STUDIES ON ORGANOGENESIS

I. THE ABILITY OF ISOLATED BLOOD CELLS TO FORM ORGANIZED VESSELS IN VITRO

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PLATES 13 TO 15

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Blood cells, when placed for incubation in culture flasks containing a mixture of blood plasma and Tyrode solution, are capable of constructing highly organized, tubular processes that resemble, in some respects, the blood capillaries of the organism. It is the purpose of this communication both to describe these structures and to disclose the conditions under which they are formed.

Materials and Procedures

The cells are taken from blood that has been drawn from the carotid of adult chickens according to the procedures regularly employed in the preparation of plasma. The blood is collected in plain glass tubes and centrifuged immediately. No anticoagulant is used. After centrifugation, and the subsequent removal of the cell-free plasma yield, the thin layer of leukocytes (buffy coat) that rests above the red cells is coagulated by the addition of a drop or two of embryo tissue juice. After a little later, this layer can be removed as a disc and cut into small fragments for the preparation of the cultures. Each fragment consists of a layer of white cells, together with such red cells as remain adherent to them. After a brief period of irrigation with Tyrode solution, the fragments are placed in micro flasks in a medium consisting of 0.2 cc. of adult chick plasma diluted with twice that amount of Tyrode solution. They are then placed for incubation at 37°C.

1 This material is rendered cell-free by freezing for two periods of 15 minutes each at -50°C.

2 This flask (Carrel, A., Compt. rend. Soc. biol., 1930, 105, 826) is 30 mm. in diameter and 5 mm. in depth. It has a straight neck, 5 mm. in diameter. At the union of the neck with the chamber, there is a slight constriction which prevents the medium from running into the neck. The walls are of sufficient thinness (0.18-0.10 mm.) to permit the study of cultures with a 3 mm. oil immersion lens. At the termination of an experiment, the cultures may be fixed and stained in situ.
EXPERIMENTS AND RESULTS

Early Stages in the Development of the Tubules.—As a rule, the tubules (Figs. 1–4) begin to be formed as soon as the cultures have been prepared. At one or more points on the margin of the implanted fragments (Fig. 4), a few red cells become dislodged and break away. Their places are taken by those from behind. If the proximal impact is great enough, these cells are also pushed out into the medium. This makes for a general streaming. Each cell that is forced out follows in the wake of those that have gone before. They may proceed in single file, or abreast of one another, but always over exactly the same route. This route may take any direction. It may become so branched as to form a system of great complexity (Fig. 1). But as long as the cells in the lead are being pushed forward by those from behind, they will continue to advance through the medium until it coagulates. The moment coagulation occurs, the pathway assumes the nature of a tunnel-like passage. Moreover, this passage will remain open and unobstructed, despite the fact that the cells in transit may be widely separated (Figs. 11 and 12). Its boundaries become the interphase between the fluid plasma within and the coagulated plasma without. By manipulating the flask, the cells that are enclosed can be made to flow in either direction. If coagulation occurs before the proximal outflow has ceased, its lumen may become densely packed with cells. Even after the medium has coagulated, however, the force of the outgoing cells may still be so great that a large spherical expansion is formed at its distal end (Fig. 3). At other times, the channels may be completely ruptured.

It has already been stated that the marginal outflow is initiated by the red cells. Red cells have smooth surfaces and easily shift their position. Without them, the phenomenon does not occur. Leukocytes, on the other hand, adhere to one another and to solid structures. While capable of independent locomotion, they may, as in the present instance, be swept along in a current of red cells. The outflow will not occur, however, if the buffy coat has become too firmly coagulated before the fragments have been prepared and placed in the flasks. Under these conditions, the red cells are held so securely in place by the fibrin meshwork of the explant itself that they are rendered incap-
able of movement. The greatest number of tubules are to be obtained from a thick fragment of relatively loose texture and one that contains an abundance of red cells.

It should also be pointed out that the tubule formation occurs independently of, and begins prior to, the general outward migration of leukocytes (Figs. 4 and 5). In fact, the two phenomena depend upon entirely different environmental conditions. It has been found, for example, that when the medium is composed of serum, the cells flow out irregularly from all sides, and no tubules are formed. In the presence of plasma, however, the outflowing cells may take every conceivable form, ranging from broad, fan-like disseminations through short, stalky, bud-like projections to long, slender ones such as those just described. It has also been found that the development of the tubules is not suppressed by substances that enhance cell activity unless they induce, at the same time, the immediate coagulation of the plasma. Thus, embryo tissue juice prevents their formation by producing immediate coagulation (Figs. 6 and 7). Their development is not hindered, however, by the addition of tryptic digests of fibrin. Although these latter substances stimulate the leukocytes to enormous activity, they do not hasten, to any appreciable degree, the coagulation of the fibrin network. It is, of course, possible to delay, to some extent, the onset of coagulation by retaining the cultures for a time at room temperature. This, as a rule, makes for the development of a larger number of tubules and for tubules of greater length than it is possible to obtain by immediate incubation.

Later Stages in the Development of the Tubules.—All that has occurred up to this point may take place within 15 to 20 minutes after the cultures have been prepared. As soon as the surrounding medium has become firmly coagulated, however, no further change occurs either in the length of the tube or in its diameter (Figs. 9 and 10). So far, in fact, the cultures have no definite walls. In the majority of cases, the walls begin to be laid down after from 5 to 8 hours’ incubation (Fig. 8). They are formed by the activity of living cells, or cell products, particularly the thrombocytes, that have been deposited along the course of the tubules. Wherever there are gaps between the cells confined in the tubules, these minute bodies may be seen to
spin out fine, hair-like filaments at the interphase between the lumen of the tubule and the surrounding coagulum (Figs. 11 and 12). These filaments are of the nature of fine mesenchymal fibrillae. Sometimes, they become very dense (Fig. 13). They take on a deep blue coloration when stained with Heidenhain's azan mixture, and become black when treated according to the Bielschowsky-Maresch silver impregnation procedures (Figs. 21 and 22).

Out in the surrounding medium, the thrombocytes may form similar strands, but here at random (Figs. 14 and 15). Where they are well isolated from one another, these strands are usually very short and are arranged radially (Fig. 14). The majority of them, however, tend to agglutinate. Very often, the agglutinated masses become joined together by numerous, parallel threads (Fig. 15).

After a few days, the red cells within the capillary-like formations become progressively phagocytized by the macrophages (Figs. 10 and 19). Eventually, these in turn may escape into the surrounding medium, sometimes through definite breaks in the walls, but more often by way of their proximal ends. After some days, the tube may be completely empty. In the meantime, however, any number of macrophages may come to rest on its outer surface (Figs. 16, 21 and 22). In this position, they bear striking resemblance to the much discussed cells of Rouget. Occasionally, their undulating membranes become so fused with one another as to give the impression of an unbroken syncytium. If the cultures were fixed and stained at this moment, the walls of the vessels would appear to be nucleated.

Sometimes the fibrous wall is incompletely formed, the strands being laid down over a single portion of the original passageway. At other times a single cord of fibrous material may extend along one side of it (Fig. 18). Invariably, however, its lumen remains open and filled with fluid. When the wall is complete, it is possible to inject the tubule with a micro pipette. With increasing pressure, the vessel becomes distended. Eventually, of course, it will burst.

DISCUSSION

The present experiments have shown that it is possible for the cells of the circulating blood, when placed for incubation in a suitable
substratum, to provide themselves with organized vessels of their own creation. In the beginning, the formation of the organized structures is a purely physicochemical phenomenon. It is dependent upon the consistency of the medium, the character and thickness of the original fragment and the surface peculiarities of the red cells. As soon as fragments containing a loose arrangement of red cells and leukocytes have been placed in a plasma mixture that does not coagulate immediately, certain of the red cells, as the result of physical pressure exerted upon them by neighboring cells, initiate a general outflow from the central mass into the surrounding medium. This leads to the formation of definite channels, or passageways, through the uncoagulated plasma. Eventually, the medium coagulates. Despite this, however, the plasma contained in the tubular channels remains fluid. Its properties have been altered, in some way, by the advancing blood cells. Then, as soon as the tubules have been formed, the thrombocytes that have been deposited at the interphase between the coagulated plasma without and the fluid medium within, proceed to lay down a fibrinated wall. They build this wall in exactly the same way that they produce at random short strands of fibrillae in the outer medium. Finally, the wall becomes covered by a membranous layer of leukocytes. Here again, the leukocytes manifest a common, well known property. They will cover a cotton fiber in quite the same manner (Fig. 17).

It follows, then, that the phenomenon as a whole is composed of a series of events that are both physicochemical and physiological in their nature. These various events are separate and distinct phenomena. They must, however, occur in a definite sequence. If, for example, the blood cells fail to stream out from the central explant before the medium coagulates, no tubules are formed. The streaming

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8 Two years ago, Hueper and Russell (Hueper, W. C., and Russell, M. A., Arch. exp. Zellforsch., 1932, 12, 407) reported the appearance of similar structures in a certain percentage of 'leucocyte' cultures, some of which had been prepared in much the same manner as those referred to above. The observations reported by these authors, however, are so completely at variance with those presented here that it does not seem advisable to attempt a comparison of the two accounts. Moreover, no pretence was made by them to determine the conditions under which the phenomenon occurred.
has been found to depend upon the physical features of the explant and the cells that comprise it; the rapidity with which the medium coagulates, on the other hand, depends upon the physicochemical nature of the various substances that go into it and also upon the temperature at which it is placed. Then again, the marginal outflow from the explant into the medium may carry only red cells. While this will insure the formation of the fluid channels, or passageways, through the plasma coagulum, the channels will not become endowed with fibrillar walls unless the red cells are accompanied by leukocytes and thrombocytes. Assuming, however, that the tubules have been formed and that they have developed fibrillar walls, the possibility of their being covered, eventually, by a cellular membrane depends entirely upon the nutritive quality of the medium.

The formation of these structures is looked upon as a definite example of organogenetic development. The phenomenon takes place whenever a certain group of cells is placed in a certain type of medium. Given the proper set of environmental conditions, its occurrence is inevitable. It is true, of course, that the structures formed are only slightly analogous to the blood vessels formed in the organism. At the same time, their manner of formation has been such as to suggest that formative processes occurring in the organism might also depend upon just such an ordered sequence of physicochemical and physiological events.

**SUMMARY AND CONCLUSIONS**

1. Isolated blood cells, when placed for incubation in a plasma substratum, are capable of constructing highly organized, tubular processes that project from the explanted cell mass into the surrounding medium.

2. The tubular structures have fibrillar walls that may be covered, eventually, by a membranous layer of leukocytes. Their lumina contain blood cells suspended in fluid.

3. The formation of the tubules is initiated by the red cells. The leukocytes, and more particularly the thrombocytes, are responsible for the construction of the walls.

4. The phenomenon occurs only in the presence of plasma, the coagulation of which has been slightly delayed. Once the surrounding
medium has become firmly coagulated, no further change occurs either in the length of the tubules or in their diameter.

5. The development of the tubules is not suppressed by substances that enhance cellular activity unless they induce, at the same time, the immediate coagulation of the medium. The addition of embryo tissue juice prevents their formation by producing early coagulation.

6. The phenomenon as a whole is dependent upon the physico-chemical nature of the medium, the character and thickness of the explanted fragment, and the physical and physiological peculiarities of the cells that comprise it. It is the expression of various physico-chemical and physiological events that have occurred in a definite order, or sequence.

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EXPLANATION OF PLATES

With the exception of those reproduced in Figs. 21 and 22, all of the photographs were made from living, unstained preparations. Eastman safety process films were used throughout.

PLATE 13

Fig. 1. Culture 5308-4. Tubule formations in a colony of blood cells photographed after 24 hours' incubation in plasma and Tyrode solution. The cells in the background are leukocytes that have migrated out from the same explant. X31.

Fig. 2. Culture 5589-3. Portion of a tubule after 24 hours' incubation. The wall, clearly defined where it is present, is still incomplete. Note the miscellaneous character of the enclosed cells. X198.

Fig. 3. Culture 4048-8. Tubule from a culture incubated for 30 hours. Note the spherical expansion at the distal end. X99.

Fig. 4. Culture 4078-1. Tubule formations after 1 hour's incubation. The structures extend well beyond the zone of migrating leukocytes. X28.

Fig. 5. Culture 4078-2. Tubule formations after 8 hours' incubation. By this time, the zone of outward cell migration extends well beyond the tubules. X20.

Fig. 6. Culture 4092-10. Tubule formations in a colony of blood cells cultivated in plasma and Tyrode solution. As usual, no embryo tissue juice was added. Photographed after 5 hours' incubation. X17.

Fig. 7. Culture 4092-3. Colony of blood cells cultivated in plasma, Tyrode solution and embryo tissue juice. In contrast to the culture shown in Fig. 6, no tubules were formed. Photographed after 5 hours' incubation. X17.
Fig. 8. Culture 4048-7. Portion of a tubule after 6 hours' incubation. In this section of the tubule, a few red cells are to be seen. A spherical expansion at its distal end (top of figure) is not shown. ×164.

Plate 14

Fig. 9. Culture 4048-7. The distal portion of a tubule photographed after 30 hours' incubation. The red cells contained in the distal expansion are evenly distributed. ×105.

Fig. 10. Culture 4048-7. The same as Fig. 9 after 3 days' incubation. By this time, the red cells have become phagocytized by the leukocytes. The several black blotches represent the location of phagocytes that are gorged with red cells. The proximal portions of the tubule are almost completely empty. ×105.

Fig. 11. Culture 4078-2. Portion of a tubule photographed after 8 hours' incubation. That section of the passageway lying between the two groups of enclosed red cells is open and unobstructed. Although the wall has not yet been formed, a single fibrous band may be seen extending along the bottom of the tubule. ×365.

Fig. 12. Culture 4078-2. Portion of another tubule from the same culture after 8 hours' incubation. As in the case shown above, this tubule extends across the entire width of the photograph. The fibrous wall is in process of formation. Only those cells that are clearly in focus are contained within the tubule. ×365.

Fig. 13. Culture 4097-2. Portion of a tubule after 5 days' incubation. The fibrous wall is completely formed and is very dense. ×328.

Fig. 14. Culture 5016-3. Red cells, leukocytes and thrombocytes from the outer medium of a culture incubated for 24 hours. The thrombocytes have thrown out fine fibrillar strands that radiate in every direction. Just above the center of the figure a macrophage is seen clinging to one of the heavier connecting fibers. ×310.

Fig. 15. Culture 5016-3. Agglutinated masses of red cells, leukocytes and thrombocytes from another portion of the same culture after 24 hours' incubation. The various groups of cells are connected with one another by numerous fibrillae. ×310.

Plate 15

Fig. 16. Culture 4048-8. Portion of a tubule after 5 days' incubation. Its surface is completely covered by leukocytes. Their thin, undulating membranes have become fused, giving the appearance of a syncytium. At the bottom and to the right of the figure, one of the cells lying on the surface of the tubule may be seen in relief. ×207.

Fig. 17. Culture 4087-10. Two sections of a single cotton fiber that has served as a supporting structure for free wandering leukocytes; from the outer medium of a culture of blood cells after 6 days' incubation. ×198.

Fig. 18. Culture 4048-9. Portion of a tubule whose cellular contents have
either died or migrated out from its lumen. Only a section of the tubule has been enclosed by a fibrous wall. Although the original channel branched out to the right (at the bottom of the figure), no wall was formed. Photographed after 8 days' incubation. ×185.

Fig. 19. Culture 4048-7. The distal portion of the same tubule shown in Figs. 9 and 10, after 8 days' incubation. ×185.

Fig. 20. Culture 4048-7. The distal portion of a tubule that has obtained a well formed, fibrous wall. The extreme tip of this tubule is of very small diameter. Photographed after 9 days' incubation. ×185.

Fig. 21. Culture 5925-3. Portion of a tubule from a culture fixed in Ringer-formol after 5 days' incubation and stained according to the silver impregnation procedures of Bielschowsky-Maresch. Note the complex, net-like structure of the fibrinated wall. ×422.

Fig. 22. Culture 5925-3. Another tubule from the same preparation shown in Fig. 21. A number of leukocytes have become incorporated in the fibrous wall. ×422.