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In a former paper L. Loeb, 97, described the changes occurring in the pigmentation of regenerating black skin on the ear of the guinea-pig, and after transplantation of pigmented skin into white skin. In this paper we give the results of a study of the conditions in regenerating frog skin. We shall consider principally the chromatophores and their origin in the epidermis, and add some notes on histological changes involved in the regeneration. We also give a brief statement of the results of some experiments with atropine and pilocarpine solutions.

These later experiments were undertaken with the intention of investigating the influence of different substances upon the growth of tissues in higher animals. In earlier experiments one of us (Loeb) had investigated the influence of narcotic substances like alcohol and chloroform upon the regeneration of the tail in tadpoles. In these experiments atropine and pilocarpine were chosen, because Matthews, 02, had more recently found that pilocarpine accelerates the development of fertilized ova of Asterias somewhat.

Methods and Material.—A patch of skin, 5—8 mm. long and 3—5 mm. broad, usually elliptical, but sometimes oval in outline, was removed from a black area on the dorsal surface of the left shank of each frog. The animals were in apparently good condition and mostly very active. Before operation they were kept at the laboratory for several days in large battery jars containing water about 1-1½ inches deep.

After the removal of the patch of skin, the frogs were divided into three series and placed in jars. The animals of one series were in jars containing tap water; another series was given a solution of atropine sulphate and a third lot of frogs had a solution of pilocarpine hydrochlorate. Both solutions had one part of the salt to 10,000 parts of water. A few animals also were placed in solutions of 1-1000 strength.

The solution and the tap water for all these series were changed every day for the first week and every other day thereafter. At the end of periods varying from ten hours to five weeks, frogs were killed and the tissue about the wounds removed.

Corrosive-acetic was used for fixing and the material was embedded in celloidin. Serial sections were made and stained in Delafield's hæmatoxylin and eosin. Altogether, series of sections from 62 frogs were examined.[1]

Observations.—No constant effects on regeneration have been noticed for the atropine and pilocarpine solutions used. Regeneration of both epithelium and connective tissue seemed to take place equally well in either the weaker atropine, the pilocarpine solutions, or the tap water. There was no marked difference in the number of mitoses when these solutions were used. In some cases there was a little evidence of possibly greater activity in regeneration, for some cases, in the pilocarpine solution than in the atropine. This difference was very slight and possibly accidental. In one series of experiments the number of leucocytes immigrating into the wound was decidedly larger in the pilocarpine solutions. This difference, however, was not observed in a second series. Frogs kept in either the weaker or the stronger solutions of pilocarpine did not behave differently from those in tap water, even at the end of four or five weeks; they were nearly all equally active. The animals placed in the stronger atropine solution were very stupid and helpless at the end of the first day or two, apparently being partially paralyzed, and none lived longer than five days. The weaker solution seemed to have a somewhat similar but much milder effect in a few cases.

In the former series of experiments carried out in the spring of 1901, and referred to above, tadpoles, whose tails had been cut, were kept in 1-6 per cent solutions of alcohol and in weak chloroform water up to six days after the operation; the control animals lived in tap water. These experiments were undertaken in order to ascertain whether or not these narcotic substances delay and weaken the movements and the growth of the cells. No marked differences in regeneration were observed between the different sets of tadpoles, with the possible exception of slight differences in the rapidity with which the epithelium covered the wound.

Regeneration of the Epidermis.—Barfurth, 91, observed in the Salamander a rapid movement of epithelium over a wound before any in-

[1] A part of these investigations was carried on with the aid of a grant from the Research Fellowship Fund of McGill University.

crease in the number of mitoses could be seen. We have made the same observation in the case of wounds in frog skin. The movement of epithelium is not limited to one layer of cells but involves several. The lower portions of the cells in the deepest layer of the epidermis move first toward the wound, and the cell-axes are occasionally rotated almost as much as 90° in the process, so that they come to have a horizontal position in place of the former vertical direction. This movement of the epithelium begins during the first hours after the operation and is completed within one to three days. There is no distinct increase in the number of mitoses, however, until after the second day.

The rapidity with which the epithelium moves over the wound after operation, together with the absence of any increase in the number of cell divisions normally occurring, indicates that the movement is not due to cell-proliferations [2] but to other causes.

Some observations made by L. Loeb on regenerating tadpole epithelium seem to indicate considerable tension in the regenerating epithelium which may be more or less responsible for the movements. He found papillæ which had ben formed apparently through the folding of the upper layers of regenerating epidermis, though they may have been the result in some cases of a degeneration of epithelial cells. These papillæ may occur within a few hours after the operation or a few days later.

The changes determining the movements affect the cells nearest the wound first, but are later extended to the epithelium farther away. The epithelium moves only in contact with solid bodies.

Mitoses occur not only in the deepest layers of the epidermis but also as high as the fifth or sixth layers, whereas in regenerating guinea-pig's skin, they were found almost exclusively in the two lowest layers.

Within forty-eight hours after the wound is made hypertrophy is seen. Individual cells enlarge and become more and more numerous.

We almost invariably find degeneration of epidermal cells connected with this hypertrophy. Such degeneration is also seen in the tissue which has advanced farthest over the wound, and is also found in the deepest layer of the epithelium in cases where many leucocytes penetrate this tissue. The nucleus of degenerating cells usually becomes kayorrhectic and the cytoplasm homogeneous, staining well with eosin. The degenerative changes accompanying the hypertrophy may be found even

[2] The proliferation of cells in the regenerating epithelium takes place both by mitosis and amitosis. Amitosis was first described for regenerating mammalian epithelium by L. Loeb, 98; later by Marchand, 01; and Werner, 02; and Nussbaum, 82, has observed amitosis in regenerating epithelium in the cornea of amphibia.

in cases where the connective tissue underlying the hypertrophied epithelium has regenerated perfectly. These degenerations are accompanied by cell-inclusions, which are sometimes almost indistinguishable from red blood corpuscles. It was found by L. Loeb, 02, that epithelial cells in regenerating mammalian skin do actually take up blood corpuscles and other solid particles.

In one case, 14 days after the operation, a development of epithelial pearls had taken place in the regenerating and hypertrophied epithelium of a frog which had been kept in a solution of atropine. Epithelial pearls could also occasionally be seen in the guinea-pig epithelium which was growing in agar. We believe that these morphological changes do not indicate a tendency of this epithelium to assume a carcinomatous character, an interpretation which has been given to similar formations by certain investigators.

It was not uncommon to find processes of the regenerating epithelium penetrating the coagulum beneath the wound. They may advance in different directions, either in one layer or in several layers of cells. Often the fibrin fibers are merely bent inwards by the advancing epithelial cells, but they are sometimes actually perforated by the epithelium.

This penetration of the fibrin may occur within twenty-four hours after the operation, and processes in the epithelium may be observed in the fibrin as late as ten days. The cells in these processes multiply mitotically, and mitosis occurs in the epithelium lying directly on the coagulum, also, just as was the case in the experiments of Loeb for epithelium penerating coagulated blood-serum and agar.

Regeneration of the Cutis.—Though regeneration in the epidermis begins within a few hours after the operation, it does not appear in the cutis until the fifth day. When once started, however, the regeneration is frequently rapid. After six days, or a day or so from the beginning of regeneration in the cutis, a small defect may be entirely filled by connective tissue and capillaries; at later periods it was sometimes impossible to detect the wound. The position of the former wound was recognized in one case at the end of three weeks only through the presence of small mononuclear cells (lymphocytes?) in the connective tissue. In another case, taken thirty-four days after the operation, masses of small round cells in the cutis indicated the previous existence of a wound; the connective tissue had not regained its typical structure.

In a number of cases where the defect in the cutis was comparatively large there was either no regeneration of connective tissue or it was more or less incomplete. Though, in many cases, a variable number of leucocytes were frequently present in the fibrin, this was not always the case,

and it seems unlikely that such failures in regeneration were due entirely to infection by micro-organisms. Those connective-tissue cells that advance into the fibrin quite frequently degenerate; they swell up and their nuclei are destroyed by chromatolysis.

In the case where the cutis did not heal, masses of leucocytes were found in the epidermis at some places. It has not been possible to decide whether this condition was due to an invasion of leucocytes into the epithelium after which a destruction of epithelial cells followed, or whether on the contrary a degeneration of the epithelial covering, caused by imperfect healing of the connective tissue, was the primary factor, resulting, secondarily, in an immigrating of leucocytes.

A number of small gland tubules were seen under the regenerating epidermis in three wounds, at the end of the third week in two cases, and, after 34 days, the third one had gland cells dividing mitotically. In these cases only the most superficial portion of the cutis had been removed with the epidermis. In the skin adjoining the wound, typical large glands were present. It seems likely that we have here a regeneration of glands, but, as in these cases only a small part of the cutis had been removed with the epidermis, it has been impossible to determine whether or not such glandular regeneration starts from gland cells left in that part of the cutis not removed, or in the epidermis itself.

The Chromatophores of the Regenerated Tissue.—There has been no unanimity of opinion concerning the origin of chromatophores, or pigment-bearing cells with ramifying processes, in the epidermis. The earlier views, that they are immigrated leucocytes, or common connective-tissue cells that have invaded the epidermis, have been more or less generally abandoned. At present two views are held, either (1) that all chromatophores of the body are of common mesodermic origin, or (2) that the chromatophores of the epidermis are simply modified epithelial cells. This latter view has been held by a number of writers, including Kodis, Jarisch, Post, Kromayer, and ourselves, Loeb, **97**, Strong, **02**. According to the first view, all chromatophores, at a certain stage of embryonic development, are differentiated from ordinary connective-tissue cells, and a part of them grow secondarily into the epidermis. These are called melanoblasts by Ehrmann, **96**, the main exponent of this idea. Ribbert, **01**, holding the same opinion, believes that the pigmented tumors, arising from pigmented nævi of the skin, are composed entirely of such cells, and accordingly calls them *Melanomata* to designate their genetic difference from other tumors.

One of the aims of our studies was to compare the behavior of the chromatophores and the pigmentation of the regenerating frog skin with the pigmented skin of the guinea-pig during regeneration.

The pigmentation of frog skin differs considerably from that of the guinea-pig. In the frog cutis there is usually a well-marked layer of chromatophores, which are generally much larger and frequently more branched than the epidermal chromatophores, whereas the guinea-pig cutis has no well-developed chromatophores and its pigment is distributed irregularly in masses or clumps. The dermal chromatophores of the frog are separated from the epidermis by considerable connective tissue, and the epidermal chromatophores are usually situated higher up in the epidermis than is the case with the guinea-pig.

L. Loeb, 98, distinguished four stages in the development of pigmentation in the regenerating black skin of the guinea-pig. These were not observed in the regenerating frog skin.

As in the case of the guinea-pig, we find no evidence of an immigration of dermal chromatophores into the epidermis of the regenerating frog skin.

The epithelium, which moves over the wound soon after the operation, carries chromatophores and ordinary pigmented epithelial cells. These chromatophores are usually found to be without processes. During the first two weeks similar chromatophores are frequently observed in the regenerating epithelium. They may still be found during the third week, especially in the central part of the regenerated epithelium. Under these conditions they may appear as ordinary pigmented epithelial cells; they carry, however, more pigment than the latter. Kromayer has also observed chromatophores without processes near the margins of wounds in amphibia.

The number of well-developed chromatophores with large processes increases gradually in the regenerating epithelium, and they become especially numerous near the margins of the regenerating area. Chromatophores without processes were found, however, even at later periods, in the hypertrophied epithelium where cell-degeneration occurred.

Chromatophores divide mitotically during regeneration in frog skin. Two chromatophores were found in mitosis *at places where ordinary epithelial cells were also dividing mitotically,* one at fourteen and the other at nineteen days after the operation. One showed processes but the other had none.

In regenerating epithelium, the chromatophores are not arranged in as regular a manner as in ordinary epithelium. During the first two weeks many chromatophores of the epithelium covering the wound and occasionally also of the adjoining epithelium, are carried into the upper part of the epidermis and are frequently cast off. Sometimes the chromatophores are pushed into the lower layer of the epithelium, *and even*

farther into the underlying fibrin; they never come from the underlying tissue into the fibrin. Under these conditions they usually lose their processes. Near such places the fibrin may be entirely free from connective tissue. In some cases, taken at different times during the first three weeks, the skin adjoining the wound was unequally pigmented, and the regenerating epidermis often varied correspondingly in pigmentation. On a side where the epithelium adjoining the wound was more heavily pigmented, there were more chromatophores in the regenerating epidermis over the wound than at another place where the adjoining side was less pigmented.

Instead of the common arrangement of pigment on the outer side of the nucleus, which is characteristic of normal epithelial cells, we often find an irregular arrangement of the pigment in the cells of regenerating epithelium, which is probably due to the turning of the cells in the movement over the wound.

Chromatophores do not appear in the cutis until after two or three weeks, though regeneration begins here at the end of five days. In fact, the sub-epidermal part of the wound is filled with connective tissue before any chromatophores are to be seen in it.

It is therefore evident that the chromatophores of the regenerating epidermis cannot possibly come from the regenerating dermis. Dermal chromatophores are sometimes found at early periods, *i. e.,* after the fifth day, projecting slightly into the wound where they were probably carried passively by the advancing fibroblasts. They remain, however, near the margin of the wound.

Occasionally we found small cells bearing pigment granules in the fibrin or in the newly-formed connective tissue. They are leucocytes or young connective-tissue cells. The chromatophores of the dermis were not regularly arranged at the end of thirty-four days; they were missing at some places, and at other points they were situated deeper than is the case normally. They appear, occasionally, in increased numbers at the margin of the wound where they are sometimes surrounded by masses of small round cells.

In the experiments with wounds in tadpole skin, referred to previously in this paper, the regenerating tissue was taken at periods varying from a few hours to six days after the operation. The chromatophores of both the epidermis and the cutis showed characteristics like those that have just been described for the frog.

In the case of transplanted guinea-pig skin, pigment is produced by epidermal cells and is not directly the product of material carried to the

cell by the blood or lymph. An unpigmented cell is surrounded by the same nutrient as a pigment producing one; yet the latter only forms pigment. The pigment forming epithelium may be transplanted to a place where formerly unpigmented cells were situated, and it will continue to produce pigment, though the blood supply must remain the same. The production of pigment by these cells can in no way be compared to the formation of pigment in connective-tissue cells which are in contact with extravasated blood.

The evidence furnished by these studies against Ehrmann's hypothesis of the mesoblastic origin of epidermal chromatophores may be summarized as follows:

(1) We find no indications of a migration of pigmented or pigment-producing cells of any kind from the dermis into the epidermis.

(2) Chromatophores were observed to multiply by mitosis in the epidermis, and they are found regularly in regenerating epithelium long before any dermal tissue has regenerated in the space below.

SUMMARY.

1. Solutions of pilocarpine, atropine, and alcohol in which the animals lived constantly had little influence on regeneration.

2. The rapid movement of epithelium over the wound soon after cutting the skin is not due to cell proliferations. It is more probable that a tension, either previously existing or called into play by the wound, is the cause.

3. Cells divide both by mitosis and by amitosis in the regenerating epithelium. In regenerating frog epidermis mitoses are found in higher layers of cells than in guinea-pig skin.

4. Epithelial cells move in all directions into the sub-epithelial coagulum, and they may break through fibers of fibrin.

5. If the wound is large, the sub-epithelial clot may remain imperfectly organized, and some connective-tissue cells may degenerate. There is often very little regeneration of connective tissue below the wound as late as three weeks after the operation.

6. The chromatophores in the epidermis of frog skin behave in regeneration as ordinary epithelial cells and not as the chromatophores of the cutis. The former regenerate rapidly and the latter very slowly. During regeneration, epithelial chromatophores may be found in the coagulum underneath the epidermis.

7. There is no evidence of an ingrowth of chromatophores into the epidermis from the cutis, and the epithelium of the regenerating patch

of skin is fully pigmented before any pigment appears in the cutis below. The pigment of the epidermis is found in cells whose origin is strictly epidermal.

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