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RESPONSE OF BASOPHIL CELLS OF THE ANTERIOR PITUITARY OF THE RAT TO INSULIN STRESS

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INTRODUCTION

The anterior pituitary has been an object of histological interest for more than seventy-five years. This interest has been directed largely toward a study of the distinct cell types and the hormonal secretions of each type. Two general methods have been used in studying the anterior lobe of the pituitary. The first method attempts to correlate the changes in the proportional number of cells in a particular physiological state, the second, to discover directly the secretory activity of the hypophysis by an analysis of the morphological changes within the cells themselves. Since 1940 various staining techniques have been developed which permit students to combine the two approaches.

The present study was begun to obtain information from a study of the effects of insulin on the basophil cells of the anterior pituitary which would clarify somewhat our understanding of types and functions of these cells. To determine variations in proportional number and morphology of the subtype basophils under the influence of insulin stress, and attempt to correlate these changes with secretory activity was the primary objective of this study. In order to accomplish this, a reanalysis of experimental approaches and staining procedures was necessary.

The establishment of anterior lobe changes with physiological disturbances in experimental organisms and in certain pathological conditions initiated a series of researches whch to this date have attempted primarily to relate the specific cells to specific secretions of the hypophysis. The results of these experiments indicate that the basophil is responsible for the secretion of thyrotrophin (TS), adrenocorticotrophic (ACTH), follicle stimulating (FHS), and luteinizing (LH) hormones. Results from previous experiments also indicate that there are at least two, and possibly three, sub-type basophils which are responsible for these sceretions. However, these studies are not in complete agreement as to the specific secretions of each sub-type of baso-

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phil. In the present study an attempt has been made to create a physiological condition in rats by insulin injection, which would result in both morphological and cytological changes in the basophilic cells. It was hoped that the results obtained in this study would help clarify the somewhat conflicting reports as to specific function of the sub-type basophils of the anterior pituitary.

The types of cells are as follows:

Basophil. This term refers to the property of cells to stain blue with aniline blue in the Mallory or azan techniques and to those cells which are PAS positive. It will not be used in this paper to denote true basophilia as revealed by an affinity for basic dyes.

Beta cells. These are heavily granulated basophils, which stain magenta using the PAS technique.

Delta cells. These cells stain a dark blue color with the iron-PAS technique. They can be distinguished easily from the beta cells by their dark blue color, smaller size and oval shape.

Gamma cells. Because the iron-PAS technique is more discriminative than earlier pituitary staining methods, some cells classified as chromophobes by former procedures have been found to contain granules. These lightly PAS-positive cells are gamma cells. Their final color is usually a shade of purple due to combined staining with iron-ferrocyanide and PAS.

REVIEW OF THE LITERATURE

A survey of the literature shows that the two major cell types of the anterior pituitary, chromphobes and chromophils, were described as early as 1884 by Flesch (1884). Schoeneman (1892) further divided chromophils into acidophils and cyanophils (basophils). These three cell types remained the only well-established cell types of the normal gland until Romeis (1940) identified the two types of basophils, the beta and delta cells. Romeis applied a kresazan technique. In 1950 Halmi applied an elastic tissue stain, aldehydefuchsin, in combination with a modified azan technique, to the pituitaries of rats and distinguished between two classes of basophils. Halmi designated these basophils beta and delta cells after the earlier terminology of Romeis. He concluded that from this experiment that the delta cells were the most likely source of FSH and TSH and that the beta cells were a possible source of ACTH.

McManus (1946) and Hotchkiss (1948) described new techniques of staining with periodic acid followed by Schiff's reagent (PAS) which permitted the demonstration of glycoproteins in histological sections.

Purves and Griesbach (1951) applied the PAS technique to sections of the anterior pituitary and were able to prove that the basophilic cells are glycoprotein-producing cells. They were able

to demonstrate two glycoprotein-producing cells which differed in location, morphology and staining intensity. One of these cell types was an oval or rounded cell which was stained intensely by PAS and localized to the lower surface of the anterior pituitary and to the upper surface adjacent to the pars intermedia. This type was found to be inhibited by estrogen and to be the source of castration cells in gonadal deficiency and was designated as the gonadotroph. The second glycoprotein-producing cell was polyhedral in shape and occupied the central region of the anterior lobe. The intensity of the PAS reaction in these cells was found to be correlated with the thyrotrophic content of the gland. This cell type is inhibited by thyroxine administration and responds to thyroxine deficiency by producing thyroidectomy cells. These cells were designated as thyrotrophs.

The thyrotrophs corresponded in location and shape to Halmi's beta cells; however, they were designated different functions. Halmi (1952) considered the delta cell to be responsible for the secretion of both thyrotrophic and gonadotrophic hormones, whereas Purves and Griesbach (1951, 1959) thought each was secreted by a separate cell.

Halmi (1952) later made a comparative study in which he applied the PAS technique used by Purves and Griesbach and his own aldehydefuchsin procedure to adjacent pituitary sections. Results from this study showed that Halmi's delta cell was synonymous with the gondadotroph and his beta cell with the thyrotroph of Purves and Griesbach. In this study Halmi also confirmed the results of Purves and Griesbach as to the secretory function of these cells. Since 1951 other workers have confirmed these results, using a wide variety of staining and experimental procedures. (Adams & Swettenham, 1958; Burt & Velardo, 1954; Pearce and Everson, 1952; Wilson, 1958).

Ezrin, Swanson, et. al. (1958) applied an iron-periodic acid-Schiff's technique to the pituitary gland of humans and identified a third sub-type basophil, the gamma cell. This third subtype basophil has not been described in the literature which deals with work on rat pituitaries. However, Purves and Griesbach (1951, 1954) called attention to a centrally located and peripherally located gonadotroph, which differs both in structure and in function, and one of which may, and probably does, correspond to the gamma cell of the human pituitary.

The hormones of the anterior pituitary are protein in nature. The chemical composition of at least six hormones of the anterior pituitary is fairly well established. (Ezrin, Swanson, et. al., 1958). Growth hormones and prolactin are simple proteins and adrenocorticotrophin is a polypeptide. The follicle-stimulating hormone, the luteinizing hormone and thyrotrophic hormoes are glycoproteins. Four of these hormones, follicle-stimulating, luteinizing, thyrotrophic and adrenocorticotropic have been associated with the basophil of the anterior pituitary.

MATERIALS AND METHODS

Forty-six young adults (175 gm. - 225 gm.) and four immature (75 gm. - 100 gm.) rats of the Sprague-Dawley strain were used in this study. All animals had been fed the same standard diet and kept at a constant temperature.

Aseptic technique was used during all operations which were performed under ether anesthesia. Insulin was diluted 3 parts normal saline to 1 part protamine zinc insulin, U-40, and injected intraperitoneally.

All animals were sacrificed by decapitation. The brains and pituitaries were removed and placed in fixative within 10 minutes after death.

Fixatives tried during the study were 10% formalin, 10% formol saline, formol sublimate, Bouin's, and Bouin's with 0.5% trichloroacetic acid. Bouin's with 0.5% trichloroacetic acid proved to be the most satisfactory and the results presented in this paper are those obtained using this fixative, unless it is otherwise designated. The pituitaries were fixed for a period of 24 hours and then washed overnight in water. They were removed from the water, dehydrated through alcohol and xylol, and embedded in 58° paraffin.

Sections were cut at 5 microns in the horizontal plane and mounted in serial sections on clean glass slides. Sections which were selected for study were taken from a standard level in the pituitary, approximately 150 microns from the inferior surface. At this level the pars intermedia and the beginning of the pars nervosa were visible at the anterior edge of the section.

In the course of this study, selected staining procedures were used in order to study the various staining reactions of the basophil and to permit a wider comparison between results obtained in this study with those of the literature. However, the results discussed in this paper were obtained by using a modification of the periodic acid-Schiff procedure which was developed by Ritter and Oleson (1950).

Iron-PAS staining technique (modified from Ritter-Oleson)
The details of the technique are as follows:

1. Decerate sections through xylol and hydrate through alcohols to water.
2. Treat for one hour in 5 ml. glacial acetic acid, 15 ml. of water and 20 ml. of colloidal iron. (Muller's colloidal iron oxide reagent)
3. Wash well in distilled water.
4. Treat for 20 minutes in equal parts of 2% potassium ferrocyanide and 0,14M hydrochloric acid.
5. Wash well in distilled water.
6. Oxidize for five minutes in 0.5% aqueous periodic acid.
7. Rinse in distilled water.
8. Treat for fifteen minutes in Schiffs' reagent.
9. Rinse for three minutes in each of three changes of sulphurous acid rinses.

10. Wash under running tap water for ten minutes.
11. Stain for one minute in 0.5% aqueous orange G.
12. Without rinsing, transfer directly to 1.0% aqueous phosphotungstic acid for thirty seconds.
13. Rinse in 1.0% acetic acid.
14. Dehydrate, clear and mount.

Solutions:

Preparation of Muller's colloidal Iron Oxide Reagent (Mowry, 1958)

Bring 250 ml. of distilled water to boil. While the water is still boiling, pour in 4.4 ml. 29% ferric chloride solution and stir. When the solution has turned dark red, remove from heat and allow to cool. This reagent is dark red, and remains clear and stable for many months. If the water is not kept boiling during ferric chloride addition, the conversion to colloidal (hydrous) ferric oxide will be incomplete and results of its use will be faulty. In the staining procedures, the stock solution of colloidal iron is diluted just before use as follows:

Glacial acetic acid, 5 ml.
Distilled water, 15 ml.
Stock colloidal iron, 20 ml.

Schiffs' reagent was prepared according to Lillie's directions. Add 1.06 gm. of basic fuchsin and 1.96 gm. of sodium metabisulphite to 100 cc. of 0.15 N hydrochloric acid. Shake at intervals for two hours. Add 500 mg. of fresh activated charcoal (Norite). Shake for one to two minutes. Filter twice. The solution is ready for immediiate use. Store in a refrigerator.

Sulphurous Acid rinse to make one liter:
Sodium metabisulphite 4 gm.
Hydrochloric acid 10 ml.
Distilled water 1000 ml.

CONTROLS:

(a) Males. The results of observations on the anterior pituitary of eight young adult, normal male rats shows that the pattern of distribution and shape of the two major basophils, the beta and delta cell, is entirely different. The two cells are often encountered next to each other, however; the beta cells were more numerous in the core of the gland, whereas the delta cells were more numerous at the periphery of the gland. The majority of the beta cells were polyhyedral, giving the impression of being compressed by other cells, while the average delta cells were smaller and oval in shape. Nuclear detail could not be observed by using the iron-PAS procedure, however the aldehyde-fuchsin technique showed that the delta cells often had pyknotic or kidney- shaped nuclei, without prominent nucleoli. The nuclei of the beta cells were mostly vesicular and provided with a large nucleolus. Vacuolated cells were seldom seen in the normal gland, those observed were more frequent in the delta cells. The gamma cells, the slightly PAS positive basophils were more numerous than either the beta or delta cells but they had no consistent pattern in size, shape or distribution.

(b) Females. The only significant differences in the normal female pituitary were: (1) a lower delta cell count, and (2) more of the delta cells present were located at the periphery of the gland.

ADMINISTRATION OF INSULIN:

A total of twenty young adult rats was used for this study. Of those, five males and five females received 15 units of U 40 insulin each and were sacrificed immediately after going into shock. Five males and five females were given daily doses of 2 units of insulin each for a five-day period and sacrificed approximately four hours after the last injection.

The animals which received 15 units of insulin lived approximately 3½ hours after injection. Observation of the anterior lobe showed that there was no significant numerical change in any of the basophils. Morphological changes were observed only in the delta cells where vacuolation was slightly more frequent in the treated animals. The most obvious changes were the prominent "signet ring" cells and an increased number of pyknotic nuclei in the delta cells. Nuclear observations were made using the aldehyde-fuchsin technique.

The second group of insulin-treated animals showed an increased number of delta and beta cells over those of the controls; the most significant increase occurred in the delta cells. The delta cells which contained vacuoles were more numerous and the "signet ring" cells were still present. In almost every case the nuclei of the delta cells were pyknotic. There was no obvious change, qualitatively or quantitatively, in the gamma cells. Males and females seemed to show the same response to insulin in both groups.

DISCUSSION

The cell types: By the iron-PAS staining technique, the following basophils were identified.

1. *Beta cells.* These cells are heavily granulated basophils. They stain magenta with the iron-PAS technique. They are polyhedral in shape.

2. *Gamma cells.* These cells are lightly PAS positive and are classified as chromophobes by less discriminative staining procedures. They are colored a light blue and contain very fine granules.

3. *Delta cells.* These are dark blue in color and have coarse granules throughout the cytoplasm. They are smaller than the beta cells and are oval in shape.

Beta and delta cells could be easily recognized by their characteristic shape and distribution. Beta cells are large polyhedral cells usually found in localized aggregations, more abundant in the interior portion of the gland. In contrast, the delta cells are smaller, oval in shape, and are more abundant at the periphery of the gland. The gamma cells vary more in size and distribution than either the beta or delta cells. They may be found scattered singly throughout the gland or occur in localized aggregations.

The exact mechanism of the iron-periodic acid Schiff reaction is still in doubt, but there is general agreement that treatment with periodic acid oxidizes polysaccharides to polyaldehydes which in turn, react with Schiff's reagent to produce a red-colored compound (Wilson, 1958). One limitation of the iron-PAS method is the problem of a nuclear stain. A convenient nuclear stain of intense but contrasting color that leaves other structures consistently unstained has not been found. However, if a nuclear stain is desired, Weigert-Lillie hematoxylin seems to give the best results and is the one most often used.

In agreement with Halmi (1952), the beta cell granules were stained a dark purple shade with aldehyde-fuchsin. The delta cell granules show an affinity for light green which resulted in a light green colored cytoplasm. Excellent contrast between these two cell types was achieved. However, the over-all picture was not as satisfactory as that obtained by the iron-PAS technique.

The PAS technique alone, used by Purves and Griesbach, was applied to several sections of the pituitary and the results were compared with the other stains. Both of the basophils which they described were easily located, however, the nuclear stain had somewhat reduced the differential quality of the cytoplasmic stain.

The effects of insulin on cell types. Insulin is an inhibitor of ACTH and its administration should affect ACTH secretion by the pituitary. Insulin is widely used to produce non-specific stress which should also cause an increased output of ACTH.

An increased output of gonadotrophin is known to occur following the administration of some specific and non-specific stress agents and during certain pathological conditions. It would seem likely, then, that insulin would also cause gonadotrophic hypersecretion.

The metabolic processes of the thyroid would be affected by insulin administration, which should indirectly, through thyroxin, alter the thyrotrophic output of the pituitary.

In view of the above facts, it seems probable that a study of the anterior pituitary following insulin injection would involve changes in all of the sub-type basophils. It was with this in mind that insulin was selected for this study.

The cell changes which occurred in the anterior pituitary following insulin injection have been observed before; however, previous investigations have always associated these changes with a specific physiological state.

Following insulin injection, numerous "signet ring" cells were formed from delta cells. Signet ring cells were first observed by Schleidt (1940) in response to gonadal deficiency. Addison (1917) described these changes in the basophils of the castrate rat and they have been studied intensively in this species since then. Engle (1929) suggested that this vacuolation was probably the source of the increased gonadotrophic content of the hypo-

physis. Other workers believe it to be the first stage in vacuole formation. Severinghaus (1937) showed that the prominence of the "signet ring" was directly proportional to the cells' secretory activity.

Results from this study seem to indicate that the "signet ring" precedes vacuolation in the delta cells, and occurs prior to any increase in number of the cells following insulin stress. It would seem then that the appearance of signet ring cells is the first phase of gonadotrophic secretion and that the formation of vacuoles and an increase in delta cells indicate extensive gonadotrophic production. However, Purves and Griesbach (1951) showed that an increase occurred in number and vacuolation of gonadotrophs within two to three days following castration and that the signet ring cells first appeared several weeks after castration.

Studies in this laboratory on pituitaries from rats which had been castrated four weeks previous showed no "signet ring" cells, but an increase in delta cells and vacuolation was noted.

Pituitaries from the rats which received daily insulin injections resembled very closely those from a six-to eight-week castrate. The delta cells had increased in number and many of those present were vacuolated and had prominent "signet rings." There was no indication as to which occurred first in these cells, the vacuoles or the "signet rings," since no animals were sacrificed prior to the fifth day.

In comparing the results from this experiment with those from the literature which deal with the delta cell and gonadotrophic secretion it seems obvious that insulin causes a hypersecretion of gonadotrophic hormone. Since "signet rings" occurred before vacuoles when a lethal dose of insulin was given it would seem that the gonadotrophic hormones are separated at a faster rate following insulin injection than during castration experiments.

Another interpretation of the function of the delta cell is that it produces ACTH. ACTH cannot be measured in systemic venous blood; however, there is good indirect evidence that ACTH is released very rapidly even after a fairly minor stress. ACTH is a polypeptide and in itself would not give a positive PAS reaction; however, its secretion has been attributed to the basophils and it has been suggested (Ezrin, Swanson et. al. 1958) that it may be linked in the cell with a carbohydrate-containing complex. If the changes which occurred in the delta cell following insulin injection are the result of ACTH secretion it would seem that the delta cell contains a store of this hormone and that it is readily secreted during extreme stress conditions.

The results obtained from this experiment are not sufficient to tie ACTH secretion to the delta cell, nor does the literature available furnish fact which would permit this.

The beta cell showed very little response to insulin in the

present study. A slight increase in the number of these cells was noted after daily insulin injection; this indicates that there is a response, but that it is much slower than observed in the delta cells. An increase in beta cells has been noted in cases of both thyroxin deficiency, and hyperthyroidism. In cases of thyroxin deficiency, an increase in beta cells is parallelled by thir vacuolation which was not noted in this experiment. If a hyper-thyroid condition is produced the beta cells increase temporarily; and, if the condition persists, they will disappear altogether. The temporary increase is probably due to some non-granular secretory forms of beta cells replenishing their thyrotrophic content and becoming granular forms again. This probably accounts for the increase which occurred in this study. If a hyperthyroid condition persists and there is no need for thyrotropin, the beta cells will finally disappear.

It has been concluded from studies on human pituitaries that the gamma cell is an actively secreting beta, delta, or alpha cell which has become degranulated through secretory activity. If this is the case in rats, there was probably a variation in the number of gamma cells in this experiment which was not obvious enough to recognize without differential counting.

CONCLUSION

An iron-PAS staining technique was applied to sections of the rat pituitary and three sub-type basophils, beta, delta and gamma, were identified.

Beta and delta cells increased in number following insulin injection. The delta cells showed changes in structure similar to those found after castration. No changes could be obesrved in the gamma cell.

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News of Tennessee Science

(continued from page 59)

baker, who will devote full time to his duties as Director of the School of Arts and Sciences.

Dr. John Warren has been appointed Associated Professor of Biology at TPI. He heceived the Ph.D. in Microbiology and Botany at Ohio State University in 1950, and was on the faculty at Duke University for six years. He has done extensive research on plant diseases in the tropics for the past four years.

Three new members have been added to the Department of Mathematics; Drs. Cecil G. Phipps and William A. Small as Professors, and Reuben C. Hood as Assistant Professor.

Dr. Small received the B.S. Degree from the U.S. Naval Academy, and the A.B., A.M., and Ph.D. from the University of Rochester and Alfred (New York) University and served as head of the mathematics department at Grinnell (Iowa) College.

Dr. Phipps has taught at the University of Minnesota and at the University of Florida. He has the M.A. and Ph.D. from the University of Minnesota and the B.A. from Montana State University.

Mr. Hood has the B.S. from Georgia Tech and the Master's Degree from Duke University. He recently retired from the Air Force with the rank of Major-General. His posts whilie in service included air attache in Rio de Janerio, Brazil, commadant, Command and Staff School, Air Uni-

(continued on page 85)