DEMONSTRATION OF EPITHELIAL MOVEMENT BY THE USE OF VITAL STAINING, WITH OBSER- VATIONS ON PHAGOCYTOSIS IN THE CORNEAL EPITHELIUM

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FOUR FIGURES

Though the cornea of the adult frog is thin and transparent enough for the observance of epithelial movement, it is not an easy matter to note in detail every part of the process. For this reason certain vital stains were used in the present experiments.

In the amphibians, vital staining can be produced either by introducing the dyes into water in which the animals swim, or by injection, though the latter procedure is not practicable in the case of corneal epithelium, owing to its lack of blood-vessels. The stained tissue may then be explanted. Another method is to add the dyestuffs to the culture medium containing the unstained tissue.

Several papers have appeared dealing with the different phases of this subject (Lewis and Lewis, '15; Russel, '14).

For the purpose of the present work it is necessary that the dyes should not have any deleterious effect upon the epithelial cells in order that they may remain capable of maintaining their normal activity. The staining must be produced easily and last for a considerable time.

That the epithelium of amphibians (especially larvae) is tingeable by various dyestuffs without affecting the organism or tissue elements to any marked degree has been shown by various observers (Fischel, '01). Whether vital staining may be employed in order to demonstrate moving epithelium in the culture is, however, another question.

A. METHODS OF VITAL STAINING

1. Methods of staining by immersing the whole animal in water containing dyes

a. Neutral red. Neutral red, which was first used by Ehrlich for vital staining, was considered as probably being the most suitable for the present purpose, and this proved to be the case.

In the majority of the experiments "neutral red for vital staining (Ehrlich)" was used, and the frogs were subjected to various concentrations of the dye for various lengths of time.

The experiments may be divided into two groups. In the first the animals were subjected to a relatively concentrated (1:20,000) neutral red solution for a short time (one to five hours), and in the second they were kept in a more dilute solution (1:100,000 to 1,000,000) for a much longer period (one-half to four days). The frog was then carefully washed in running water, the tissue removed and prepared in a manner similar to that used in previous experiments (Matsumoto, '18). Both modes of staining are adapted to the study of the movement of epithelium.

Though the frog could be stained an intense red without doing it any apparent harm, still the dye proved to be more or less harmful to the cells. In fact, an earlier degeneration of the explanted tissue due to the injury of cells in such preparations was frequently seen. As it was desirable to stain the tissue in such a manner that the granules would be just evident enough for observation, weak solutions (such as 1:100,000 to 1:200,000; ten to twenty-four hours) were preferable. The use of the more rapid staining, at times, brought on the disadvantage of having the granules appear unevenly distributed.

The arrangement of the neutral red granules shows a more or less marked difference in the several cell layers of the corneal epithelium. Observing from the upper (outer) surface of the corneal tissue in the culture, we see the following:

1. The outermost layer (fig. 1, *d*) consists of flat polygonal cells with large nucleus and unstained fine refractive granules in the cytoplasm. These cells, which may be present all over the

original epithelial surface or may be absent here and there, do not usually exhibit neutral red granules and show no activity.

2. The next layer (fig. 1, *c*), which is the uppermost layer in some preparations, consists of cells which contain in the cytoplasm the most minute red granules, almost uniform in size. These appear in abundance after prolonged intense staining. The contour of the individual cells is hardly visible at first. In some instances, especially when the rapid method of staining was employed, larger granules appeared in the cells so abundantly that they almost filled the cell body, leaving only a clear round or oval space in the center, occupied by the nucleus.

3. The basal cells (fig. 1, *a*), which are evidently smaller than those of the upper layers, exhibit, as a rule, distinct and much larger granules, not very uniform in size, and often more numerous on one side of the nucleus than on the other.

4. Between the two latter layers frequently another layer of cells (fig. 1, *b*) is visible. These are intermediate in size between the cells of the basal layer and those of the layer above, and as regards the arrangement of the colored granules show a resemblance to the cells of the second layer.

Furthermore, between the second and third layers there were found a number of deeply red stained bodies, round or irregular in shape, some of which showed a more deeply stained red spot (one of these is shown in fig. 1, *a*, and another in fig. 2, *a*). The origin of these bodies is unknown.

When the culture is observed several hours or more after the preparation, the cells of the upper and basal layers are easily distinguishable according to the distribution of granules in their cytoplasm, and this becomes more marked as the tissue becomes more translucent. The individual granules were sometimes observed to coalesce gradually, changing in shape.

When the tissue is intensely stained, the cells of the connective tissue and of the posterior endothelium also show distinct granules which are usually not so abundant and are more or less distinguishable from those of the epithelium. The nucleus remains unstained throughout.

Cultivated in vitro, the corneal epithelium, when stained with neutral red, showed a condition entirely similar to that seen in unstained preparations. The cells exhibited practically the same degree of activity, though at times they degenerated a little earlier than in the control preparation.

By this method, the cell movement along the tissue, which took place, as a rule in the fluid or semifluid culture medium, could be easily detected. That the spreading epithelium was, as a rule, two and sometimes three layers thick, was clearly demonstrated (fig. 2, *a*), while in an unstained preparation it was rather hard to determine whether they were one or two cells in thickness.

As the epithelium spread out and the individual cells became very flat and thin, a change in the arrangement of the granules took place. Then the granules of the basal cells, which were rather irregularly distributed at first, frequently assumed a crescent-shaped arrangement (fig. 2, *a*). If the staining was light, the tiniest red granules in the cells of the surface layer became faint relatively earlier. As a rule, the hyalin processes of the cells on the border did not exhibit any red granules. In general, after all activity of cells ceased, the red granules faded out and fatty granules increased, whereas the nucleus remained unstained. The same was true of the isolated epithelial cell.

So far as our observations go, there was in general a direct relation between the cell activity and presence of neutral red granules, although of course the intensity or richness of the granules was not always parallel with the cell activity.

b. Some other dyes. In the corneal epithelium subjected to 'Nile blue sulphate,' the granules were beautifully stained. This dye was found, however, to be injurious to the cells, and even the solution 1:200,000 in most instances caused more or less injury to the epithelium, though not enough to interfere immediately with the cell activity, since in many cases the cells continued to move. Disintegration of cells took place earlier, and a preparation which showed intense granulation never lived so long as the control culture (unstained or stained with neutral red). In addition to this, in the preparation subjected to the dye, fine violet crystals appeared in the cells or in the medium. Nile blue,

therefore, does not seem to be a very favorable dye for the corneal epithelium.

Neither is 'methylene blue (rectif.)' suitable for the purpose, as it fades too easily.

So far as our observations go, 'gentian violet' did not give a satisfactory result. Even the weakest solution, which stained the cells, caused their death.

'Brilliant cresyl blue 2 b' and 'methyl green' did not stain the granules clearly, nor did 'indigo carmin,' 'toluidine blue,' or 'litmus' give positive results.

2. Staining of the cornea by administration of dyes in the conjunctival sac

Arnold ('00) demonstrated neutral red and methylene blue granules in the frog cornea by conjunctival administration of the dyes in the form of fine powder. In this way typical granules appeared in the cells, often causing injury. No experiments of this kind are recorded in the present paper.

3. Staining of excised cornea

In some experiments the cornea was first excised from the animal and then stained. The tissue, after removal, was immersed in physiological saline solution containing neutral red, freshly prepared, so that the epithelium became intensely red, and could later be cultivated after thorough washing. The technique is simple and is useful in some particular instances. Usually, however, crystals appeared in the culture, when this method was employed.

4. Staining by addition of dyes to the culture medium

Both neutral red and Nile blue, freshly dissolved in salt solution, were experimented with. They are easily soluble in pure water, but not so easily if the water contains any salt (NaCl). The appearance of crystals and the unevenness of the staining proved this method to be an unsatisfactory one. Staining of nucleus and granules by the use of gentian violet failed. Methylene blue stained granules, but it could not be employed for the purpose on hand.

B. PHAGOCYTOTIC PHENOMENON OF THE CORNEAL EPITHELIUM

Though the experiments on this line have not yet been completed, some interesting phenomena will be described here.

It was noted in the course of this work, that not infrequently in the preparations of cornea, where the pigment of the iris or chorioidea was accidentally mixed with the epithelium, the cells of the latter, originally not pigmented, took up the pigment, becoming gorged with it. This was easily demonstrable, but more markedly when the pigment of the eye was finely teased and put into the culture medium with the corneal epithelium. The cells of the moving border, especially, took up the dark pigment abundantly.

That the melanin granules are really taken up in the cytoplasm of cells, was clearly demonstrated in the preparations which were first vitally stained with neutral red (fig. 3). That those pigmented cells were not the originally pigmented epithelium of eye, accidentally mixed in the preparation, is beyond doubt. The epithelial cells were able to take also melanin which was previously heated or boiled.

The arrangement of the pigment granules in the cytoplasm was characteristic, resembling that of the neutral red granulations. The cells of the basal layer were often abundantly packed with the pigment, and those of upper layers were able to ingest it, too.

If in the epithelial cells which are originally not pigmented, abundant melanin is taken up, it is extremely difficult to distinguish them from the original pigment cells.

The possibility of epithelial phagocytosis has been considered by Riehl ('84); somewhat later, Rabl ('96) also took up the question and found that the epithelium of adult salamanders (*S. maculosa*) is able to take up carmin injected subcutaneously. In cultures of carcinoma and sarcoma, Lambert and Hanes ('11) demonstrated that carmin granules are taken up by the cells. Carrel and Burrows ('11) observed a similar phenomenon in cultures of sarcoma.

The melanin problem represents one of the most interesting questions in dermatohistology and experimental dermatology and is a much discussed subject. I should not go, of course, so

far as to say that the so-called 'Einschleppungstheorie' of melanin is generally accepted, though the fact stated above shows the possibility of its correctness.

Though the observations here recorded have been made only upon cells in culture, still it may not be impossible that the same occurrence takes place in vivo under certain conditions. At any rate, the phagocytic action toward melanin of epithelium of the adult frog in culture is here definitely shown.

Therefore, it is not quite safe, in the culture of pigmented epithelium, such as iris, to consider the pigment granules as the peculiar possession of that epithelium, because the original non-pigmented epithelium can ingest pigment granules. The study of a complex and peculiar cell, such as the pigment cell, requires great care in the methods used and in conclusions drawn.

Carmin, which was ground into fine powder, was also taken up into the cell bodies, which then showed beautiful carmin granulation. For this purpose the use of tissue, vitally stained with Nile blue, offered great advantage.

The arrangement and distribution of the granules was entirely similar to that of the melanin; the particles were arranged with considerable uniformity around the periphery of the cytoplasm.

As figure 4 shows, corneal epithelium, cultivated in plasma (or serum), containing both melanin and carmin, exhibits beautiful melanin and carmin granulations which can be preserved safely. Examination of such preparations in serial sections demonstrated the intracellular and perinuclear position of the granules. That they are in the cell bodies may be placed beyond doubt even by the careful observance of fresh preparation. Furthermore, such preparations have the advantage of enabling the observer to watch how cells with ingested granules may become detached from the main mass and move off in the culture medium.

Fine granules of Chinese ink or minute foreign bodies accidentally mixed in the culture were also taken up by the epithelium.

In regard to this most interesting phenomenon of epithelial phagocytosis, our present knowledge is very deficient. A strict differentiation of the epithelial movement from that of leucocyte

may not be possible in the case of the tissues of the frog living in vitro. Not only in their movements, but also in the phagocytic phenomena (with respect to foreign bodies) do they show a certain degree of resemblance.

The question of the said phenomena in the warm-blooded animals, as well as their bearing upon bacteriology and serology, will be considered later.

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C. SUMMARY

The epithelium of the cornea of the frog which was vitally stained with neutral red and Nile blue exhibits characteristic granules in the cytoplasm.

If properly stained, the granules exist through the entire period of cell activity, without practically affecting the cells, and facilitate the observance of cell movements.

Phagocytic phenomena of the corneal epithelium with reference to melanin, carmin, etc., are definitely demonstrated.

The distribution and arrangement of melanin and carmin granules, if finely powdered, show a certain degree of resemblance to that of neutral red and Nile blue.

LITERATURE CITED

- ARNOLD 1900 Granulabilder an der lebenden Hornhaut und Nickhaut. *Anat. Anz.*, 18, 45.
- CARREL AND BURROWS: 1911 Cultivation in vitro of malignant tumors. *Jour. Exp. Med.*, 13, 571.
- FISCHEL 1901 Untersuchung über vitale Färbung. *Anat. Hefte*, 16, 417.
- LAMBERT AND HANES 1911 Characteristics of growth of sarcoma and carcinoma cultivated in vitro. *Jour. Exp. Med.*, 13, 495.
- LEWIS AND LEWIS 1915 Mitochondria and other cytoplasmic structures in tissue cultures. *Am. Jour. Anat.*, 17, 339.
- LOEB 1912 Growth of tissue in culture media and its significance for the analysis of growth phenomena. *Anat. Rec.*, 6, 109.
- MATSUMOTO 1918 Contribution to the study of epithelial movement. The corneal epithelium of the frog in tissue culture. *Jour. Exp. Zool.*, vol. 26.
- OPPEL 1912 Causalmorphologische Zellenstudien. V. Mitteilung. *Arch. Entw.-Mech.*, 35, 371.
- RABL 1896 Untersuchungen über die menschliche Oberhaut und ihre Anhangsgebilde mit besonderer Rücksicht auf die Verhornung. *Arch. Mikr. Anat.*, 430.
- RIEHL 1884 Zur Kenntnis des Pigmentes im menschlichen Haar. *Viertelj. Derm. u. Syph.*
- RUSSEL 1914 The effect of gentian violet on protozoa and on tissues growing in vitro, with special reference to the nucleus. *Jour. Exp. Med.*, 20, 545.
- ZIELONKO 1874 Über die Entwicklung und Proliferation von Epithelien und Endothelien. *Arch. Mikr. Anat.*, 10, 351.

PLATE 1

EXPLANATION OF FIGURES

1 Experiment 202. C. 11. Neutral red granules in different layers of the corneal epithelium (adult frog) cultivated in plasma, 12 hours old. *a*, Granules in the basal cells; *b*, cells of a layer often recognizable between *a* and *c*; *c*, granules in the cells of upper layer; *d*, cells of the uppermost layer, which show no activity. $\times 450$.

2 Experiment 206. A. 3, showing neutral red granules in the moving epithelium, *a*, endothelium, *b*, and below, those of connective-tissue cells; *c*, 24 hours old. Culture in plasma. Movement (from left to right) of the ameboid border of the epithelium, *a*, on the endothelial surface *b*, is demonstrated. That the moving epithelial membrane consists at least of two layers of cells is readily confirmed by the types of granulation. Note the crescent-shaped arrangement of granules in the basal cells. On the right, *b*, those of the endothelium are represented; the contours of the cells are not visible. Connective-tissue cells (*c*), the contours of which are visible, show granules in their fine processes, too. As a rule, the hyaline processes of the epithelium do not show any granules (see also figs. 3 and 4). The tissue was intensely stained (four days in the dye 1: 200,000). $\times 250$.

3 Experiment 226. A. 2, showing phagocytic phenomenon of the corneal epithelium, which are previously stained with neutral red; 24 hours old. Resemblance of arrangement of melanin granules to that of neutral red is noticeable. *a*, Drawn from a part of epithelium, moving on the endothelial surface of cornea; *b*, epithelial cells of actively moving border. $\times 450$.

4 Experiment 236. 12. Phagocytosis of the epithelium to melanin and carmin; 18 hours old. Culture in autoplasm, in which fine powdered carmin and fresh melnin of iris were added. Drawn from a part of moving epithelial rim. $\times 450$.