Electron Microscopic Study of the Perivascular Region of the Pineal Gland of the Early Post-Hatching Domestic Fowl with Emphasis on Exogenously Produced Lymphocyte Emigration

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ELECTRON MICROSCOPIC STUDY OF THE PERIVASCULAR REGION OF THE PINEAL GLAND OF THE EARLY POST-HATCHING DOMESTIC FOWL WITH EMPHASIS ON EXOGENOUSLY PRODUCED LYMPHOCYTE EMIGRATION

by

CHRISTOPHER N. CASCIANO

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF LOYOLA UNIVERSITY OF CHICAGO IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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INTRODUCTION

The observations and descriptions of the pineal gland by early researchers were based, not so much on scientific theory, but rather, were influenced by philosophical concepts. The study of the pineal gland can be traced back about 2000 years. Authors, such as Hero­philos of Alexandria (325-280 B.C.), mentioned that the epiphysis could function as a sphincter, controlling streams of thoughts. They also suggested that the epiphysis might regulate the flow of substances between the third and fourth ventricles of the brain. It should be noted that the medical thinking at this time placed emphasis on the ventricles rather than the cellular matter as the functional parts of the brain.

Galenos of Pergamon (130-200 A.D.) termed the epiphysis, konarion, because of its pine-cone like shape in mammals. Galenos, however, did not ascribe to Herophilos' concept of the pineal, stating that it was merely a lymph gland. Descartes (1596-1650) following the concepts of ancient Greek philosophers, claimed that the epiphysis was the seat of the soul. He felt the blood contained very fine particles which, in his view, were separated from the blood by the epiphysis, to be transformed into the esprits animaux or animal spirit, which was the psychic and somatic activating principle of the organism.

The ideas of the ancient philosophers, concerning the localization of the mental abilities within the ventricles being mediated somehow by the pineal, lasted for many centuries. In fact, it was probably DeCyron (1907) who was the last author to believe that the epiphysis
would regulate the flow of the cerebral spinal fluid in the aqueduct of Sylvius. This fluid previously being considered by many to be the organ of the mind.

In the years following Descartes, fewer and fewer authors accepted the concept of the soul being located within the epiphysis of the ventricular systems. It was slowly, but generally, realized that the relationship between the metaphysical soul and physical body was no simple problem to be solved. In fact, after Kant, it was impossible to say the "soul" would be located in any definable space.

After the time of Descartes, interest in the epiphysis subsided and many thought it to be a rudimentary organ. It was not until the discovery of endocrine organs that the study of the gland was again rekindled. In more recent years, with the technological advances in chemical analysis, histological techniques, and observation machinery such as the electron microscope, the pineal could no longer be classified as rudimentary, but rather, as a functional multipurpose gland.

In the hands of a growing number of workers, electron microscopy is providing a wealth of new structural information relating to functional relationships within pineal systems. Particularly significant is the fact that electron microscopic evidence combined with physiological experiments now more adequately describe cytological interrelationships in such areas as: innervation (Kappers, 1965), vascularization (Beattie and Glenny, 1966), lipid chemistry (Quay, 1961) and lymphocyte formations (Spiroff, 1958).

By using the high magnification capability of the electron microscope, a more detailed study of the avian pineal perivascular region could be realized.
The purpose of this investigation was to provide insight into several basic questions relating structure to function in the pineal. Results of the following study of the pineal gland suggest a possible synthetic interrelationship between secretory cells of the gland and emphasizes the probable exogenous origin of the lymphoid nodule found to be evident during the early post-hatching period of the domestic chicken.

Are these lymphocytes which make up the nodule endogenous to the pineal gland? Do they originate from within the gland from mesenchymal cells such as those found in the connective tissue, or do they come from some exogenous source, such as the thymus, when upon entering, proliferate and form nodular centers? While the final proof may require isotopic tracing studies of lymphocyte migration through the systemic circulation, this study relies on photographic observation to elucidate the answers to these questions.
REVIEW OF THE LITERATURE

The pineal gland is a small pear-shaped body lying between the cerebral hemisphere and cerebellum with its apex directed ventrad. It is a consolidated organ of epithelial parenchyma and encapsulated by connective tissue which is continuous with that of the dorsal meninges (figure 2), (Quay, 1965).

The pineal gland, or the epiphysis cerebri, as it is sometimes known, develops from the roof of the diencephalon and in vertebrates, the site of pineal development is consistently a median region at the posterior commissure. The neuroepithelial proliferations forming the pineal consist of cords and follicles of cells invested in embryonic mesoderm of meningeal mesenchyme gives rise to the stromal tissue, which consists of connective tissue and contained blood vessels (Quay, 1974a).

In recent years the biochemistry of the gland has become the subject of intense research. It is believed that the indoleamines secreted by the gland are involved in the regulation of biological rhythms.

More attention has been given to indoleamines than to any other group of pineal compounds. Lerner (1958, 1959, 1960), while attempting to isolate the pineal constituents responsible for blanching of amphibian skin, isolated and identified melatonin (5-methoxy-N-acetyltryptamine). The concentrations of 5-hydroxytryptamine (5-HT) were found to have high levels in the pineal, especially those in mammals (Giarman, 1959). Axelrod and Weissbach (1960) isolated the enzyme responsible for the O-methylation of 5-hydroxy-N-acetyltryptamine, which is responsible
for the biosynthesis of melatonin. Melatonin was brought into prominence as a possible antigonadotropic pineal hormone by Wurtman (1964).

Pineal 5-HT and related compounds are known to be secreted in a cyclic fashion during the solar day. The amount of each substance secreted at any given time appears to be closely correlated with the intensity of environmental illumination (Menaker, 1972).

Lipids occurring in the pineals of various species have been examined by several investigators. Quay (1961a) demonstrated that lipids occur in pineal glands of mammals and birds and that the amounts vary according to species, age, and a great variety of other conditions and treatments. No lipid compounds have been isolated or identified which are peculiar to pineal tissue (Quay, 1961b). Available information suggests that lipid is used primarily as an energy source in general cellular metabolism and constitutes the structural component of cellular membranes. Little is known of the biochemical pathways involved in pineal lipid metabolism, however, these are probably similar to those already elucidated in other cell systems such as liver.

Enzymes, such as hydroxy-indole-0-methyltransferase and succinate dehydrogenase, are believed to be important in the synthetic activities of the pineal gland and may be essential in the metabolic control of the gland. Scattered reports on pineal enzymes have appeared, but these have been restricted to mainly histochemical techniques (Gusek, 1968 and Arvy, 1963).

Great advances have been made in recent years in our knowledge of many pineal biosynthetic and metabolic activities. However, with the possible exception of melatonin secretion, little is known about how
these activities relate to the release of secretory material, aside from the apparent release of melatonin. Enzymes involved in the control of pineal protein synthesis have been neglected.

Even though the pineal of fish, amphibians, reptiles, and mammals have been the subject of many investigations, little information is available on the avian pineal gland. Reports of histological and structural changes which occur in the avian pineal during development and early growth are few in number. In the earlier literature, Hill (1900) and Cameron (1903) provided useful accounts of the gland's basic anatomy. Recently, the development and structure of the pineal gland of *Gallus domesticus* has been described by Spiroff (1958) and the post-hatching period has been examined by Romieu and Jullien (1942a, b, c). Ultra structural studies have been carried out in the rat (Gusek and Santoro, 1961a), bovine system (Anderson, 1968), human fetus (Maller, 1974), and adult chicken (Fijie, 1968). Finally, various aspects of development, anatomy and innervation of the pineal gland of several species of birds have been reported by Ward (1963), and Quay (1965).

The pineal gland has been found to be highly vesicular. The vesicular cells are secretory in nature and are found to contain glycogen, glycoproteins, RNA, acid mucopolysaccharides, and neutral lipids. It is thought that the vesicular tissue which is most prominent during days 15-17 of incubation is related to some secretory roles at a relatively early period of development (Campbell and Gibson, 1970). For the adult chicken, Fijie (1968) describes three types of cells: (1) pinealocytes; the most numerous parenchymal cell, flask or pear-shaped, with large spherical nuclei in the basal half; (2) supporting cells
EXPLANATION OF FIGURE 1

Figure 1 Vasculature of the pineal gland: Pineal body P, Artery A, Vein V, Cerebellum CE, Stalk S, and Cerebrum C.
inserted among the pinealocytes with extending micro-villi into the lumen: and (3) glial cells; small in size, uncommon, and containing many filaments.

Spiroff (1958), using light microscopy, notes that in the chick embryo, the epiphyseal mass becomes surrounded by an intricate vascular network by the fifth day of development. Capillaries filled with blood cells are present among the follicles by the sixth day. By day twelve, capillaries encircle the follicles, but never penetrate them. It is within these capillaries that lymphocytes are thought to gain entry into the pineal stroma.

In Gallus domesticus (Beattie and Glenny, 1966) the pineal is supplied by the posterior meningeal artery which arises as a branch of the cranial ramus of the left internal carotid. It ascends the anterolateral face of the pineal stalk, where it ramifies at or near the base of the gland proper, to form a circulus vasculosus or rete pinealis. Efferent vessels emerging from the antero-dorsal aspect of the pineal curve along the posterior medial surface of the cerebral hemispheres and join the internal jugular vein (figure 1).

There is little known about possible lymphatic vessels within or adjacent to the chick pineal. However, in birds, more frequently than mammals, lymphoid tissue is found in the stroma of certain species. In the domestic chicken, pineal lymphoid tissue is characteristically present, its distribution and composition being age dependant. Romieu and Jullien (1942a) were first to observe the presence of lymphoid tissue in the chicken. Their contention was that the lymphocytes of the meninges and cerebral spinal fluid owe their origin to the pineal lymphoid nodule. In the chick, Spiroff (1958) found patches of lymphoid
cells in or near the walls of superficial pineal blood vessels as early as four days post-hatching. Such cells tend to reduce in number beginning with the fourth month, with the occasional survival of some pineal lymphoid tissue (figure 3) into adulthood. Quay (1965) found that in one year old chickens there is nearly always a lymphoid nodule within either the capsule or the internal connective tissue. The anatomical position of the nodules is otherwise variable. Some such nodules contain an active germinal center where production of lymphocytes may be occurring. From the germinal center they may be able to pass into the blood vessels which drain the pineal body.
MATERIALS AND METHODS

Twenty-one pineal glands were examined. One through six day and ten day post-hatching domestic chickens, purchased from Spafas Inc., Norwich, Conn., were used. Three glands from each stage were sectioned. The chickens were kept under continuous illumination by incandescent lighting. This was done to maintain a constant body temperature during the respective post-hatching periods. The heads were removed by scissor decapitation. The skulls were trimmed and placed into a 4.5% Veronal buffered gluteraldehyde fixative at 4 degrees centigrade (Pease, 1968). After primary fixation for 24 hours, the pineal glands were removed and washed in Veronal buffer for 12 hours. The pineals were then cut in half and placed in Palad's buffered 2% osmium tetroxide fixative (pH 7.4). After 1.5 hours, they were removed and placed in 0.5% uranyl acetate stain for one hour (Maller, 1974). Uranyl acetate staining was carried out at this time rather than after sectioning because the Jem-50 electron microscope has a maximum magnification of 5000X and heavy metal post-staining at this relatively low magnification was not thought necessary. The glands were rinsed in distilled water and brought through successive solutions of 50%, 75% and 95% ethanol for five minutes; followed by three successive 15 minute washings in 100% ethanol. The specimens were placed in propylene oxide and stored over-night at four degrees centigrade. They were then rinsed in a fresh solution of propylene oxide for 5 minutes. Small quantities of epon resin mixture (Mixture A - Epon 812, 62 ml and DDSA (dodecenyl succinic anhydride), 100ml; and mixture B - Epon 812, 62 ml, and MNA (methyl nadic anhydride), 89 ml (Pease, 1968) was added successively, while stirring continuously,
at 7 minutes, 30 minutes and 45 minutes respectively. The epon was then drained and undiluted resin mixture was added to the specimens and stirred for one hour. The specimens were then placed in polymerizing capsules and incubated in an oven at 60 degrees centigrade for 40 hours, and allowed to cool for 24 hours. The specimens were sectioned (purple sections, eg, 1500 - 2000 A) on a Sorvall MT-1 ultramicrotome. Glass knives were used. The sections were examined and photographed with 35mm film which was placed within a JEM-50 electron microscope. The photographs were printed on R.C. polycontrast paper.
RESULTS

Cellular Composition of the Pineal Cells Located in and Around Blood Vessels.

Six distinct cell types were observed in and around the blood vessels of the pineal gland. They include; interstitial cells and the dark secretory cells of the stroma, light pinealocytes found in the medulla of the gland; and endothelial cells, connective tissue cells, and smooth muscle cells of the blood vessel wall itself.

Stroma - Interstitial Cells: Interstitial cells were generally found to possess a lobular, highly irregular nucleus (figures 5 and 6). The nuclei demonstrate a well defined nuclear membrane and prominent nucleolus. The cytoplasm is rather scanty and extends throughout the interstitial fibrous network. Cellular organelles, such as mitochondria, can be seen throughout the cytoplasm. The chromatin of the nucleus is dispersed and stains lightly. The cell membrane is not as densely stained as is the nuclear membrane.

Stroma- Dark Secretory Cells: In close proximity to many of these interstitial cells are what appear to be lipid-containing, darkly nucleated cells. These cells (figures 4, 5, 6, and 7) have a very densely stained irregular nucleus. The nuclear membrane is well defined and the presence of a prominent nucleolus was absent. The chromatin of the nucleus is diffuse; however, it appears to clump noticeably toward the periphery of the nucleus. The cytoplasmic area is relatively
small in comparison to that of the nucleus. The plasmalemma is well defined and does not demonstrate cytoplasmic extensions into the interstitial network and perivascular spaces as seen in the interstitial cell. The dark cells are often found to have large lipid inclusions, particularly during the stages immediately after hatching (figures 5 and 6). These inclusions are seen to distend to such a degree as to compress the nucleus against the limiting plasma membrane. It was also observed that lymphocytes found in the lumen of the blood vessels during day one and two also demonstrate these large lipid vesicles (figure 7). These large lipid vesicles may result from the high lipid content of the blood during development and just after hatching. The high lipid content in the blood is a result of the large amounts of lipid laden yolk making up the diet of the embryo prior to hatching (Romanoff and Romanoff, 1949).

These small droplets of neutral fat appear to be restricted to dark cells in the perivascular connective tissue (figure 6). They are generally scattered in the loose connective tissue, especially in close proximity to the blood vessels (figures 4 and 7). The cytoplasm in some cells appears to surround the droplet (figure 5). The individual dark cells, for the most part, are surrounded by a network of fibrous tissue. It may be noted that new fat cells in the embryo always appear along the small blood vessels and are accompanied by cells of undifferentiated mesenchymal nature (Clark and Clark, 1940).

The dark cells observed from day one through day six, demonstrate the gamut of developmental stages of the classical fat cell. Figures 4, 5, and 7 demonstrate a dark cell that may be considered an early stage of fat cell development. Note that there are many lipid inclusions within the cytoplasm. As lipid content increases, the droplets
coalesce to form one large lipid droplet (figure 6). By six days, the lipid appears to be metabolized and lipid inclusions diminish considerably (figure 13).

**Light Pinealocytes:** Observations made toward the medullary region of the gland, demonstrate less stromal cells and an increase in the number of light pinealocytes (figures 8, 9 and 11). The light pinealocyte appears to be the principle glandular cell of the pineal gland. The density of these cells increases dramatically toward the central region of the gland (figure 12). The pinealocyte (Fijie, 1968) demonstrates a rather well defined nuclear membrane. It confines a nucleus which is for the most part large and uniformly rounded (figures 9, 11 and 12).

The light pinealocytes contain a prominently stained nucleolus, and the chromatin is dispersed and lightly stained throughout the nucleus. The plasma membrane has, in contrast, poorly defined limits. There is a tendency for the light pinealocytes to have extended terminal offshoots (figures 9 and 12) (Romijn, 1973). What appears to be organelle complexes are interspersed within the cytoplasm, especially within the non-extended region (figure 11).

The cytoplasm of the light pinealocytes appears scattered with particles in the matrix (figure 8). These wisps may be due, to a limited extent, to distortion from over exposure to the high intensity electron emission from the electron gun, causing some spreading of the tissue. Organelles, which might include mitochondria and endoplasmic reticulum (figures 9 and 11) appear to be interspersed within the cytoplasm. The pericellular tissue in the region of light pinealocytes (figures 8 and 9) appears to have little organization. A vague floc-
culent material fills the pericellular spaces which contain widely varying numbers of connective tissue components such as mitochondria and golgi complexes (figure 8).

**Vessel Wall:** A longitudinal section through a blood vessel found in the stalk region of the gland (figure 13) demonstrates the well defined, sometimes irregular, nuclei of the endothelial cells and the intricate network of connective tissue fibers which surround the vessel. A perivascular corona of autonomic axons and terminals can be seen to surround a pre- or post-capillary blood vessel (figure 16). In contrast to the more peripheral regions (figures 15), there is little perivascular space which is characteristic of vessels found in the more densely compacted regions of the pineal stroma. Pineal connective tissue cells are well defined and are separated from the endothelial cells by basement lamina (figure 16). It was observed that no lymphoid tissue was seen in the perivascular tissue through the first two days post-hatching. The only lymphoid tissue seen up to day three was within the lumen of the blood vessels (figures 7 and 10).

**Lymphocyte-Leucocyte Tissue:** A thorough search through one day pineal glandular tissue, especially in the peripheral vascular regions, gave no indication of the presence of lymphoid tissue. The only lymphoid tissue found was located within the blood vessels proper (figure 10). In two day old chicks, no discernable lymphoid tissue or activity could be seen within the glandular or connective tissue. The first appearance of a white blood cell in close proximity to the connective tissue was observed on day three post-hatching (figure 17). A leucocyte (Lucas and Jamroz, 1961) with a small nucleus, having highly condensed chro-
matin and easily identifiable nucleolus, was observed within the stroma. The cell was clearly distinguished from the previously described cells and may have gained entry into the perivascular tissue by amoeboid movement through an endothelial pore. Days four, five and six (figures 18 through 24) demonstrated increased leucocytic activity throughout the entire vascular system of the gland, and in particular, in the peripheral region in close proximity to the stalk. Note in figure 18 the presence of an endothelial cell and its nucleus which lines the lumen of the vessel. Also present is a previously described connective tissue cell. In the lumen of the vessel, adhering to the endothelial wall is a leucocyte with a small, darkly stained nucleus. Distinguishable from the endothelial and connective tissue cells are lymphocytes with relatively large, round and irregular or indented nuclei. The nucleus is relatively darkly stained and moderate amounts of condensed chromatin toward its periphery. The cytoplasm appears to be bound by a defined membrane. Lymphocytes appear to migrate into the perivascular region. Several of these cells were observed to have foot-like processes, which were inserted into the connective tissue of the stroma (figures 10 and 23).

By day six, extensive leucocytic presence was seen, both within the connective tissue as well as in the blood vessel lumen (figure 19). Figure 20 demonstrates a leucocyte (day 4) passing through an endothelial gap. It would appear that this particular perivascular region is void of any other lymphoid-like tissue. The only lymphocyte would be that which is moving into the tissue. It may be suggested that this may be how lymphocytes gain entry into the connective tissue of the pineal gland stroma. Figures 21, 22 and 23 of five and six day glands demonstrate
large aggregations of lymphoid tissue well entrenched in the peri-vascular spaces of the gland. Figure 20 shows a lymphocyte midway through an endothelial gap. It would seem to indicate that the already present lymphocytes gained entry in much the same fashion. Figure 21 also demonstrates lymphocytes in the blood vessel, others in the connective tissue, and still another which appears to be settling into the stroma to join the already present aggregate of lymphocytes.

Figures 19 and 23 (day 6) demonstrate extensive leucocytic activity within the lumen of the vessels. Pseudopodia (figure 23) projecting from the lymphocytes into gaps in the endothelial tissue can also be seen. There are also numerous leucocytes interspersed within the surrounding tissue. Figures 18 (day 5) and 19 (day 6) demonstrate the "sticking" phenomenon of activated leucocytic tissue to endothelial cells of the vessel lumen (Macleod, 1968). This is very characteristic of high leucocytic activity. In figures 24 and 26 a leucocyte appears to be migrating still deeper into the connective tissue network. Figure 25 further demonstrates this amoeboid type movement within connective tissue.

It is at the ten day post-hatching stage that the appearance of an active germinal center was first observed (figure 27). The cells were found to be similar in appearance to those found within active germinal centers of the spleen, removed from the same ten day chick (figure 28). The active germinal centers of both the pineal and spleen contained several stages and varieties of leucocytic tissue, eg., small, medium, and large lymphocytes.
EXPLANATION OF FIGURES 2 and 3

Figure 2 Light microscopic section through a day 1 post-hatching pineal gland (pi) as it is situated between the cerebrum (c) and cerebellum (ce) (X100).

Figure 3 Light microscope section of the pineal gland of a day 24 post-hatching chick. Developing lymphocyte nodule (ln) can be seen in medial region of the gland. Cerebrum (c) is situated to the left of the pineal body (X400).
Fig. 2

Fig. 3
EXPLANATION OF FIGURES 4 and 5

Figure 4  The distinguishable stroma cells located within the connective tissue (ct) between two blood vessels (v). Connective tissue cell (c) has lightly stained nucleus (n) and prominent nucleolus (nu). Darker cell (dc) has dark nucleus and lipid inclusions (li) (day 1) (X3000).

Figure 5  Two adjacent stroma cells located near blood vessel (v) in the stalk region of the gland. Connective tissue cell (c) has large irregular nucleus with no definitive plasma membrane. Adjacent is darker lipid laden cell (dc). Nucleus (n) is displaced to one side. Organelles (o) can be seen within cytoplasm of connective tissue cells (day 1) (X3000).
EXPLANATION OF FIGURES 6 and 7

Figure 6 Micrograph of dark secretory cell (dc) with small compact nucleus (n). Organelles (o) can be seen in cytoplasm of adjacent connective tissue cells (c). Lipid inclusions (li) can be seen within cytoplasm of dark cell (day 1) (X3000).

Figure 7 Lipid laden leucocyte (l) found is vessel (v). Lipid inclusion can also be seen in darker secretory cells (dc). Nucleated erythrocyte (e) can be seen in capillary (ca) (day 1) (X 1800).
EXPLANATION OF FIGURES 8 and 9

Figure 8 Two pinealocytes (p) found in parenchyma near connective tissue (ct) of pineal gland. Pinealocyte on left demonstrates well defined nuclear membrane and definitive nuclear membrane and definitive nucleoli (nu). Cytoplasm of two cells appears continuous (day 1) (X3000).

Figure 9 Pinealocytes (p) with well defined nuclear membranes. Nucleus (n) appears large with diffuse chromatin. Cytoplasmic processes (cp) can be seen extending from the nuclear portion of the cell. Organelles (o) are interspersed within cytoplasm (day 1) (X3500).
EXPLANATION OF FIGURES 10 and 11

Figure 10  Leucocyte (l) floating free in lumen of blood vessel. Note phagocytized material within cytoplasm. Endothelial cells (en) can be seen lining the vessel lumen (v) (day 2) (X1800).

Figure 11  Pinealocyte (p) found relatively close to vascular area. Note adjacent connective tissue cells (c) with their large flattened nucleus (n). Autonomic axons (a) are located in interstitial tissue. Organelles (o), secretory inclusions (s), and a network of Golgi (g) can be seen in cytoplasm (day 1) (X3500).
EXPLANATION OF FIGURES 12 and 13

Figure 12 Pinealocyte found toward the medullary area of the pineal gland. Note diffuse chromatin (cr) and double nucleoli (nu). Also visible are cytoplasmic processes (cp) (day 1) (X3500).

Figure 13 Representative section of cells found near lumen of peripheral blood vessel (v). Endothelial cells (en) line the lumen of the vessel. Erythrocytes (e) can be seen in the lumen. Connective tissue cells (c) are scattered throughout the stroma. Leucocytes (l) can be seen as well as lipid bodies (li). Secretory cell (se) is also present (day 6) (X2000)
EXPLANATION OF FIGURES 14 and 15

Figure 14 Enlargement of secretory cell (sc) found in connective tissue. Note structured nucleus (n). Secretory bodies (sb) can be seen within cytoplasm (day 6) (X8000).

Figure 15 Cross section of blood vessel (v) in loose connective tissue of extreme peripheral region of pineal gland (day 1) (X2000).
Fig. 14

Fig. 15
EXPLANATION OF FIGURES 16 and 17

Figure 16 Cross section of blood vessel. Lumen (lu) is surrounded by endothelial cells (en), basement lamina (b), and smooth muscle cells (sm). Connective tissue cells (c) along with collagen fiber network (cl) surround autonomic axons (a) and terminals (t). Sectioned erythrocyte (e) can be seen in lumen (day 2) (X2000).

Figure 17 Leucocyte (l) appears to have invaded connective tissue. Note compact nature of red blood cells (e) in lumen of vessel (day 3) (X2000).
EXPLANATION OF FIGURES 18 and 19

Figure 18  Leucocyte (1) adhering to wall of blood vessel. Lymphocytes (ly) can be seen within connective tissue. Darkly stained secretory material (se) can be seen within the connective tissue (day 5) (X2500).

Figure 19  Lymphocytes (ly) within blood vessels among erythrocytes (e). Lymphocytes are also present within connective tissue of perivascular region (day 6) (X2000).
EXPLANATION OF FIGURES 20 and 21

Figure 20  Lymphocyte (ly) seen entering through endothelial pore into connective tissue stroma (s). Note no other lymphocytes appear to be in stroma at this time in this area (day 4) (X2000).

Figure 21  Lymphocyte (ly) can be seen entering stroma by way of endothelial pore. Note that lymphocytes are already present in stroma. (day 6) (X2300).
EXPLANATION OF FIGURES 22 and 23

Figure 22  Lymphocytes (ly) can be seen densely packed within stroma as well as entering stroma. Note cell process still extending into lumen. Other lymphocyte appears to be adhering to wall of blood vessel. Erythrocyte (e) can be seen in lumen (lu) (day 5) (X2000).

Figure 23  Lymphocytes (ly) appear to be entering connective tissue which is already laden with lymphocytes. Note pseudopodia (ps) of lymphocyte as it enters stroma by way of endothelial pore (day 6) (X2000).
EXPLANATION OF FIGURES 24 AND 25

Figure 24 Characteristic amoeboid movement is demonstrated as lymphocyte (ly) immigrates into stroma through smallest of endothelial pores. Lymphocytes can be seen in connective tissue stroma (day 6) (X2300).

Figure 25 Lymphocyte (ly) demonstrates ability to squeeze through small opening in connective tissue (ct) as it migrates to its destination within the stroma (day 6) (X2500).
EXPLANATION OF FIGURES 26 and 27

Figure 26  One of five lymphocytes (ly) migrating between stroma and lumen of capillary. Lymphocytes do not appear to be abundant in this connective tissue stroma (s) region of pineal gland (day 6) (X2000).

Figure 27  Nodular area deeply embedded within the connective tissue of peripheral region of ten day pineal gland. Note several different stages of lymphocytic development: small lymphocytes (sly), medium lymphocytes (mly), and large lymphocytes (lly) (day 10) (X2000).
Figure 28 Nodular area of spleen of ten day chick. Various stages of lymphocytes can be seen in this medullary region of the spleen: small lymphocytes (sly), medium lymphocytes (mly), and large lymphocytes (lly) (day 10) (X2300).
DISCUSSION

Lipid Containing Cells

Dark cells with lipid inclusions always appear in close proximity to blood vessels, almost regularly interconnected by interstitial tissue, while the lighter pinealocytes form more distally from the more vascular regions of the gland. These observations concerning dark cell - light cell positional relationships might be relevant to those observations made by Wartenberg (1968) and Rimji (1973) on the pineal gland of the rat. They stated that a synchronization type relationship of some special activity patterns between these two cell types may exist. It would appear that the darker cells have a high affinity for soluble constituents found within the perivascular network whether their source be from within the gland proper or some external site. It was demonstrated photographically that the dark cells are able to ingest large amounts of lipid from the blood when lipid contents are high such as during embryological development. In the same way, these cells may infest partially synthesized indoleamines or lipid soluble steroids, produced by the inner, medullary light pinealocytes. The suggestion of Romji (1973) appears relevant to this point. He believes that the medulla of the pineal gland of the rat may synthesize one or more pineal specific compounds such as serotonin and melatonin by dividing their synthesis over two compartments located in different cells. The rough endoplasmic reticulum of the light pinealocyte might synthesize a presursor compound. After depletion of the compound into the intercellular space, it would subsequently be taken up by cells in close proximity (dark cells) (figures 9 and 12). These substances may then be processed by the cells resulting
in the production of pineal specific substances such as melatonin. This hormonal end product would then be released by these dark cells into the perivascular space (figure 14) and from there into the systemic circulation. This hypothesis implies that those regions of the gland deficient in dark cells would deplete its precursor substance so that it reaches the blood directly without further conversion in the pineal.

It should be noted that the rapid disappearance of the lipid inclusions within the dark cells may be due to the lipid soluble fractions (also observed by Quay (1961a) in the pineal glands of rodents) being decreased in amount by various environmental treatments of the animals prior to removal and analysis of the pineal tissue. The present experiments used incandescent light to maintain body heat and thus establishing a continuous light environment for the post-hatching chicks. This treatment may have provided the proper conditions which lead to a rapid decrease in the pineal lipid soluble fraction, manifested in the histologically demonstrated rapid decrease in lipid droplets in the pineal dark cells (Quay, 1961b). This observation indicates that the effect of continuous light on pineal lipid content is somehow mediated by the lateral eyes and central nervous system, and may be a function of light duration and intensity.

**Leucocytes-Lymphocytes** - Leucocytes in the connective tissue were not observed until day three post-hatching. This would correlate with a light microscopic study which demonstrated the appearance of lymphocytes on day four post-hatching (Spiroff, 1958). It would appear that on days one and two the only lymphocytes present are those located in the lumen of the peripheral blood vessels. By days three and four
lymphocytes begin to migrate into the connective tissue of the peri-
vascular fibrous network. Although the present evidence is suggestive
of exogenous origin of lymphocytes within the pineal. These E.M.
studies do not provide direct information on the function or source
of the migrating leucocytes.

Lymphocytes were originally thought to arise from stem cells in
the pineal (Romeiu and Jullien, 1942a) which, in turn, had arisen from
reticular cells. It was subsequently found that the thymus was neces-
sary for the normal development of lymphocytes and the lymphatic system.
It was generally thought that the thymus and bone marrow could be the
original source of the lymphocytes and the lymphoblasts or stem cells
were all descendants of cells emanating from these organs (Cooper and
Lawton, 1974). While this is true, lymphocytes can also develop from
other lymphocytes (Clark and Clark, 1940). The cells that originally
enter the perivascular tissue during these early days post-hatching,
might therefore proliferate rapidly up to sixty days at which point
they form a very distinct nodule, which after sixty days tends to
degenerate and become reduced in size (Spiroff, 1958; Quay, 1965).

One approach to this problem that might prove fruitful would be to intro-
duce into a suspected origin site of these lymphocytes, labeled thy-
midine or $^{14}\text{C}$. This might be done by direct injection of the labeled
precursor into such sites as the bursa Fabricus or thymus of the chick
just prior to hatching. The cells could then be distinguished as to
possible site of origin (Hemmingsson, 1972). Figures 18, 19, 23 and
26 suggest a mechanism by which lymphocytes might gain entry into the
perivascular space. It would appear from these micrographs that the
white blood cells apparently cease to float freely in the blood stream,
no longer keeping pace with the red cells as they pass along in the vessels, but rather adhering closely to the endothelium (figure 18). Furthermore, during this period of intense lymphocytic activity the cells not only stick to the endothelium, but also to each other (figures 19 and 26). Very soon after sticking, the lymphocytes are seen to work their way through the endothelium and perivascular structures into the connective tissue spaces. The micrographs would indicate that this is accomplished by their inserting a pseudopod into an intercellular junction of the endothelial cells (figure 23), enlarging the opening somewhat (figures 20 and 21), and eventually squeezing through it (figures 22, 24 and 26). This is analogous to lymphocyte migration into tissues in regions of acute inflammation where tissue damage has occurred (Macleod, 1972).

Once within the stroma, the lymphoid cells migrate through the tissue (figure 25) to various locations and start to form nodular areas. By day six the lymphocytes appear to be quite numerous in many of the perivascular regions, especially in the periphery of the gland in the region of the stalk. By day ten there appears to be a beginning germinal center in which are seen a variety of lymphocytic stages. At this point in development, the lymphocytic germinal centers of the pineal bear striking resemblance to those found in the spleen (figures 27 and 28). During the course of this investigation no evidence was obtained indicating that early pineal cells transform into lymphoblasts (a blast cell being defined as the earliest recognizable cell belonging to a particular cell type (Bloom and Fawcett, 1962)). It may, therefore, be concluded that all lymphocytes observed in the pineal gland at early post-hatching periods are derived from an external source and
that these cells migrate to the pineal and proliferate by cell division in germinal centers established in the gland. There is no indication that lymphocytes differentiate from toti-potent cell types such as mesenchymal, reticular connective tissue or endothelial tissue present in the pineal.

Therefore to summarize, the observations of cellular interrelationships in the perivascular region indicate that there is a synchronization of synthetic activity between the light pinealocytes found toward the medullary region of the pineal gland and the somewhat darker stained cells found in closer proximity to the blood vessels.

The lipid inclusions indicate accumulation of precursors such as triacylglycerols in the cytoplasm, which may indicate the presence of active synthesis of various products or the mobilization of various lipid soluble substrates into fuel molecules or lipid soluble steroids that are transported to other tissues by the blood.

Leucocytic activity was not observed within the stroma of the pineal until day three. At that time and in the days immediately following, intense leucocytic activity was observed, both within the connective tissue of the perivascular spaces and within the lumen of blood vessels. There was no evidence of mesenchymal precursor cells. Rather, there was observed characteristic elucocytic sticking to endothelial walls and inward migration of lymphocytes into the stroma.

On day ten what appeared to be an active germinal center, perhaps a precursor to a future lymphoid nodule was observed within the connective tissue. This observation coupled with the pattern of migration would lead one to believe the lymphocytic nodular source to be exogenous.
BIBLIOGRAPHY


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The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Biology.

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