single cell divides, there is no longer any difficulty, because two angioblasts stay together while two granuloblasts separate. This criterion is not adequate when one has only sections, but in watching the living membranes or in studying them after fixation, where every cell of an entire area can be seen in its relations to other cells, it is sufficient. Such material has obvious advantages over sections. Thus, from one cell comes a clump of four or more cells with a dense azurophilic cytoplasm, the stem cell of the monophyletic school, lying near a vessel. These cells then show the following changes. The nuclei become excentric, while the center of the cell is occupied by the centrosphere made very obvious by the development of fine granules, staining pink in neutral red, always arranged in a crescent around the centrosome. Thus, there is a nucleus on one side, a clear spot in the center of the cell and on the other side this crescent of fine granules. The granules are entirely motionless at the start, there is none of the active streaming of the granules which is always associated with amœboid movement and which must be associated with a fluid state of the cytoplasm. The cell itself, however, does move, but very slowly, directly toward the vessel. One of the cells reaches the wall, half-way between two endothelial nuclei, and then one can see the wall bend inward, until finally the cell enters the lumen. The rest of the clump line up behind the first and also pass in. Thus, these granulocytes show a specific chemotactic reaction at once. Throughout these early

stages the granules are arranged characteristically around the centrosome. Thus, the specific granulation of the red cell is arranged around the nucleus, of the granulocyte around the centrosome, while the granules of the endothelial cell are scattered throughout the cell. Even in these early stages the nuclei of many of these cells become indented, the concave side always being toward the centrosome; thus, the primitive cell may soon be regarded as a leucocyte.

In the case of the monocytes and the clasmatocytes, both of these cells can be readily found differentiating and dropping off from the endothelium, but no relation to endothelium can be made out in the case of the granulocytes. They are near vessels but never form a part of their wall. It was shown by Danchakoff [8] in 1908 that the granulocytes are extravascular in origin.

There are no eosinophilic cells on the third day. The eosinophilic granule of the chick's blood is in long rods. During the first seven days I have seen only a few in the circulation and have not found them differentiating in the area pellucida. Probably further study will bring them out, since they are known through the work of Danchakoff [8] to develop in the area opaqua of the yolk-sac. The mast cells I have not seen at all in the first seven days, and Maximow [24] found that they develop late in mammals.

From these observations one may offer the theory that the two stem cells, the angioblast with its power to give rise both to red cells and to histiocytes in the larger sense, and the granulocyte are cells whose common ancestor is a mesenchyme cell instead of a differentiated stem cell or "hæmatoblast." In other words, the cells of the blood are not so sharply marked off from the cells of the connective tissues as to have a specific, common stem cell. At least one would have to prove that the differentiated cells which normally made the syncitial masses of angioblasts could be made to develop granulocytes. The argument for the mesenchyme cell as the stem cell, for the three distinct strains of cells which contribute to the blood, is that three such groups can be isolated embryologically and that they correspond with a functional classification. At least one may say that no common differentiated stem cell has been adequately demonstrated.

LYMPHOCYTES

In these studies of the development of blood in a living form the account of the origin of the lymphocytes is very incomplete. The lymphocytes make a group of cells ranging in the mammal from the size of a red cell up to cells twice the size. Likewise in the chick the lymphocytes are the smallest cell. When the cell first appears, all of them are of the small size. The cell has a characteristic nucleus and its cytoplasm contains a few azurophilic granules, discovered by Michaelis and Wolff.[25] The living cell has a nuclear membrane which is more distinct than in any other cell, but that this criterion is a difficult one to go by can be realized readily in connection with the fact that all nuclei become distinct as a cell dies. The cytoplasm of the lymphocytes contains but few granules and they do not stain readily in neutral red, but can be made to by increasing the amount of the dye or the time of staining. From these facts it is less readily discriminated than the other types. The reactions of lymphocytes in tissue cultures have been described by Lewis and Webster.[19] In Wright's stain, the early lymphocytes are exactly as distinctive as in adult blood. The first forms are of the small variety. I have seen a few on the fourth day, more on the fifth and sixth. In the blood smears, they occur in small clumps. The chromatin of the nuclei is very dense and has a peculiar violet reaction with azur-eosin. I have never found any indication of their differentiation in the area pellucida, thus it may be that they form only within the embryo itself rather than in the yolk-sac. However, a more extensive study of the yolk-sac may bring them out. All of the evidence from the study of this cell in the adult is that it differentiates extra-vascularly from reticulum. The only evidence, then, of significance in these studies in regard to this cell is that it occurs later than the other two groups and hence should not be regarded as a stem cell. Thus, from these studies, I would stress the use of the three names of white cells as specific for the three distinct groups, the leucocytes, the monocytes and the lymphocytes.

In these studies it is very plain that each white blood-cell, as it first appears, is differentiated. The red cells pass through a long stage of maturation. The first megaloblasts can be told as early stages of the red cells by a specific granulation, but the cell itself passes through a long series of stages, the erythroblasts, before it is the erythrocyte of the adult blood. The monocytes are a phagocytic type, like the cells of the adult, before they leave the wall of the vessel, the endothelium itself being phagocytic; the granulocytes develop their specific type of granulation early and soon begin to be leucocytes, and thirdly, the first lymphocytes are distinctive. When, however, all of these cells begin to divide, discrimination between all of the young cells is by no means easy, as the entire history of hematology attests. From this it can be seen readily that one must continue these studies of the development of blood in these living forms, watching especially the young cells just after division in all the stages of incubation, before one can adequately master all of the types of cells that are to be seen in bone marrow. In a drop of blood taken on the third day of incubation it is possible to tell all the cells apart, later it becomes most difficult. It is the study of the maturation stages of each group of cells by means of the eosin-azur technique that has been the great contribution of the monophyletic school. To this study must now be added certain specific criteria that come out through the method of applying dyes to living cells and we must now follow the stages of the cells with these vital dyes through the different embryonic periods.

The postulation of three strains of blood-cells on the basis of embryology fits in with the functional groups as we now know them. The endothelial or angioblastic group represents first the hemoglobin-bearing cells, and secondly, that group of the blood-cells which exhibit a special property of endothelium, namely, phagocytosis. The monocytes have this power of phagocytosis, they possess a peculiar type of motion *in situ* with very slow locomotion. The granulocytes possess a high

degree of amœboid motion, with speed and a flowing of the granules. They respond to chemotactic influences, are also phagocytic and have functions probably related to their specific granulations. The lymphocyte strain, as Murphy [36-38] has shown, are separated off physiologically by their being more sensitive to X-rays and to the emanation of radium than other normal cells. Moreover, he has shown that they are related to immunity toward certain forms of tumors as well as to certain types of infection.

The study of the blood-cells is a part of the study of the cells of the connective tissues. The erythrocytes are the only type that function only within the vessels. Of the other group from endothelium, the histiocyte in the larger sense, the vast majority make the clasmatocytes of the connective tissues, which are the mononuclear forms and the actively phagocytic forms of sub-acute infection, the resting and active wandering cells of Maximow. A few of this group make the monocytes, that is, the large mononuclear and transitional forms of the blood.

Of the granulocytes, which all differentiate extra-vascularly, the neutrophilic leucocytes pass into the vessels in largest numbers. Of the eosinophiles very many remain in the tissues while the mast cells never enter the vessels in most animal forms. By mast cell is meant a cell of the connective tissues occurring along vessels, along nerves and between muscle fibers, having a special metachromatic, basophilic granule. The so-called mast cell of human blood has been shown by Weidenreich,[39-41] to be a degenerating cell without any centrosome. The lymphocytes are for the most part extra-vascular, arising in the lymph glands and in the follicles of the spleen and in very numerous follicles in the various organs either associated with lymphatic capillaries or not. Thus, the differentiation of three strains of blood-cells, the endothelial strain, the granulocyte strain and the lymphocyte strain, that can be made out in the early stages of the chick embryo, can be shown to correspond with a functional grouping as far as we yet know the functions of the types of blood-cells. Moreover, the origin of the cells of the blood can be shown to be but a part of the study of the great groups of wandering cells of the connective tissues, the one type which functions only intra-vascularly being the erythrocyte.

The method of studying blood with vital dyes, beginning with the stages of the embryo when the cells first appear gives a very great advantage in following the maturation of specific cells and gives a chance of analyzing the complicated young forms which it is necessary to recognize in order to understand bone marrow.

The group of the red cells is characterized by a specific granulation stainable in certain vital dyes, possibly one should say precipitated by these dyes. This substance is at first throughout the cytoplasm, then in a wreath around the nucleus, then a reticular form and finally in scattered granules or droplets. The arrangement of this granulation around the nucleus should be stressed, although the substance is of cytoplasmic not of nuclear origin. Red cells with nuclear fragments, Howell-Jolly bodies, can be shown to be dying cells.

The strains of white cells, clasmatocytes and monocytes, that come from endothelium, are characterized by certain granules and vacuoles stainable in very dilute neutral red. They are scattered diffusely throughout the cells. The granulocytes are characterized by the arrangement of their specific granulation with reference to the centrosome. The lymphocytes are less sharply characterized morphologically, but have somewhat distinctive nuclei and granules stainable in azur.

This work is a part of the new subject of experimental cytology which seeks to analyze cells by means of specific criteria and to use these criteria to study the reactions of cells to normal and abnormal conditions.

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