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THE STAINING OF AMPHIBIAN LARVAE WITH BENZIDINE DYES WITH ESPECIAL REFERENCE TO THE BEHAVIOR OF THE LYMPHATIC ENDOTHELIUM

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A new point of view has developed during the last decade concerning the inter-relationship of certain cells of the blood and connective tissue; we have had no satisfactory classification for such cells heretofore. A number of morphologically heterogeneous endothelial and connective tissue elements have been grouped together into one class by the possession of a common physiological function—that is, the ability to ingest, aggregate, and store in their cytoplasm the submicroscopic particles afloat in colloidal sols and solutions of the acid azo (benzidine) dye-stuffs. To cells having this power Goldmann (1) has given the name pyrrhol-cell. Evans and Schulemann (2) have shown that the storage of the high molecular dyes by these cells is merely a manifestation of an ability to ingest and store foreign material, existing in the form of fine physical subdivision.

The pyrrhol-cells of the adult organism are of three general types. The first type, those which are truly endothelial in nature, line the capillaries of the liver, the venules and sinuses of the spleen, the haemal-lymph nodes, and the true lymphatic glands. The second type, which are more or less fixed cells, includes those widely distributed elements designated variously as adventitia, resting wandering cells, or clasmatocytes; and those colonies of cells closely akin to them, which are found in the omentum in which situation they are referred to as taches laiteuses. To this group belong also those interesting elements designated as reticulum-cells, which occur in the pulp of bone-marrow, spleen and lymph-glands. Thirdly, we find the free macrophages, which consist of the host of round, mono-nuclear cells inhabiting the serous cavities, the mono-nuclear cells occurring so abundantly at times within the lymph-sinuses of the lymphatic nodes and occasionally in the spleen and liver; and which under experimental conditions are

found in large numbers in the latter places and even in the peripheral circulation.

The histogenesis of these various elements has become, with our increasing knowledge of their importance in the adult economy, a fascinating and tempting problem for anatomists. With the discovery of the special affinity of these cells for the diazo-dyes, it seemed as though we were on the threshold of solving the mysteries of the origin of blood and endothelium, and their relation to mesenchyme and the adult connective tissue types.

Technical difficulties soon arose however, and our dream still remained unrealized. The placenta was shown to be impermeable to vital dyes, and the direct injection of dye into the embryo to be impracticable. Introduction of the dyes into avian embryos was fraught with even greater difficulty, and repeated attempts have ended in failure. There remained no alternative but to try to introduce the dye by some means into the embryos of one of the cold-blooded vertebrates.

The object in attempting to stain amphibian larvae would be: to determine the fate of benzidine dyes when introduced into the body of the embryo, to discover which elements possess the power to phagocytise high-molecular particulate matter and to ascertain the distribution of these elements; and furthermore to show what relation these embryonic phagocytes might bear towards the pyrrhol-cells of the adult. Staining these embryos might assist us to a knowledge of the genetic interrelationship of the sessile pyrrhol-cells, and the wandering phagocytes of the mesenchyme and the serous cavities, or to shed light upon the embryology of types of cells (Kupffer cells, taches laiteuses) concerning which we know practically nothing.

Amphibian larvae, immediately after hatching, seemed suitable subjects for such an investigation although feeding experiments on the adult animal have led us to expect very little from absorption of the dyes through the alimentary tract, and the delicate organisms seemed unfit for such drastic measures as injection of the vital stain into the peritoneal cavity. So the most reasonable method appeared to be, to introduce the dye-stuffs into the water in which the larvae swam, and trust to its being absorbed through the respiratory tract or through the epidermis, which in amphibia is known to be very permeable.

For this purpose, eggs, of the common species of frogs and salamanders in the vicinity of Baltimore, were collected in April, 1916, and brought to the laboratory where they were cared for in balanced aquaria.

The species used were Rana temporaria, Hyla pickeringii and Amblystoma. The main experiments were carried out with Rana temporaria, repeated trial having demonstrated a greater immunity to the toxic action of trypan-blue, the benzidine dye used in the experiments, for this than the other two species. The author wishes to emphasize here, that trypan-blue is not the physiologically inert, non-toxic substance some writers have claimed, but that even comparatively weak solutions are definitely toxic, as will be clearly brought out by the following experiments. I have also found the dye to be definitely toxic to fish and adult amphibia when given intraperitoneally in 1 per cent solution with perfect technique.

Immediately after hatching, the larvae were transferred from the aquaria to finger-bowls containing graded strengths of freshly prepared trypan-blue solutions, made up with ordinary tap-water. In the initial experiment the strongest solution was 1 : 50. The others in decreasing strengths were: 1 : 100; 1 : 200; 1 : 400; 1: 800; 1 : 1600; 1 : 3200. The larvae were observed twice daily, morning and evening, for a few moments, as a routine, on a glass-slide in a small amount of water. The animals in the 1 : 50, 1 : 100 and 1 : 200 solutions were all dead and partly macerated the morning following the initiation of the experiment. Nearly all the larvae in the 1 : 400 solution were dead and the remainder died within the next twenty-four hours. After repeating the experiment with these strengths, the use of a solution more concentrated than a 1 : 600 was abandoned. In the 1 : 800, 1 : 1600 and 1 : 3200 solutions the animals remained alive indefinitely from two to four weeks and seemed to be little affected by the toxic action of the dye.

On the fourth day of immersion of the animals in the 1 : 800 and 1 : 1600 solutions, faintly discernible traces of trypan-blue were to be seen, in certain cells in the tail. On the fifth and the sixth days it became apparent that the trypanophilic cells, which were gradually becoming stained, lay either immediately adjacent to the course of the lymphatics, or were the cells of the lymphatic endothelium themselves. Observation of the living tadpoles on the seventh and eighth days, in a chamber such as described by Clark (3), left no doubt, but that the trypan-blue granules were within the cytoplasm of the endothelial cells of the lymphatic capillaries. Under the low power of the microscope the lymphatic system of the tail, including the caudal trunks, was brilliantly outlined in blue.

Under the high power of the microscope, in a Clark chamber, the

nuclear areas of the lymphatic endothelial cells were visible at irregular intervals along the capillary wall. These areas appeared relatively free from trypan-blue, the dye corresponding in its distribution in the cell with the granular zone of the cytoplasm; and extending in both directions, it gradually faded away into the delicate strand of protoplasm outlining the wall. The vital dye was of uniform intensity, and the granules equally numerous, no matter whether the cell was situated near the sprouting tip of a growing vessel, or lining an established channel.

After careful observation it became evident that the lymphatic endothelium had been stained quite specifically. The endothelium of the blood-vessels, many of which were distinguishable because they contained rapidly circulating blood-corpuscles, showed not the slightest particle of vital stain. Careful examination, of both mesenchyme and wandering-cells, for the slightest trace of the blue dye, was in vain; the specificity of lymphatic endothelium for the dye was complete.

It is evident, that, in the specific affinity of lymphatic endothelium in amphibian larvae, we have a means at our disposal for settling conclusively the long controversy, concerning the growth of lymphatics, in which studies on the lymphatics of amphibian larvae have played such a prominent rôle in the past.

It may not be out of place here to discuss briefly the conflicting ideas about the mechanism of the growth of lymphatic vessels, which have been advanced and are now championed, by the modern students of angiology. Three views have assumed the most important places among the numbers of theories which have been evolved to explain the development of new lymphatic vessels.

1. That lymphatics may result from a transformation of blood-vessels, and that extension of the lymphatic system results from the coupling on or addition of these changed blood-channels.

2. That the endothelial cells of the lymphatics may arise from cells of the extra-vascular mesenchyme.

3. That after the initial outgrowth of lymphatics from the veins, in the form of lymph-hearts has occurred, neither mesenchyme cell nor blood-vessel endothelium contributes any further to their growth; but that they are an independent tissue, growing by division of primary lymphatic endothelium, and invading the embryonic body, as a vine climbs a trellis.

These three views, as they apply to the tadpole, can be subjected to a crucial test in the light of the discovery that trypan-blue is an

elective stain for amphibian embryonic lymphatic endothelium. Experiments necessary to this end are being prosecuted in this laboratory. Even at the present time, while these experiments with vital dyes are only in their initial stages, it is possible by their help to draw certain fundamental conclusions, which may aid in directing the study of lymphatic growth.

For example, the results of the application of this method show that the Mayer-Lewis anlagen are not modified blood-capillaries but true lymphatics, since they store vital azo-dyes in their endothelium. This is in accord with the knowledge gained from the injection and reconstruction methods, which make it seem probable that the Mayer-Lewis anlagen are parts of a continuous lymphatic vessel.

The view maintained by a second group of writers, namely, that lymphatics grow by the conversion of extraintimal and perineural spaces into lymphatic channels, by the transformation of mesenchyme into endothelium, clearly becomes untenable, since mesenchyme and lymphatic endothelial cells are shown by their reaction toward the dye to be biologically different; and conversion of one into the other becomes extremely improbable.

The observations made so far upon vitally stained larvae, substantiate and add additional proof to the interesting observations by E. R. Clark (4) on the growth of lymphatics in the tadpole's tail. The author is able to confirm Clark's view that these lymphatics grow only by sprouting, and by sending out fine protoplasmic processes, which gradually become definite lumen-containing sprouts. Furthermore the author also agrees with Clark in maintaining that blood-vessels, and mesenchyme and wandering-cells, never contribute to the formation of lymphatic endothelium in this locality; but that each of these tissues has an independent, characteristic existence.

The staining of the larvae became so intense by the eighth day that even to the unaided eye a blue color was evident in the tail. A diffuse blue stain of the peritoneal cavity became apparent about the third day; but it seemed plausible to attribute this to the presence of dye in the intestinal contents, especially as a plug of faecal material stained deep blue could always be seen in the anal gut.

The animals were fixed at successive intervals from the third until the tenth day in 10 per cent neutral formalin. The material was dehydrated, imbedded in paraffin, cut, and stained with Mayer's carmalum.

Study of the sections seems to show conclusively that the epidermis does not serve as a portal of entry for the dye, since there are no visible

traces of trypan-blue within or between the cells of this tissue. It seems more probable that the stain gains admission to the body through the alimentary canal, because in certain regions of the gut, the entire intestinal mucosa and submucosa are colored a deep diffuse blue and occasionally definite granules of dye are distinguishable in these layers. The embryonic intestine behaves radically differently towards the dye than does that of the adult, which under no circumstance, has been known to absorb the dye or to have visible particles of stain in the cells of the mucosa. Large amounts of the blue are aggregated in the cytoplasm of the vacuolated, mucoid epithelial cells, which line certain regions of the gill-pockets (notably those adjacent to the respiratory mucosa) and it is interesting to note the rich supply of lymphatics in this region, all of whose endothelial cells are loaded with trypan-blue. The respiratory epithelium appears to be practically free from trypan-blue granules, although in some instances areas may be found in which these cells have become actively phagocytic towards the stain.

As yet it is impossible to state positively whether the staining of the intestine represents an attempt on the part of the organ to absorb the dye, or whether this coloration is evidence of an excretion process or whether, as seems most probable, the absorption and excretion of the dispersed dye are not proceeding simultaneously through the intestinal epithelium. However, the mucous nature of the epithelium of the gill-pouches makes it seem probable that in this region we may be dealing with the excretory phase of vital staining.

A distinct attempt on the part of the body to excrete the dye, is evidenced by the remarkable appearance of the kidneys. The epithelial cells of many of the tubules are uncolored or contain only traces of the dye, whereas in other tubules the cells seem loaded to their full capacity. It is possible to find normal cells in all stages of transition between the two conditions. In still others there are degenerative changes, where judging from the accumulated stain, the cells are apparently taxed by the tremendous influx of foreign material, beyond their physiological limit. The epithelium in this instance is swollen to three or four times its normal height, the lumen of the tubule nearly obliterated, and the cytoplasm filled with clumps of dye and numerous vacuoles of varying sizes. It is not improbable to suppose, that the toxic action of stronger solutions of diazo-dyes upon poikilothermal vertebrates, can be accounted for by an identical, but more extensive degeneration of renal parenchyma, following an overtaxing of the kidney.

It is clear from microscopic study of the sections that the dye has

been stored by three types of tissue as follows: by the lymphatic endothelium in its entirety, the Kupffer cells lining the sinuses of the liver, and by groups of large, round, mono-nuclear pyrrhol-cells occurring in the mesentery and omentum, not unlikely homologues of the taches laiteuses of higher vertebrates.

There is no phagocytic activity shown toward the dye by any type of cell elsewhere in the organism. There are no trypanophylic clasmatocytes in the connective tissue, nor are there any free "macrophages" discernible, either in the serous cavities, or in the blood-stream. There is no vital stain in the spleen or blood-forming tissue, and the circulating blood-elements do not contain a trace of the dye.

The lymphatic system throughout the organism is conspicuous by the brilliant trypan-blue granules displayed in the cytoplasm of its endothelium. Here, as in the living specimens, there is no difficulty in distinguishing lymphatics from the blood-vessels because of the absence of dye in the endothelium of the latter.

The nuclei of the lymphatic endothelium are small, flattened, lense-shaped, or somewhat irregular, and stain very deeply with carmalum; they never contain dye-granules, while the cytoplasm is conspicuous by the quantity of vital stain which it exhibits. The dye however appears to be in larger clumps and masses than in the living cell, where it is distributed in much finer and more diffuse particles. Besides the trypan-blue the cells contain fine pigment-granules, which are also visible in vivo, and which would appear to be normal for this species. The vital stain is limited in the stained section, as in the living cell, to the granular zone surrounding the nucleus, and does not extend far into the finer protoplasmic filaments of the sprouting tip of the lymphatic vessel, or into the strands of protoplasm extending from nucleus to nucleus and forming the capillary-wall. One receives the impression here, as well as in the living, that the dye granules have a tendency to remain near the more physically inert nucleus, rather than to enter into the plastic and markedly amoeboid pseudopods of the cell.

The liver is characterized in all the sections studied by the large quantity of dye which it contains. Here it is the endothelium of the vascular sinuses, the Kupffer cells, which house the dye. In the amphibian larvae the number of these cells relative to liver-parenchyma would appear to be very great. The liver cells themselves contain appreciable amounts of dye, a phenomenon seen in other animals at times; and possibly attributable to the fact that these cells absorb some of the dye in the presence of an excess in the blood-stream, which

cannot be cared for by the cells, whose chief function it is to phagocytise particulate matter.

The third place of storage of the dye-granules, in the larval amphibians, is in the colonies of pyrrhol-cells in the mesentery and omentum. Some of these cell-aggregations seem to be true homologues of the taches laiteuses of higher vertebrates, others are merely accumulations of primitive wandering-cells peculiar to amphibian larvae in this locality. These cells are round and relatively large, having a single, round, eccentrically placed nucleus. Under no circumstance does the nucleus store the dye, but the cytoplasm, besides a quantity of brownish-yellow pigment-granules, displays an enormous amount of dye in clumps of differing dimensions and intensity.

The above results apply to all of the species studied, although the results with Hyla pickeringii were not nearly as satisfactory as with Rana temporaria, owing to the fact, explained above, that the toxicity of the dye towards the latter was very much greater, the animals succumbing before a brilliant vital stain was obtained. Amblystoma, although surviving longer than the other species in 1 : 1000 and 1 : 1200 solutions of the dye, had several drawbacks, which militated against their use. For example, the study of the lymphatics in vivo was impracticable, although their endothelium has the same affinity for trypan-blue, because of the dense, opaque chromatophores and melanophores which characterize this species of amphibian, and also on account of the slow rate of absorption of the dye, the endothelium frequently showing no trace of dye before the twelfth or fourteenth day.

The question now arises as to the significance of this rather striking distribution of trypan-blue in amphibian larvae. Leaving out the intestinal tract, which is concerned only in the transmission of the dye to its final destination, and also the kidney, where there is merely an attempt on the part of the body to rid itself of an excess of foreign material, the only cells found storing the dye are the lymphatic endothelium (throughout its entire extent), the specialized endothelium of the liver capillaries, and colonies of pyrrhol-cells in the mesentery and omentum. May we regard the lymphatic endothelium of the amphibian larva as an intermediate stage in the phylogeny of the pyrrhol-cell? Could we but vitally stain the amphibian embryo at a still earlier period, it would not be startling to find the blood-vascular endothelium similarly stained. This is borne out by the fact that, after experimental injury to the blood-vessel wall, its endothelial lining-cells apparently resume a more primitive and embryonic function, as evidenced by the absorption of the vital-

dye by the damaged endothelium. It would seem plausible, then, to infer that this phagocytic potential may be common to all endothelium in very early embryonic life, and that it may be lost progressively by the large majority of endothelial cells during their later development, and finally retained only by scattered groups of endothelial cells in the liver, bone-marrow, spleen and lymph-glands. What relation the fixed phagocytes in adult connective tissue bear to this primitive phagocytic endothelium is not at this time clear; but it is to be hoped, that the additional work now under way, on amphibian and other embryos, may eventually settle this much discussed question.

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THE PHYSIOLOGY OF THE MAMMALIAN AURICLE

II. The Influence of the Vagus Nerves on the Fractionate Contraction of the Right Auricle

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I. INTRODUCTION

It is generally conceded that stimulation of the vagus nerves causes a marked reduction in the amplitude of auricular contraction in mammals. Experiments similar to those reported by MacWilliam in 1888 (1) have been frequently reduplicated by experimental investigators. When the movement of a point on the auricular surface (usually the appendage) is transmitted to a recording lever or a tambour transmission system, the amplitude of the recorded curves becomes gradually smaller during weak vagal excitation until, in some instances, complete stoppage occurs. Upon cessation of stimulation the reverse process follows; the beats gradually increase in amplitude until a range equal to or exceeding the normal occurs.

In the foregoing paper (2) it was pointed out that not only does the "suspension curve" not represent a true record of auricular shortening, but the myogram recorded by an efficient myocardiograph from two approximating points is not necessarily an index of the contraction process as it affects the individual units of auricular muscle. Evidence was presented which favored the conception that the contraction process spreads over the auricle in the wake of the excitation wave in such a manner that the portions nearer the sinus node begin to relax before the more distal portions have ceased to contract. The interval that any unit of cardiac tissue remains in the contracted state was designated as the *fractionate contraction*. When the approximation of two distant points is recorded, the shortening, designated as the *mechanical contraction*, represents the algebraic sum of fractionate contractions and relaxations between these points.