THE BEHAVIOR OF THE LEUCOCYTES DURING COINCIDENT REGENERATION AND THYROID-INDUCED METAMORPHOSIS IN THE FROG LARVA, WITH A CONSIDERATION OF GROWTH FACTORS.

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I.

INTRODUCTION.

Various aspects of the problem of growth stimulation of tissues have been emphasized recently. From experiments with tissue cultures Carrel (1) has obtained results showing that leucocytes have a growth-promoting function, that they give off substances which stimulate the growth of adjacent cells. These growth-promoting substances he has named trephones (2). On the basis of the specific differential distribution of leucocytes in thyroid-treated tadpoles, the writers (3) have concluded that, if leucocytes have a growth-promoting function, this function is probably to be ascribed to the lymphocytes; that the granulocytes, on the contrary, probably give off substances that aid in the breaking down of tissues.

In a recent series of studies on cancer, Burrows (4) finds the following conditions favorable and probably essential to growth: a relatively crowded condition of cells, a relatively reduced condition of vascularization, and absence of intercellular materials. These spatial conditions, he believes, favor the accumulation of certain products of normal cellular metabolism. Among these metabolites is to be found the fundamental stimulating agent causing increase in growth rate of adjacent cells. On the other hand, Burrows finds that a loose arrangement of cells, much vascularization, and the presence of intercellular materials are conditions which prevent the accumulation of cellular metabolites, and are, therefore, conditions which inhibit growth.

In a number of earlier studies upon amphibians the writers have discussed the rôle of various types of blood cells both in relation to changes in metabolic rate and in relation to local processes of progressive and regressive change (Jordan and Speidel (3, 5, 6)). Some of these conclusions may be summarized as follows:

1. Granulocyte production is stimulated by the products of tissue lysis (proteolysis). Eosinophilic granulocytes are stimulated by that type of proteolysis occurring normally in the *intestine* of a frog larva undergoing thyroid-induced metamorphosis, and occurring pathologically in infections by certain worms and protozoa. Neutrophilic granulocytes are stimulated by that type of tissue lysis occurring normally in the *tail* of a frog larva undergoing thyroid-induced metamorphosis, and occurring pathologically in infections by many types of bacteria. Both eosinophils and neutrophils are believed to exert a lytic effect upon the adjacent tissues, thus preparing them for phagocytosis by the macrophages (monocytes) or for elimination by a sloughing process.

2. The lymphocytes, functioning in the frog as blood mother cells for the erythrocytes, the granulocytes, the thrombocytes, and the macrophages, are stimulated by the various factors that underlie the normal production of the definitive types of blood cells. These would include prominently the stimuli of proteolysis and of increased production of cellular metabolites, especially carbon dioxide. The lymphocyte is also the particular type of leucocyte of chief importance in the growth-promoting effect upon adjacent tissues.

3. Erythrocyte production is stimulated by some product of normal cellular metabolism, probably carbon dioxide.

4. Macrophage production is called forth by the residue of tissue autolysis.

5. Thrombocytes are interpreted as abortive erythrocytes. They develop only from the smallest lymphocyte progenitors.

6. Basophilic granulocytes are interpreted for the most part, if not entirely, as immature stages in the differentiation of eosinophils.

The tissues of the frog larva offer excellent material for the study of blood cells in relation to progressive and regressive changes. In a tadpole in which metamorphosis is initiated by thyroid administration, certain regions, *e.g.* tail, intestine, undergo rapid regressive changes; other regions, *e.g.* limbs, undergo rapid progressive changes. It occurred to the writers that coincident progressive and regressive changes might be readily induced experimentally, by simultaneous thyroid administration and removal of a part of the tail. The removal of a portion of the tail of a normal tadpole is quickly followed by regeneration of the lost part. This means an acceleration of the growth mechanism in that region. Treatment of the animal with thyroid extract not only inhibits growth in the tail, but it initiates the process of degeneration. In the conflict between the mechanism for regeneration and the mechanism for degeneration our purpose was first, to obtain data regarding the behavior of the leucocytes, and, second, to add further data regarding the conditions determining growth.

Some experiments were also performed on the limbs, consisting of simultaneous thyroid administration and removal of a portion of the hind limb. In this case it is clear that both reactions, that of growth of the hind limb and that of regeneration of the lost part, are of the progressive kind.

After our experiments had been completed it was found that Romeis (7) had made a study of the effect upon regeneration of thyroid and thymus. His study, however, is purely from the macroscopic viewpoint, and in no way touches our results upon the behavior of the blood cells or the factors concerned in growth.

EXPERIMENTS AND OBSERVATIONS.

The frog larvæ used in the experiments, forty-eight in number, were collected on December 4, and the experiments started immediately. The size, as indicated by the total body length, ranged from 27 to 80 mm., most of the tadpoles being of intermediate size. Approximately one-half the tail was removed, a hot knife being used. Thyroid administration was accomplished by placing some dried extract of sheep thyroid in the water with the tadpoles.

The animals were divided into seven lots as follows, each lot containing large, medium sized, and small animals:

Lot 1.—Eight tadpoles (Nos. 309–316). Kept in water with thyroid extract starting Dec. 4. One-half of tail removed 2 days later, Dec. 6. Four died; Dec. 8, 11, 12, 13, respectively. The other four were killed; Dec. 8, 11, 12 (two).

Lot 2.—Ten tadpoles (Nos. 319–328). Kept in water with thyroid extract starting Dec. 4. One-half tail removed the same day, Dec. 4. Three died; Dec. 11, 13 (two). The other seven were killed; Dec. 6, 8 (two), 10 (two), 11, 13.

Lot 3.—Ten tadpoles (Nos. 329–338). One-half tail removed on Dec. 4. Kept in water with thyroid extract starting 2 days later, Dec. 6. Two died; Dec. 11, 13. The other eight were killed; Dec. 7, 8, 9, 10, 11, 12, 13 (two).

Lot 4.—Five tadpoles (Nos. 339–343). Control animals. Tail left intact. Kept in water with thyroid extract starting Dec. 4. Two died; Dec. 11. The other three were killed; Dec. 6, 8, 10.

Lot 5.—Five tadpoles (Nos. 344–348). Control animals. Kept in water without thyroid extract. One-half tail removed Dec. 4. No deaths. The five animals were killed; Dec. 6, 8, 10 (two), 13.

Lot 6.—Five tadpoles (Nos. 349–353). Control animals. Kept in water without thyroid extract. Tail left intact. No deaths. The five animals were killed; Dec. 6, 9, 12, 13 (two).

Lot X.—Five tadpoles (Nos. 355–359). Kept in water with thyroid extract starting Dec. 4. One-half tail and a variable portion of both hind limbs removed the same day, Dec. 4. Three died; Dec. 11, 13 (two). The other two were killed; Dec. 7, 11.

In addition a few tadpoles from another lot were used for a study of the early changes following tail removal, at the end of 7 hours, 18 hours, and 43 hours. Direct observations were also made on the living regenerating tail at many stages in its growth period.

Mortality.—The mortality was high in the lots that were subjected to thyroid treatment. The operation of tail removal apparently somewhat decreases the ability of the animal to withstand the thyroid treatment, and reciprocally the thyroid administration decreases the ability of the animal to recover from the operation.

Regeneration.—Regulation of the tissues in the cut region quickly takes place. An almost immediate cessation of circulation in the vicinity of the cut region occurs. The cut muscles retract somewhat, carrying skin and connective tissue with them, thus bringing about protrusion of the notochord. Autolysis in the cut region is initiated. In a normal tadpole, untreated with thyroid 7 hours after the tail removal, there is a thin epidermis of two cell layers completely covering the cut. After the first few days of the experiment it was obvious from macroscopic observation that the rate and amount of regeneration were greatest in Lot 5 (tadpoles which were fed no thyroid), next greatest in Lot 3 (tadpoles which were fed thyroid 2 days after the removal of the tail, next greatest in Lot 2 (tadpoles which were fed thyroid beginning the same day that the removal of the tail occurred), and least in Lot 1 (tadpoles which were fed thyroid beginning 2 days before the removal of the tail). Microscopic examination of the tissues in the regenerating zone confirmed these observations. In absolute amount new growth took place to the extent of about 5 mm. in a representative individual of Lot 5 in 9 days. In Lot 1, there was regulation of the cut surface, but almost no new growth.

In all cases the regeneration consisted chiefly of new epithelium, connective tissue, and to a less extent notochord. Muscle regeneration was noted in late stages, especially in Lot 5.

Vascularity of Regenerating Zone.—A comparison of the degree of vascularization among the first five lots revealed that it was greatest in Lot 1, next in Lot 2, next in Lot 3, and least in Lot 5. It is furthermore apparent that in Lots 1, 2, and 3, all thyroid-treated, the vascularity increases with time. In Lot 5 the most rapidly growing portion, the tip, remains the least vascularized. The other portions, with the slowing up of growth and the advance of differentiation, become progressively characterized by increase in vascularization.

Crowding of Cells in Regenerating Zone.—The mesenchyme cells were especially active in all animals in which regeneration was occurring. A comparison of sections revealed that they were most closely crowded in Lot 5, next in Lot 3, next in Lot 2, and least in Lot 1. To a less extent similar conditions obtained in regard to the epithelial cells and the cells concerned in the regeneration of the notochord.

The Leucocytes of Regenerating Zone.—The types of leucocytes that were most conspicuously present soon after the tail removal were neutrophils, and to a less extent lymphocytes and early stages in the process of differentiation of erythrocytes from lymphoid hemoblasts. In those animals not treated with thyroid (Lot 5) the leucocytes became less numerous with the advance of the regenerative process and the blood picture returned to a more normal condition. This was clearly the condition after 6 days in one animal in which about 3.5 mm. of new tail had been regenerated. In the thyroid-treated individuals the vascularity is somewhat greater at the end of 2 days, with the same types of leucocytes in evidence; neutrophils, lymphocytes, and young red cells. In later stages, with the great increase in vascularity, other types of leucocytes become abundant, especially the macrophages. There may also be seen a number of hemoblasts, connective tissue basophils, and a few eosinophils. These basophils are apparently early stages in the development of the eosinophils, before the granules have fully "ripened." The macrophages are the chief agents, in the removal of tissue dêbris, being especially conspicuous in the transportation of pigment. A few eosinophils from the blood vessels may appear in the later stages.

The Epithelium.—As has already been mentioned the epidermis plays the chief rôle in closing over the wound, both surface and underlying layers taking part in the process. Within 7 hours a thin epidermal membrane has spread over practically the entire cut surface.

In the stratified epithelium of the normal tadpole the surface layer of cuboidal cells is rather sharply demarcated from the several subjacent layers. The cells of the underlying layers are usually characterized by the presence of conspicuous coarse threads of mitochondrial nature. These are never seen in the surface layer. In newly regenerated skin, both in normal and thyroid-treated animals, these mitochondrial threads are not present. After about a week or so a few threads may make their appearance. In the hind limbs, with the rapid growth following thyroid administration, the mitochondria disappear almost entirely.

In each of the cells of the surface layer there is present both in normal and thyroid-treated animals a definite crescentic condensation of cytoplasm located always on the proximal side of the nucleus. This body or apparatus stains somewhat more deeply than the rest of the cytoplasm. It may become quite prominent in the cells of the regenerated zone. It appears to represent a remnant of the mitochondria. At the distal surface of the cell may be seen a number of indistinct granules, presumably degenerating pigment granules. The complete history and significance of these cytoplasmic structures in the epithelial cells of tail and limbs will be considered in detail in a separate paper.

Miscellaneous.—Lot 3 affords excellent examples showing the operation of the conflicting processes of regeneration and degeneration. The regenerative impetus gained by the removal of tail 2 days before thyroid feeding persists for several days at least before checked by the thyroid. Once the thyroid extract does take effect, however, the rapidly growing region undergoes more rapid degenerative changes than do the more stable regions outside of the regenerating zone.

The individuals of Lot X present merely a typical thyroid picture in the limbs, possibly somewhat accentuated. The characteristic lymphocyte layer is present beneath the epidermis. A few neutrophils may also be seen in the epidermis.

A comparison of the epidermis of tail and limb shows some sloughing in both regions and some multiplication of the basal cells; both processes, however, being more pronounced in the limbs. In later stages there appears on the surface in both regions a dense membrane, consisting of a single layer of squamous cells. The suggestion presents itself that this membrane is to be interpreted in terms of a protective reaction against the thyroid in the water.

INTERPRETATION AND DISCUSSION.

The Leucocytes.—The neutrophil is quite conspicuous in all of the experimental animals. Found chiefly in the dermis and underlying tissues, its presence is interpreted as associated with proteolysis. Its function is conceived to be chiefly that of giving off a secretion which furthers the process of tissue lysis. In this manner it acts as a forerunner of the macrophage, preparing tissues undergoing degeneration for their removal by the macrophages. A slight amount of phagocytosis of very small particles may also be accomplished now and then by the neutrophils, as shown by injection experiments with India ink, but there is little or no evidence for this in the process of tail degeneration. Since the neutrophils are present especially in the tail of all thyroid-treated animals, in which degeneration has been initiated, it is hardly likely that these leucocytes have any growth-promoting function.

Not much additional light is thrown by these experiments upon the function of the lymphocyte. The lymphocytes are present in the process of regeneration of the lost portion of the tail. They are not present, however, in sufficiently great numbers to warrant the conclusion that they are of prime importance in aiding the new tail growth. This condition is in direct contrast with the condition in the limbs of thyroid-treated animals where they are present in great numbers. Doubtless those present in the regenerating tail have some growth-promoting function, but this activity must be small compared to their corresponding activity in the limbs of thyroid-treated animals.

Zeleny (8) has concluded that regeneration in the tadpole tail is centrally controlled. His data may be supplemented by our observation that the leucocytes have no important local effect in aiding and maintaining the process. Carrel (2) recognizes that regeneration may be brought about by various mechanisms; that leucocytic trephones may not be necessary in all cases for bringing about the new growth. Here the stimulation might be initiated by substances set free from disintegrating muscle cells.

The eosinophil is absent from the tail almost entirely in these experiments. A few may appear in the later stages. That this absence is not due to a scarcity of eosinophils in the entire animal is shown by an examination of the hemopoietic centers. Eosinophils are produced in great numbers in the intertubular regions of the kidney. Large numbers of eosinophils are attracted to the intestinal region, presumably by the particular type of tissue lysis and the sloughing process occurring there. This interesting differential distribution of eosinophils and neutrophils to the intestine and tail has been noted by us before (3). Both cells are believed to function in much the same way, *i.e.* to give off a secretion which causes, or at least aids, regressive change (proteolytic change). Until more light is thrown upon the exact function of these cells it can only be surmised that the eosinophil reacts to certain types of tissue lysis, the neutrophil to certain other types.

Macrophages are very numerous in the later stages in the thyroid-treated animals. This type of cell is the chief agent for the removal of pigment and other tissue débris produced by the primary degenerative processes. Hemoblasts of varying sizes appear in the tail after thyroid treatment in somewhat more than normal numbers. These hemoblasts are not localized in the tail any more than in other regions of the body. Their presence is merely a part of the general response of the body tissues to meet the tremendous demand for erythrocytes for the maintenance of the new high level of metabolism induced by the thyroid administration.

A number of basophilic granulocytes, evidently of connective tissue origin, make their appearance in later stages. The significance of their presence is not fully understood but there is evidence that they may differentiate into eosinophils. They are provisionally interpreted as precursors of eosinophils. The suggestion presents itself that the connective tissue has been stimulated to give rise to eosinophils in the tail, since this region may suffer from lack of these cells, with the strong competitive attraction for eosinophils by the intestine.

Growth Stimulation.—Our observations are of interest in connection with the recent views of Burrows (1923 (4)) on the problem of the stimulation of cells to growth. As has already been pointed out his conclusion is that the spatial conditions favorable for the stimulation of growth of cells are (1) a relatively crowded condition, (2) a relatively reduced blood supply, (3) a relative lack of intercellular substances. The fundamental stimulating agent he believes to be something given off by cells in the normal process of metabolism. A crowded condition with reduced blood supply affords a favorable basis for the accumulation of this stimulating substance. A looser relation of cells with greater vascularity, on the contrary, makes for a removal of this stimulating substance with resultant decrease in growth stimulation.

Our findings harmonize well with this conception. There is least vascularity and greatest crowding in the animals of Lot 5, those in which regeneration was most rapid. Next in order as regards amount of regeneration comes Lot 3, with somewhat greater vascularity and somewhat less crowding; then Lot 2, and Lot 1. In Lot 1, with least regeneration, the vascularity is greatest and the crowding of cells least. It is also of interest that in Lot 5 (not treated with thyroid) the vascularity varies somewhat in the different regions of the zone of regeneration. At the very actively growing tip the vascularity is least and remains so. At the ventral and dorsal fin expansions near the place of the original cut the vascularity becomes fairly normal; as great as before the cut. *Growth* here largely ceases and *differentiation* sets in. Relatively little vascularity then, is associated with growth; somewhat greater vascularity with differentiation and cessation of growth. In Lots 1, 2, and 3 the vascularity increases with time, correlated with decrease in regenerative activity. Our observations from these four lots show that there is a regular graded series of growth reactions, the amount of growth being correlated directly with the degree of crowding and inversely with the degree of vascularization. This supplements upon experimental animal material the results of Burrows on tissue cultures.

It may be noted also that in the limbs of a tadpole under thyroid treatment this correlation is not always so close. A few days after the thyroid administration the limbs become tremendously vascularized, though at the same time rapid growth processes are taking place. At this stage the opposing conditions of great vascularity and crowding of cells obtain.[1] In later stages, the vascularity becomes proportionately less.

One other point may be mentioned. The cells taking part in the regenerative process following removal of the tail are functioning at a higher metabolic rate presumably than those outside the regenerating zone. The addition of thyroid should, then, according to the work of Child (9) affect the region of new growth more readily than other more stable regions. Microscopic examination shows this to be the actual case.

SUMMARY.

1. Coincident progressive and regressive changes are induced in the tail of the frog larva by simultaneous thyroid administration and removal of a portion of the tail. The rate of growth of the regenerating tail is greatest in animals not treated with thyroid, next greatest in animals treated with thyroid 2 days after tail removal, somewhat less in animals treated with thyroid simultaneously with tail removal, and least in those treated with thyroid 2 days before removal of the tail.

2. The leucocytes chiefly concerned are neutrophilic granulocytes, lymphocytes, hemoblasts, and lymphoid phagocytes (monocytes, macrophages). The neutrophils further tissue lysis, the lymphoid phagocytes remove tissue débris. The lymphocytes are apparently not numerous enough to exert any important growth-promoting influence. The hemoblasts represent merely a part of the general reaction to thyroid treatment. Eosinophils are absent except for a few that appear in the late stages. Basophils of mesenchymal origin are present in the later stages of thyroid-treated animals. These appear to differentiate further into eosinophils.

3. The rate and amount of growth are correlated directly with the degree of crowding of cells, and inversely with the degree of vascularity. The greatest crowding and least vascularity occur in animals

[1] Even here, however, most rapid growth occurs in the non-vascular and the relatively less vascular areas of the limbs, that is, in the epithelium, and the axial preosseous and premuscular regions, respectively.

not treated with thyroid, somewhat less crowding and greater vascularity in those treated with thyroid 2 days after tail removal, still less crowding and somewhat greater vascularity in those treated with thyroid at the time of tail removal, and finally, least crowding and greatest vascularity occur in animals treated with thyroid 2 days before tail removal. These results support the views of Burrows (4) upon the mechanism of growth stimulation of cells.

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