THE DEVELOPMENT OF PURE CULTURES OF FIBROBLASTS FROM SINGLE MONONUCLEAR CELLS

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PLATES 9 TO 11

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Fibroblastic or fibroblast-like transformation of blood mononuclear leucocytes in tissue culture was first reported by Awrorow and Timofejewskij (1). Carrel and Ebeling (2), working with chick buffy coat explants, believed the transformation to be an adaptive phenomenon of the monocyte to an unfavorable environment, since the fibroblast is a more resistant cell. Fischer (3) found that special conditions, such as adding dead muscle tissue to the cultures, were necessary for fibroblastic metamorphosis of chick mononuclear cells. Maximow (4), in a detailed study of fibroblastic growth from guinea pig, monkey, rabbit and chick buffy coat explants, observed that there were certain species differences and that guinea pig non-granular leucocytes transformed into fibroblasts most readily. Bloom (5) also described polyblastic and fibroblastic mutations of rabbit lymph cells in tissue culture.

While studying the reaction of cells from infected animals to bacterial products (6), it was again observed (Figs. 1 and 2) that luxuriant growths of fibroblasts frequently developed from guinea pig buffy coat explants. Beginning fibroblastic growth usually occurred 4 to 6 days after explantation. Further analysis indicated that there was a definite correlation between the age of the animal, cellular type and fibroblastic growth potentialities of the explants. Buffy coats from younger animals contained a higher percentage of mononuclear cells, particularly lymphocytes, and also developed greater fibroblastic growths.

The object of the present study was to determine whether pure cultures of fibroblasts derived from single cells could be developed by

using mononuclear cells as progenitors. This seemed feasible because fibroblastic transformations occurred readily, and suitable suspensions of individual mononuclear cells could be made with minimal trauma.

Unsuccessful efforts, chiefly by Fischer (7), to grow pure cultures of fibroblasts from single cells were ascribed by him to unsatisfactory environmental conditions or to manipulative trauma of the cells. Burrows (8) stated that while single cells may move in plasma medium they do not grow, and expressed the opinion that cells can grow only when crowded together in an environment where accumulation of certain normal metabolic products acts as a stimulus for proliferation.

EXPERIMENTAL

Preparation of Mononuclear Cellular Suspension.—Several methods for obtaining a mononuclear cellular suspension were tried and discarded before a satisfactory plan was perfected. Cells aspirated into a fine capillary pipette from minced buffy coat failed to grow, probably because of the excessive trauma entailed in the manipulation. A mononuclear exudate consisting almost entirely of monocytes and clasmatocytes was then produced by the injection of paraffin oil intrapleurally into medium sized guinea pigs. The exudate, removed aseptically by opening the thorax of the pig 5 days after the oil injection, contained many mononuclear cells filled with ingested oil droplets; and although some of them apparently proliferated in tissue cultures this method was discarded for one yielding more normal exudative cells.

The best mononuclear cell incitant for our purposes was the intrapleural injection of low melting point wax. Normal guinea pigs weighing between 300 and 400 gm. were lightly etherized. A small incision was made through the clipped skin of the right chest on the lateral aspect in the mid-thoracic region. Using a medium caliber needle 0.5 cc. of fluid parowax (a widely used commercial wax for household purposes, melting point 48–50°C.) was injected into the pleural cavity. At body temperature this wax congeals into a soft pliable mass. Caution was taken not to inject the wax while too warm. The injection was performed quickly and with minimal trauma, since, as indicated later, the avoidance of even slight hemorrhage into the pleural cavity was necessary for satisfactory experimental conditions. After 3 to 14 days the mononuclear exudate was removed; the most satisfactory time was found to be 5 to 7 days, because after more extended periods various amounts of amorphous debris accumulated, probably due to degenerated cells, and this seemed to affect proliferation of isolated cells adversely. The animal was killed by a sharp blow on the head and exsanguinated as completely as possible by severing the neck vessels. After the heart had ceased beating the thorax was opened under aseptic precautions and great care was taken to prevent red blood cells from entering the pleural cavity. No macroscopic

exudate was visible about the pliable mass of wax. The right pleural cavity was then gently irrigated with 1 or 2 cc. of Tyrode's solution and the suspension removed. A properly prepared cellular suspension was practically water-clear and contained few gross particles. A cell count of the suspension was then made and serial dilutions in Tyrode's solution were so arranged that 0.1 cc. of the suspension contained from one hundred to several thousand cells.

Supravital studies of the cells with neutral red and Janus green, according to the method described by Sabin (9), showed them to be almost entirely mononuclears chiefly of the monocytic type with a smaller percentage of lymphocytes and a few clasmatocytes. The proportion of lymphocytes ranged between 5 to 20 per cent, the higher percentage resulting from more prolonged pleural irritation. The monocytes were of the normal resting type with large neutral red rosettes and presented no other noteworthy characteristics. Most of the cells were free, but they occasionally adhered to one another in clumps. Rarely, desquamated serosal cells were found in groups or small sheets and they presented the usual polygonal shape with small oval or round nuclei. Polymorphonuclear leucocytes were seldom observed except when the cellular suspension was blood-tinged.

Culture Media.—Plasma was obtained from normal medium sized guinea pigs by heparinizing and centrifuging chilled cardiac blood. 0.5 cc. of a 1 to 700 dilution of heparin in Ringer's solution was used for each 4 cc. of blood. The separated plasma was again centrifuged at high speed to remove erythrocytes. Plasmas showing even slight hemolysis were not used.

The tissue extract was derived from the spleens of the normal guinea pigs that had been previously bled by cardiac puncture. The spleens were freed of adherent fat, finely minced with scissors and suspended in Tyrode's solution to make a 10 per cent tissue extract. The suspension was agitated by aspirating with a pipette and allowed to stand for ½ hour, then centrifuged at high speed for 10 minutes to throw down the gross particles. The supernatant slightly turbid fluid which still contained many blood platelets was further centrifuged for 20 minutes at high speed to remove the thrombocytes, and then appeared quite clear and free from cells. In some experiments the splenic extract was also repeatedly and rapidly frozen in a salt-ice mixture and thawed to obviate the theoretical objection that isolated living cells may still have been present in the extract after prolonged centrifugation.

Carrel's micro flasks were found most suitable for photographic purposes. After repeated trials the following amounts of media and cells in each flask were determined to be most favorable: 0.9 cc. of heparinized plasma, 0.1 cc. of suspension of mononuclear exudative cells in Tyrode's solution and 0.5 cc. of 10 per cent splenic extract. Each flask was shaken to mix the contents thoroughly, then stoppered, following which coagulation soon occurred. The number of mononuclear cells in different flasks varied from one hundred to several thousand. Cultures were set up and placed in an incubator regulated for 37°C. within a short time after removal of the cells from the animal.

The isolated cells, designated for microscopic study, were identified under low power magnification by dots of India ink or black enamel paint applied with a fine pen at suitable locations on the outer surface of the flask. The cultures were placed in a warm chamber during microscopic study and except when being photographed were protected from strong light with a green filter.

Cultures observed for a week or more were washed with Tyrode's solution and renourished with 0.5 cc. of 10 per cent guinea pig splenic extract twice a week. For subcultures, colonies of cells were excised in the usual manner and transplanted into Carrel micro flasks containing 1.0 cc. of 50 per cent guinea pig plasma in Tyrode's solution and 0.5 cc. of 10 per cent guinea pig splenic extract.

A Leitz "micca" photomicrographic attachment for an ordinary microscope was used for recording the changes observed in the cultures. The magnifications obtained by different combinations of lenses were 25, 40, 90 and 200, the last being secured with a water immersion lens.

RESULTS

Types of Cellular Development.—Under suitable experimental conditions some of the isolated mononuclear cells divided by mitosis and proliferated to form colonies of morphologically typical fibroblasts. The development of different cells in tissue culture seemed to be conditioned to a certain extent at the time of explantation, for within a few hours after the cultures were made, distinct differences could be noted in many of the isolated cells: some were dark and very granular with distorted nuclear and cellular outline; obviously they were dying or dead, for they soon fragmented and disintegrated completely. By far the greater proportion of the cells hypertrophied, extended pseudopodia and undulating membranes, and became typical migrating macrophages (Fig. 22). These cells varied greatly in size, rapidly changed their shapes and usually migrated actively. After several days some of them became less active, retracted their pseudopodia, assumed round or irregular polygonal shapes, became filled with dark coarse granules or droplets and eventually degenerated. Others of the macrophage type maintained their healthful appearance and activity for much longer periods. The occasional small clump or sheet of desquamated serosal cells showed no signs of activity and did not proliferate. Admixed red blood cells usually disintegrated within 2 days after explantation.

A smaller percentage of the cells slowly developed into spindle or stellate forms with a varying number of long, pointed, branching,

protoplasmic processes. The nuclei were usually oval or round and contained several nucleoli. These cells did not migrate but altered their shape by rearrangement of their processes. Many of them were marked for identification, photographed and observed for evidences of cellular division. Cells thus selected, however, only occasionally underwent division, but usually degenerated.

Selection of Isolated Cells.—A much better technique with greater chances for success was to search for isolated single cells in the process of mitosis (Figs. 3 to 7), since we were then assured of some proliferation, if only of slight extent. It was discovered that the most favorable period for finding these cells in mitosis was 24 to 48 hours after explantation; although occasionally some isolated cells did not undergo mitosis until after a latent period of 3 days. Diligent and frequent observations of the flasks on the 1st and 2nd day after explantation usually revealed several satisfactorily isolated cells in mitosis in each flask. Such cells were readily recognizable from their characteristic appearance. The cell drew in its processes and assumed a nearly spherical shape; the cytoplasm condensed into large irregular dark masses surrounding a nucleus which had lost its ordinary structure of the resting state. Under a water immersion lens the breaking up of the spireme thread into chromosomes and migration of the chromosomes were frequently observed.

Rate of Proliferation.—The rate of proliferation of individual cells varied even in the same flask. In some instances after proliferation had set in, the number of cells was roughly doubled in each 24 hours. Some proliferated more slowly and others more rapidly. One isolated cell was the progenitor of seven cells in the course of 28 hours; but this rapid proliferation was not maintained. No instances of amitotic division were observed.

Criteria of Fibroblasts.—Cells presenting the following morphological characteristics were considered to be of the fibroblastic type: the cells had spindle, stellate or polygonal shapes with variable numbers of pointed or branch-like protoplasmic processes and relatively large, clear, oval nuclei containing one or several nucleoli. These cells tended to grow in solid sheets or in reticular arrangement and displayed marked cytotropism, but did not have migratory ameboid movements.

Types of Colony Formation.—The type of colony produced from various individual cells differed. Some formed solid sheets of cells (Fig. 18); others developed typical branching formation (Fig. 23). Likewise the morphology of individual cells in different colonies was variable, but different individuals of the same colony were essentially the same. Some fibroblasts had many long branching protoplasmic processes (Fig. 21); others were polygonal with few processes and grew in tight solid sheets (Fig. 24). The oval or round nuclei contained two to six large nucleoli. Probably the different types of cell colony formation can be explained in part by the supporting structure on which the cells grew; proliferation on the free surface of the clot, in the fibrin network or between the glass and coagulum would naturally have their respective influences.

Transitional cells between the typical macrophage and the stellate and spindle-shaped fibroblast were frequently encountered. Some were polygonal with nuclei similar to that of fibroblasts. Variable amounts of granules were present in the cytoplasm. Usually there was a broad, clear, peripheral zone free of granules. Pointed protoplasmic processes were very few and usually entirely absent. Such cells grew in colony formation as solid sheets (Fig. 24).

Although the fibroblasts did not migrate in the sense applied to macrophages, still in an actively proliferating colony, movements and changes in the relationship of cells to one another were often quite marked. This was especially noticeable in colonies growing in branch-like arrangement. From hour to hour the pattern changed, assuming various bizarre shapes. A cell would break one of its connections with a neighbor and later attach itself to another cell. Due to the marked cytotropism, however, individual cells of actively growing colonies derived from single cells seldom severed connections with the mother colony. There was also a marked attraction of one colony of fibroblasts for another, as shown in Figs. 15 to 18. Tongues of cells sent out from each colony soon resulted in complete fusion.

The extent of proliferation of individual cells was variable even in the same flask where the environmental conditions and isolation of the cells were essentially the same. Sometimes macroscopic colonies consisting of hundreds of cells developed from a single cell, while a neighboring cell produced a colony of only ten to twenty members

which then degenerated (Fig. 27). Other cells disintegrated after one or two divisions.

Relationship between Bulk of Medium, Number of Explanted Cells and Cellular Proliferation.—The number and size of the colonies of fibroblasts originating in the flasks containing the larger number of explanted cells was often disproportionately greater when compared with other flasks containing serial dilutions of the same cellular suspension. This suggested that there was an optimal relationship between the bulk of the medium, the number of explanted cells and the degree of cellular proliferation. For this reason, and also because the proportion of mononuclear cells which eventually developed into colonies of fibroblasts was comparatively small, it hardly seemed feasible to isolate single cells from the suspension with a capillary pipette, transfer them to culture media and obtain growth.

The occasional small clump of explanted cells proliferated rapidly. Larger clumps behaved in some respects similarly to explanted buffy coats. Shortly after explantation numerous macrophages wandered out, and fibroblastic forms appeared within 48 hours (Fig. 26). This was much more rapid than that noted in guinea pig buffy coat explants, from which the first spikes of fibroblastic growth usually did not appear until 4 to 6 days after explantation. Proliferating cells that were not well isolated but were in the vicinity of other fibroblasts showed the usual cytotropism, readily united with their neighbors and soon formed large groups.

Subcultures of Fibroblasts.—Colonies of fibroblasts originating from mononuclear cells were easily transplanted, carried through repeated subcultures and still maintained their morphological fibroblastic characteristics (Fig. 25). Attempts have not been made to carry these transplants on indefinitely; and efforts to transform fibroblasts back to macrophages (10, 11) have not been pursued.

Proliferation of isolated macrophages was not observed in these experiments; but persistent efforts in this direction were not made because the migratory character of the cells rendered detection of these changes difficult with our technique. Once a typical migrating macrophage with a broad undulating membrane approached a colony of proliferating spindle-shaped fibroblasts and became entangled in the network of cells. After 48 hours this cell lost its undulating membrane

and underwent division; it subsequently produced a small nest of transitional type of cells similar to those shown in Fig. 24, amongst the fibroblasts. Many other macrophages in close proximity to colonies of fibroblasts did not transform.

Because of the difference in the behavior of the various mononuclear cells an attempt was made to correlate their reaction to vital dyes with their subsequent behavior in culture media. This did not meet with success, as neutral red and Janus green, even in dilutions of 1 to 40,000, proved lethal for these cells.

Inhibiting Influence of Erythrocytes on Cellular Proliferation.—Early in this work when the results of individual experiments were not usually successful, analysis of the variables was undertaken with the object of removing the inhibiting factor or factors. It soon appeared that when there were many red blood cells in the mononuclear cellular suspension the growths were characterized by very slight cellular proliferation; in other words, the explanted mononuclear cells seemed definitely inhibited by the presence of erythrocytes. Disintegration of red blood cells, which usually occurred within 48 hours after explantation, was followed by degenerative changes of the mononuclear cells. The macrophages seemed more affected by the toxic products and usually disintegrated before the fibroblastic forms. Some of the larger macrophages remained viable for longer periods. The transformed fibroblasts also became very dark, developed vacuoles and coarse granulations and their processes became attenuated; proliferation was much inhibited and usually entirely suppressed, and in a few days the cells eventually fragmented.

Several experiments were undertaken to analyze the apparent inhibition of disintegrated erythrocytes on cultures of isolated mononuclear cells. Normal heparinized whole guinea pig blood in a final dilution of 1 to 2,000 had a definite inhibitory effect on growth of mononuclear cells, as described in the preceding paragraph. On the other hand, the soluble products of a 1 to 2,000 dilution of hemolyzed, stroma-free, guinea pig blood produced no inhibition but possibly stimulated growth slightly. The direct effect of erythrocytic stroma alone could not be adequately tested because of the difficulty encountered in resuspending it. The results indicate, however, that the toxic or inhibiting factor of disintegrating red blood cells resides in the stroma.

With improved technique, particularly by avoiding traumatic hemorrhage in the pleural cavity when injecting the wax, satisfactory results were usually obtained.

DISCUSSION

The transformation of guinea pig non-granular leucocytes and mononuclear exudative cells into fibroblasts was again demonstrated. The experimental conditions most favorable for this transformation seemed to be the same as those optimal for the cultivation of other guinea pig fibroblasts. Special conditions such as those described by Carrel and Ebeling (2) and Fischer (3) in their studies of fibroblastic transformation from chicken buffy coats were not necessary in the case of guinea pig cells.

From a study of the development of individual isolated mononuclear exudative cells in tissue culture it is suggested that the type of development or transformation of each cell is more or less conditioned at the time of explantation, because different cells, in apparently the same environment, developed differently, and usually maintained their characteristics. Under the conditions of these experiments most of the cells behaved as typical macrophages. A smaller proportion took on fibroblastic characteristics which persisted. Whether these two divergent cellular morphological groups originate from two different types of cells, such as the monocyte and the lymphocyte, or whether they represent merely different developmental stages of a common cell type was not ascertained in these experiments. The latter conjecture seems most probable in view of the fact that cells in various transitional stages between the macrophage and the typical fibroblast were frequently seen.

It is known that optimal environmental conditions vary in the case of different types of cells. Plasma is the optimal medium for macrophages, while high concentrations of tissue juice are toxic for them. Fibroblasts, on the other hand, thrive in media containing relatively large amounts of embryonic or tissue extract. Parker (11) has observed the transformation of a long established strain of chick fibroblasts into typical macrophages by changing the nutritional environment from one containing embryonic tissue juice to a pure plasmatic medium. He, therefore, considers that the fibroblast and the macrophage represent extreme functional and structural variations of the

same cell type. It thus seems probable that the culture medium used in our experiments, due to the relatively large amount of tissue extract, was more favorable for prolonged fibroblastic proliferation than for continued growth of macrophages. Yet it should again be emphasized that fibroblastic transformation took place soon after explantation when most of the macrophages appeared to be in good condition. This suggests that the different individual cells were variously conditioned at the time of explantation.

This study clearly shows that certain single isolated mononuclear cells of the guinea pig can give rise to a pure colony of fibroblasts. Direct apposition of other cells is not necessary, at least in the case of guinea pig mononuclear cells, to supply the stimulus for cellular division as suggested by Burrows (8) and Fischer (7). It seemed possible that sufficient growth-promoting substance was present in the splenic extract to initiate mitosis in isolated cells. On the other hand, it should be emphasized that a distinct period of latency followed explantation of isolated cells before which mitosis did not occur; and cellular division usually did not begin until a lapse of 1 or 2 days, and sometimes not until several days after explantation; this indicates that the cell may have been elaborating some product into its immediate environment which made it favorable for cellular proliferation. It is also possible that comparatively far removed cells secreted substances into the medium which by diffusion reached the isolated cell and stimulated it. This possibility is suggested by the disproportionately greater proliferation in flasks containing the larger number of mononuclear cells. A definite optimal relationship between the bulk of medium, number of cells and amount of proliferation has also been shown in the case of yeast (12) and Infusoria (13). Wildiers (12) designated this cell-stimulating material elaborated by normal yeast as "*la substance enigmatique.*" Robertson (13), working with Infusoria, described an "X substance" produced by the organisms which catalyzed their reproductive rate. He also pointed out the protective effect of mutual contiguity of cells. It seems possible in the light of our experiments that a similar agent is necessary for cellular proliferation of isolated mammalian cells. Likewise it is well recognized that a condition of crowding of the cells in tissue culture is the more favorable one for proliferation, and Burrows' experiments

(8) showed that the continual removal of certain substances produced by normal cells inhibited proliferation.

A distinct cytotropism between cells of the fibroblastic type was noted, and although these cells do not possess true ameboid movement, the growth of small colonies was directed toward similar cells in the vicinity. Proliferation was definitely enhanced by this union. Rous and Jones (14) and others have also pointed out the cytotropism of fibroblasts in tissue culture. This behavior is in distinct contrast to that of the macrophages which usually migrate away from one another.

The question arises as to whether isolated animal cells from other sites of origin might have the property of proliferation in tissue culture or whether this ability is peculiar to the cells selected in this study. Possibly the wax stimulated certain cells to take on fibroblastic transformation potentialities and enhanced their proliferation. Explanted clumps of mononuclear exudative cells gave rise to fibroblastic growth much earlier than did buffy coat explants, suggesting either a stimulation of the exudative cells by the wax or an inhibition of the fibroblastic precursory cells in the buffy coat by the many red blood cells incorporated in the explants. An important contributing factor to the success of our experiments was the minimal trauma associated with the handling of pleural exudative cells. This was relatively slight as compared with techniques used in isolating single cells from other tissues.

Rather exacting conditions were found necessary to accomplish the development of pure colonies of fibroblasts from single cells, and these were in the direction of optimal conditions for guinea pig fibroblasts in general. Aside from inflicting minimal trauma on the cells, the removal of inhibitory factors from the tissue culture media was obviously necessary. The inhibitory effect of erythrocytes or their disintegration products on cellular proliferation was shown and superficially studied. Our observations are in accord with Earle's (15) experiments with the effect of light on blood and tissue cells. He showed that erythrocytes, leucocytes and fibroblasts degenerated when exposed to irradiation, and noted that the disintegration products of red blood cells increased the degenerative changes in fibroblasts. If an amount of irradiation which had practically no effect on fibro-

blasts in the absence of erythrocytes was applied to similar cultures of fibroblasts in the presence of red blood cells, marked degenerative changes took place. Earle also believed that the disintegration products of red cells possibly caused degenerative changes in leucocytes. Our results corroborated this opinion. Further analysis indicates that the toxic factor in disintegrating erythrocytes resides in the lipoid-containing stroma.

Our studies show that when environmental conditions are favorable, and when manipulative trauma is reduced to a minimum, certain isolated cells will proliferate to form pure colonies of fibroblasts. Repeated subcultures of these pure colonies of fibroblasts may be carried on. It is also suggested that with better knowledge of the requisite environmental factors together with suitable methods for isolating other tissue cells, pure strains derived from single cells of various tissues may possibly be developed.

SUMMARY

1. Most isolated guinea pig mononuclear exudative cells in tissue culture become typical migrating macrophages, but a small proportion take on fibroblastic characteristics, and produce pure colonies of fibroblasts. These fibroblasts maintain their morphological characteristics through repeated subcultures.

2. It is suggested that the subsequent development of individual mononuclear cells in tissue culture is conditioned at the time of explantation.

3. Apposition with other cells is not necessary for the initiation of mitotic cellular division.

4. There is a definite optimal relationship between the bulk of the medium, the number of explanted cells and the extent of proliferation. The presence of other cells in the vicinity enhances cellular division.

5. Mitosis in the isolated explanted cell is preceded by a latent period. The rate of division varies in different colonies of fibroblasts.

6. Admixed erythrocytes in the mononuclear suspension definitely inhibit proliferation of fibroblasts in tissue culture. The inhibiting factor in disintegrating erythrocytes is apparently present in the stroma.

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EXPLANATION OF PLATES

PLATE 9

FIG. 1. Fibroblastic growth from guinea pig buffy coat 6 days after explantation. Wandering cells are also present. × 120.

FIG. 2. Type of fibroblastic growth 48 hours after transplantation of culture shown in Fig. 1. × 90.

PLATE 10

Figs. 3 to 14 inclusive. These photomicrographs show only the cells that have arisen from the single isolated cell in Fig. 3.

FIG. 3. Photomicrograph showing degree of isolation of a mononuclear cell in mitosis (anaphase) 2 days after explantation, 10.45 a.m. × 180.

FIGS. 4 to 7 inclusive. Higher magnifications showing further stages in mitotic division. Fig. 4, taken at 10.45 a.m.; Fig. 5, at 10.53 a.m.; Fig. 6, at 11.05 a.m.; and Fig. 7, at 11.30 a.m. × 400. The indistinctness of cellular outline in Fig. 5 is due to the marked activity associated with the "boiling" stage.

FIG. 8. 9 hours after Fig. 7. Two cells in resting stage. 8.15 p.m. × 180.

FIG. 9. 3 days after explantation. Three cells, 10.30 a.m. × 180.

FIG. 10. 4 days after explantation. Six cells, 11.30 a.m. × 180.

FIG. 11. 5 days after explantation. Fourteen cells, 2.30 p.m. × 180.
FIG. 12. 6 days after explantation. Twenty cells, 10.30 a.m. × 90.
FIG. 13. 7 days after explantation. About thirty-five cells. × 90.
FIG. 14. 8 days after explantation. × 90.

PLATE 11

FIGS. 15 to 18 inclusive. Lower magnifications of the same colony shown in preceding photomicrographs. Portions of two other colonies of fibroblasts are seen sending out tongues of cells and uniting with the first colony. Several wandering macrophages are visible. Fig. 15, 9 days; Fig. 16, 10 days; Fig. 17, 12 days; and Fig. 18, 13 days after explantation. × 40.

FIGS. 19 and 20. Still lower magnification of combined colonies of fibroblasts showing continual growth. Small colony at top of photograph is degenerating. Fig. 19, 14 days; and Fig. 20, 16 days after explantation. × 25.

FIG. 21. Higher magnification of blocked area in Fig. 19, showing protoplasmic processes. × 90.

FIG. 22. Group of macrophages 5 days after explantation showing variation in size and shape. × 90.

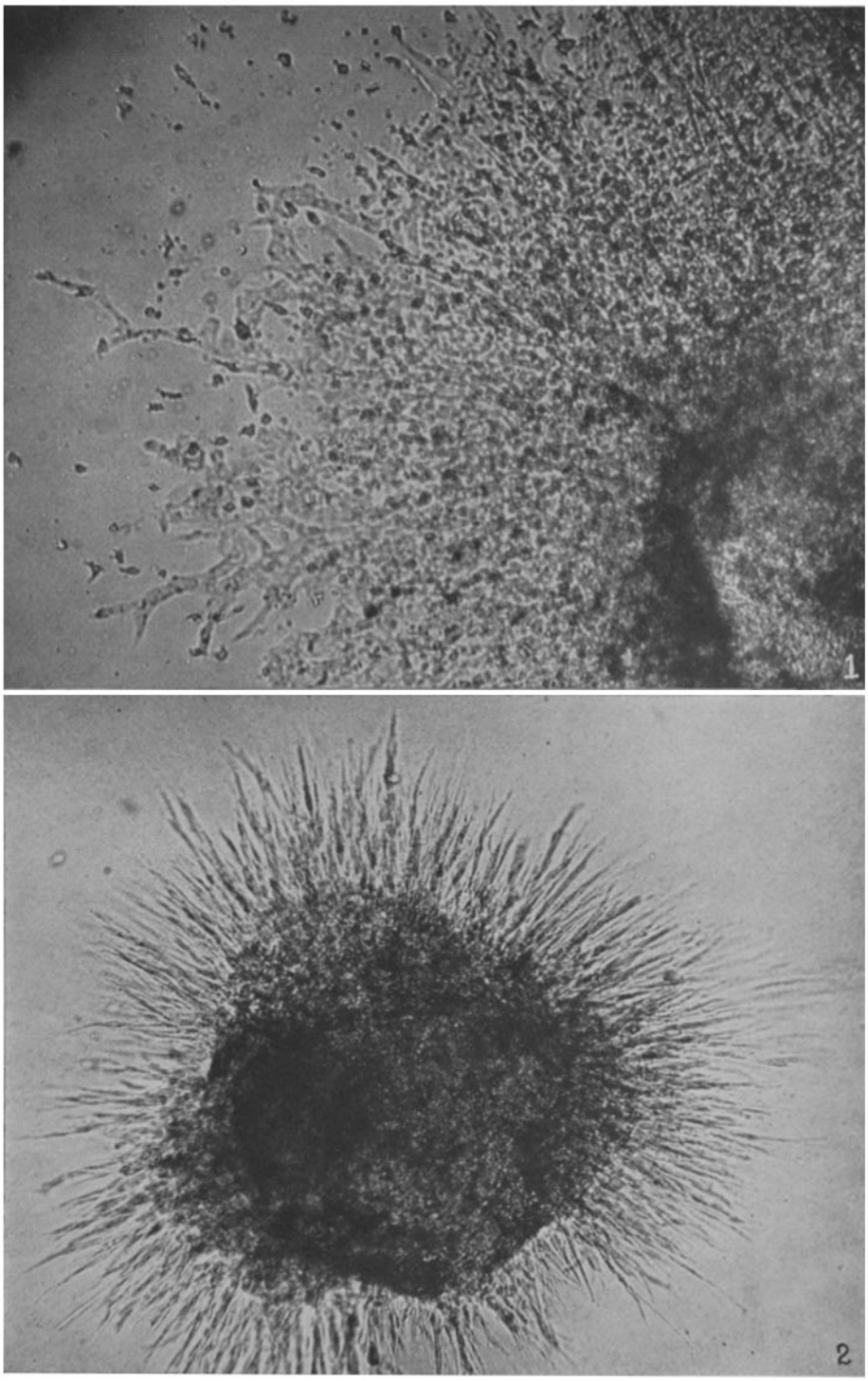
FIG. 23. Branching type of fibroblastic growth in colony 15 days after explantation. × 40.

FIG. 24. "Transitional" type of cellular growth originating from single cell 9 days after explantation. Note the absence of pointed protoplasmic processes and the presence of peripheral zone of clear cytoplasm. × 90.

FIG. 25. Fibroblastic cellular type 4 days after the second transplantation of fibroblastic growth from mononuclear exudative cells. × 90.

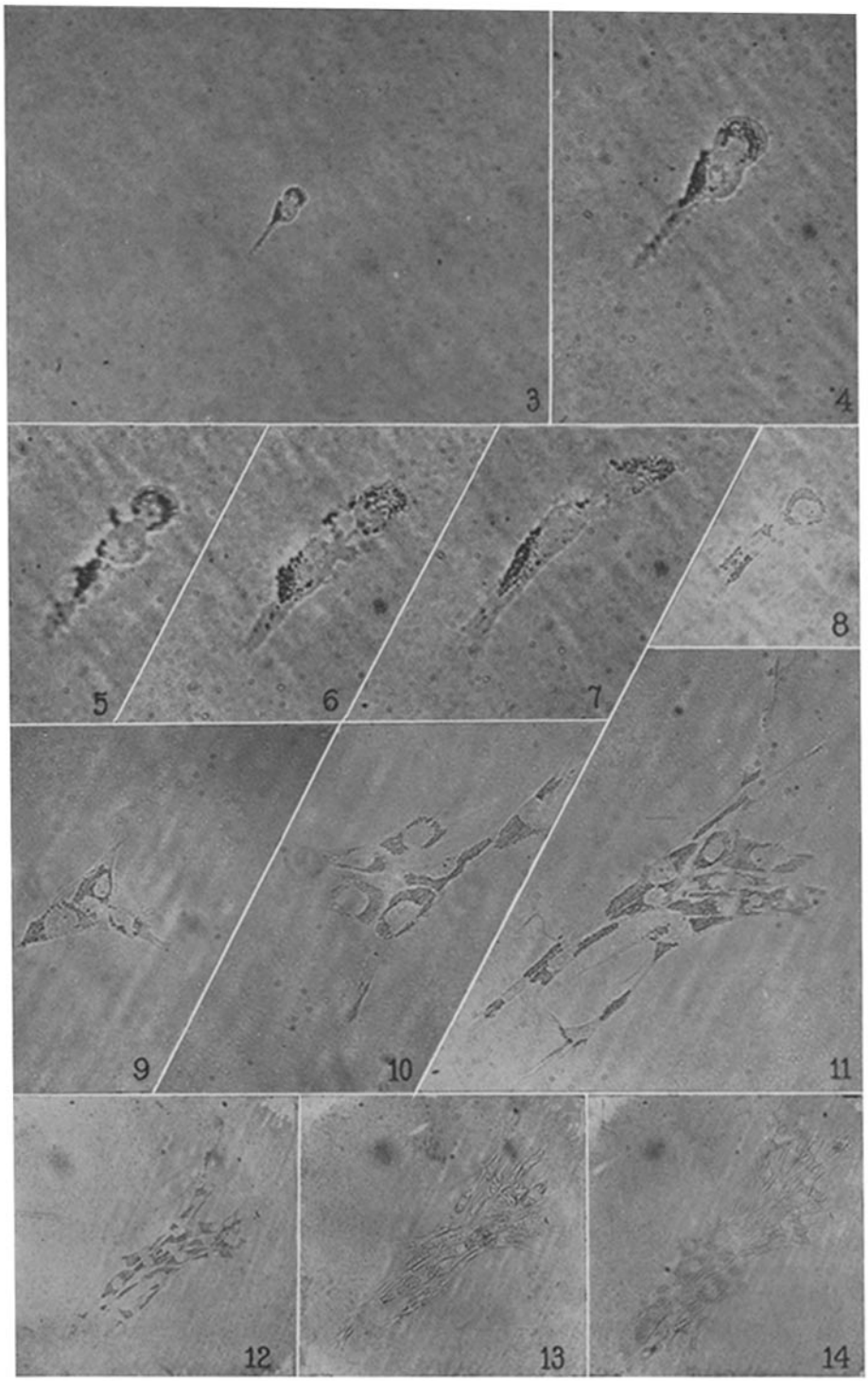
FIG. 26. Growth from a large clump of mononuclear exudative cells 2 days after explantation showing macrophages and early fibroblastic growth. × 90.

FIG. 27. Colony of fibroblasts from single cell 18 days after explantation showing degenerative changes. × 90.



Photographed by Louis Schmidt

(Moen: Cultures from single mononuclear cells)



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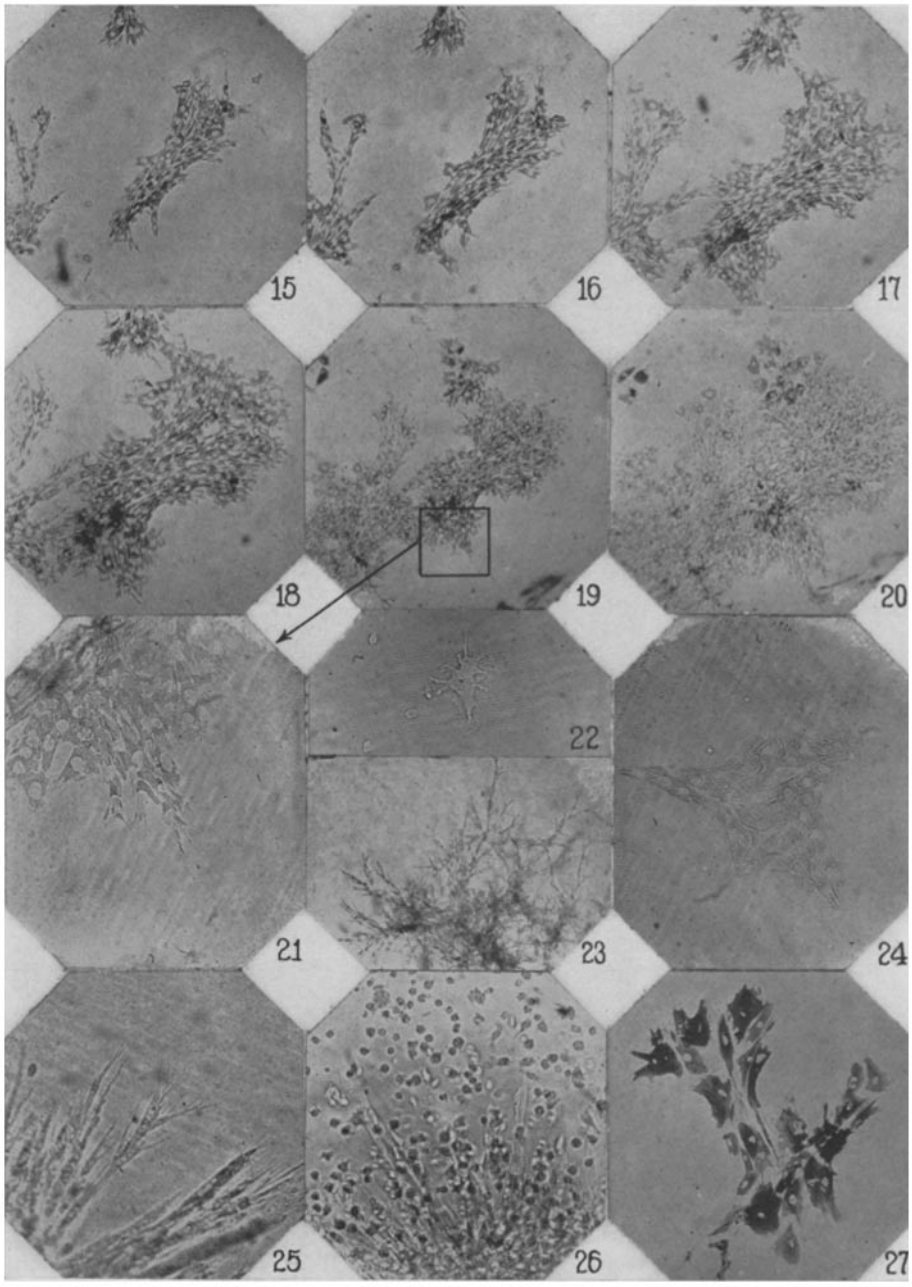

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