In Search of the Hagfish Thymus

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SYNOPSIS. The Pacific hagfish, *Eptatretus stoutii*, is capable of a variety of immunologic responses including allograft rejection and serum antibody production to soluble and cellular antigens. Interest has revived in the morphology of hagfish lymphoid tissues. The search for a thymus in young specimens has resulted in the discovery of a phagocytic and antigen-receptive cell population associated with the pharyngeal velar muscles. We suggest that a protothymus or precursor of the thymus of higher vertebrates may be contained within this velar muscle complex.

Müller (1845) and Stannius (1854) were among the first to describe a thymus in *Myxine glutinosa*, the Atlantic hagfish. This was later shown to be the pronephros. Cole (1925) described a lymphoid organ in the pharyngeal velum which he stated "may be interpreted as a thymus." His lack of conviction was supported by Kampmeier (1969) who called the same structure the anterior lymph propulser. It has been generally accepted that species of hagfish have no thymus.

Until recently, functional investigations strengthened this supposition. Papamastner et al. (1964) tried to raise antibodies to a variety of antigens in Pacific hagfish, *Eptatretus stoutii*. They also exchanged skin allografts between hagfish but failed to observe incompatibility in short-term experiments. Linna et al. (1970; 1975) studied proliferative responses of cyclostomes to stimulation with antigen in adjuvant. They injected bovine serum albumin and Freund's adjuvant into hagfish and lampreys. Using 32P incorporation into DNA as an indicator, no proliferative response was detected in the blood, intestinal mucosa (lamina propria and epithelium), or gills of the hagfish. Autoradiography of peripheral blood revealed an increase in blast cells in stimulated hags, while cell proliferation was observed in the gills and blood of lampreys. An increase in blast cell types, lymphocytes, and polymorphonuclear leucocytes was seen in lampreys. Hagfish were erroneously supposed to be immunologically unresponsive.

Other investigators have shown that with appropriate antigens, immunization schedule, and animal care, Pacific hagfish are capable of a variety of immunologic responses. Hildemann and Thoenes (1969a) demonstrated that hagfish exhibit allograft transplantation immunity which is characterized by persistent and specific immunologic memory. Pacific hagfish also produce serum antibodies of the IgM-type to the soluble antigen keyhole limpet hemocyanin (KLH) (Hildemann and Thoenes, 1969a). Serum antibodies may also be readily evoked to cellular antigens as found on sheep erythrocytes (Linthicum and Hildemann, 1970). The antibody produced against xenogeneic red blood cells is IgM-like, but distinguishable from that of higher vertebrates. Finally, bactericidins are produced in Pacific hagfish following injection of gram-negative organisms (Acton et al., 1971). A secondary response was elicited which was accelerated but not of
greater magnitude than the primary response. Given the repeated demonstration of immunologic responsiveness, we were encouraged to reexamine the question of whether or not the hagfish has a thymus or an identifiable equivalent. To this end we adopted several guidelines. First, any structure which could be accurately designated a thymus or protothyhmus should be located in the head region. Second, we assumed that hagfish might not possess an anatomically distinct structure or well-defined thymus as found in advanced vertebrates. Perhaps thymic functions are carried out by other tissues at the cyclostome level of phylogeny. However, the absence of a definitive organ could indicate that the thymus of the hagfish is an embryonic structure which disappears before or shortly after hatching. Finally, if a pharyngeal lymphoid structure were located, it should meet functional as well as structural requirements of a thymus.

GROSS DISSECTIONS AND LIGHT MICROSCOPY

In an attempt to determine the extent of the reticuloendothelial system in the head of the hagfish, a small amount of India ink was injected into a number of hagfish. Inasmuch as the size of a fish is directly related to its age, we chose the smallest specimens we could obtain on the assumption that the younger fish might have a more active thymus if one is present. A small amount of India ink was injected into the blood sinus of the tail. The sinus is part of a continuous blood and lymph sinus which runs, subcutaneously, the entire length of the hagfish. A substance injected into this space can be distributed throughout in a matter of seconds by simply stroking along the length of the body. In this manner, the ink was quickly distributed as evidenced by the blue-black color acquired by the fish. Specimens were sacrificed after 1, 2, or 3 hr, blood smears made, and gross dissections performed. The ink was quickly distributed to all parts of the body via the vascular system. Blood vessels were outlined making capillary systems visible. The liver which is normally pink, was very heavily blackened. Gross dissection did not reveal any previously undescribed organs. However, we decided to section serially some small injected hags to make sure that no lymphoid aggregates had been missed. The results can be seen in Figures 1, 2, 3, and 4. A group of muscles in the pharyngeal region of the head contain numerous ink particles. This group constitutes the velar muscles. The muscles of the velar complex in *Myxine* have been more than adequately described by Cole (1907), revealing the complicated anatomy of the hagfish head. Therefore, the following description of the extent of the velar muscles is simplified for the sake of clarity.

Two muscles have been described, the veloquadratus and velospinalis. The former is comprised of three divisions, essentially three separate muscles. The attachments of the velar muscles vary. All three divisions of the veloquadratus are located in the roof of the pharynx in a lymph sinus. They are covered laterally by a large cartilage, the palatoquadrate, which forms the third fenestra of the skull. The middle division of the velar muscle is visible through the fenestra which is covered by a tough translucent membrane. The cartilage is, in turn, covered by the parietalis muscle situated subcutaneously. This muscle is extensive and enshews the entire hagfish body. The velospinalis is the most caudal of the velar muscles and appears in the third fenestra of the skull just caudal to the middle division of the veloquadratus. In cross section, it appears just lateral to the spinal column (Fig. 4). The velar muscles may function as the occlusor of the nasopharyngeal opening, drawing water during respiratory movements into the pharynx toward the gills. The particles of ink appear to be trapped between the cells of these muscles. Since the complex is located deep to other muscles, virtually within the cranium, it was not discovered until after serial sectioning. Upon examination of previously fixed dissected specimens, the velar muscles were found to contain trapped ink. Ink particles were located throughout all portions of the muscle complex. None of the other cranial muscles contained ink and in some sections the con-
FIG. 1. Cross section of young hagfish through the nasal passage (NP) at the anterior end of the velar muscle complex (V). Note the subcutaneous vascular sinus (SS).

FIG. 2. Cross section of hagfish through the middle of the velar muscles (V).

FIG. 3. Cross section of a hagfish through the caudal end of the veloquadratus (VQ).

FIG. 4. Cross section of a hagfish through the velospinalis muscle (VS).

FIG. 5. Cross section of velar muscle (V) from a hagfish injected with India ink. The extensive vascularization of the muscle is evident. A cell containing ink particles can be seen (arrow). The section is stained with hematoxylin and safranin O. × 1200.

FIG. 6. Longitudinal section of velar muscle. Note the striking number of cells between the myofibrils. × 275.
Contrast between the ink-laden muscle and the overlying normal skeletal musculature was striking. Injection of ink into older, larger hagfish resulted in similar localization.

The next question was whether the ink was merely trapped between muscle fibers or inside cells. Light microscopy of formalin fixed tissue indicated that much of the ink was simply trapped between muscle cells. However, a small number of cells located between muscle fibers appeared to contain phagocytosed ink particles. Longitudinal sections of the middle division of the veloquadratus muscle revealed an extensive network of capillaries and numerous connective tissue cells located between loosely arranged bundles of muscle cells (Figs. 5, 6).

Blood smears confirmed that circulating phagocytes occur in hagfish blood (Figs. 7, 8). Because of the extensive vascularization of the muscle and presence of circulating phagocytes, it is possible that the ink containing cells seen in the velar muscles may be a part of the circulating cell population (peripheral blood) and not a part of the muscle cell population.

**Electron Microscopy and Autoradiography**

Therefore, two additional lines of inquiry were utilized. An electron-microscopic survey of the velar muscle of ink-injected hagfish was carried out. In addition, an autoradiographic study of $^{125}$I KLH-injected fish was done. The E. M. study supported light microscopic findings. A network of connective tissue cells most of which appear to be fibroblasts in varying stages of activity are interspersed between muscle cells (Fig. 9). Occasionally a smaller cell was found closely apposed to a muscle cell (Fig. 10). The small cell contained a large, eccentric, homogeneous nucleus. The cytoplasm was granular with a network of smooth membranes. Numerous small vesicles appear to be clustered near the junction with the muscle cell. While the cell surfaces appear to be closely apposed, it is impossible to determine the exact nature of the junction. A number of these cells were observed in various stages of degeneration.

Numerous nerves were observed in the interstitial connective tissue (Fig. 11). The most striking feature of the muscle was the extensive endothelial lining of capillaries and sinusoids. The sinusoids were full of blood cells. At times, the endothelial lining is drawn very thin. Unfortunately, none of the cells, either interstitial or in the vascular system, contained any ink particles. While disappointing, this was not surprising since the number of ink-containing cells observed with the light microscope were not numerous. However, in electron micrographs of velar muscle from one fish, we observed bacteria in the interstitial spaces (Fig. 12). The bacteria were located only in the interstitial tissue, not in the vascular system. If the bacteria were from an external source such as contaminated processing materials, they should have been evenly distributed across the sections. This was not the case. It is noteworthy that a naturally occurring foreign substance can be filtered out of the hagfish's system by the velar complex. Overlying skeletal muscle from the same fish did not contain any bacteria. Also, survey of an unwashed white cell pellet from this fish showed no signs of phagocytosed or free bacteria.

The velar muscle complex, therefore, seems to be able to filter particulate matter from the circulation. The large spaces of connective tissue and extensive vascularization are suggestive of a previously more dense cell population. However, if any past or present immunologic function is to be assigned to the velar complex, it should recognize soluble antigens. Therefore, three small (18 to 20 cm) and three large (38 to 50 cm) hagfish were injected with $^{125}$I-keyhole limpet hemocyanin (KLH). KLH obtained from Pacific Biomarine Supply Company, Venice, California 90291, was iodinated with carrier-free $^{125}$I (Radiochemical Center, Amersham) by the method of Greenwood et al. (1963). Approximately 1 mc of $^{125}$I/200 mg of protein with 100 mg/ml of chloroamine-T was allowed to react for 15 min at 4°C and 4 to 6 $\mu$ci/mg of KLH was achieved. The labelled protein in a dose of 200 mg/0.1 ml was injected into the lateral tail sinus. All fish were sacrificed at 72 hr post-injection. Blood
smears were made and tissue samples taken including velar muscle, normal skeletal muscle, liver, and gut. The tissues were embedded in paraffin, sectioned, mounted, and coated with NTB II emulsion (Kodak, Rochester, New York). Blood smears were also coated. The slides were developed and stained with hematoxylin and eosin.

The results can be seen in Figures 13 through 20. No labeled cells were observed in any of the blood smears (Figs. 13, 14). Therefore, any labeled cells in other tissues were not the result of cells just passing through via the circulation. As was expected, the liver contained the highest concentration of label (Fig. 15). It appeared to be concentrated in the cells of the parenchyma. Label present in the gut was located in the absorptive epithelium (Fig. 16). A patch of lymphoid tissue, equivalent to the spleen, in the lamprey is found in the spiral valve of the gut at the junction of the stomach and the intestine (Jordan and Spiedel, 1920). The spleen of the hagfish is distributed throughout the submucosa of the intestine. Although vascularization was extensive, no label was observed in the submucosa of the hagfish gut (Fig. 17).

Label was also absent from normal skeletal muscle (Fig. 18). However, velar muscle samples contained scattered labeled cells (Figs. 19, 20). The cells were of two distinct sizes, a small highly labeled cell containing fine granules of label (Fig. 19), and a large heavily labeled cell in which the granules were large and globular (Fig. 20). We therefore found a muscle which could concentrate particulate material and take up specific labeled antigen. The number of labeled cells roughly approximated the number of cells which took up ink particles.

**DISCUSSION**

Young specimens of Pacific hagfish, *Eptatretus stoutii*, have proved difficult to capture. This species is fond of deep water and muddy, rocky bottom conditions. Larger fish can be caught in baited traps constructed of large barrels with holes allowing water to exit while the trap is hauled in. Small fish escape via the holes as the catch is raised. Smaller traps enabled us to obtain smaller fish (17 to 25 cm long), but newly hatched specimens (5 to 7 cm long) and
FIG. 9. Active fibroblast in the connective tissue of the velar muscle. × 24,750.
FIG. 10. Small cell associated with a velar muscle cell. Note the vesicles (V) and membranes (M) within the cell. × 16,875.
FIG. 11. One of the numerous nerve fibers found in the connective tissue of the hagfish velar muscles. \( \times 22,100 \).

FIG. 12. Bacteria in the connective tissue of the velar muscle of a hagfish. Insert is a higher magnification. \( \times 26,500 \) and \( \times 34,500 \).
FIG. 13. Autoradiograph of peripheral blood from a hagfish injected with \( ^{125}I \) KLH. None of the cells take up the label. \( \times 275 \).

FIG. 14. Higher magnification of Figure 13. \( \times 650 \).

FIG. 15. Autoradiograph of liver taken from a hagfish injected with \( ^{125}I \) KLH. Many cells in the parenchyma have taken up the label. \( \times 275 \).

FIG. 16. Autoradiograph of intestinal mucosa of hagfish injected with \( ^{125}I \) KLH. Label is localized in the cells of the intestinal mucosa (arrow). \( \times 275 \).

FIG. 17. Autoradiograph of intestinal submucosa of hagfish injected with \( ^{125}I \) KLH. Despite the extensive vascularization none of the cells are labeled. \( \times 275 \).

fertile eggs or embryonic stages have not been obtained. Many large adult fish bearing eggs have been brought into the laboratory. Egg clusters are also often found in hagfish holding tanks, but the eggs are always infertile. All attempts to hatch hagfish in the laboratory have failed. We worked with the smallest (and therefore youngest) specimens obtainable, but truly decisive studies must await procurement of developing eggs or newly hatched hagfish or both. Little appears in the literature concerning the embryology of hagfishes. Dean (1899) described the Myxinoid development from egg to hatching. While accompanied by elegant drawings, the descriptions are based on whole specimens. Holmgren (1946) came into possession of two embryos and sectioned them. The specimens were not in the best of condition and their value with respect to the thymus is doubtful.

On the basis of quite limited evidence, we would suggest that a "protothymus" or precursor of the thymus of higher vertebrates may be associated with the velar muscle complex. The complex lies within a lymph sinus, traps particulate matter and contains antigen-binding cells not found in other muscles or in the general circulation. The numbers of phagocytic and antigen-receptive cells in the pharyngeal region of juvenile or older hags appear low. Given the large number of fibroblasts and the fibrocytes in this area, the non-myoid cellular population of the velar complex appears to have degenerated. If this is true, the protothymus occurs at a very early stage in hagfish development, perhaps just after hatching. Velar muscle samples from the youngest hagfish surveyed were similar to those of older hagfish with which they were compared. Since a much longer period of time is necessary to detect serum antibody production after immunization with K.I.H., repeated injections and selection of tissue samples near the peak of antibody production could yield larger numbers of labeled antigen-sensitive cells. The velar complex is located in an advantageous position to encounter antigenic materials, either particulate or soluble, in the circulation. The trapping of bacteria as well as ink particles at
this site suggests an immunologic function. Surgical excision of the hagfish "prothymus" is not possible without killing the animal. Administration of 5000 R of X-irradiation to the head area with lead shielding of the remainder of the body yielded a peripheral blood lymphopenia in hags after 5 days, but the effect of such irradiation or subsequent immunocompetence remains to be tested.

It is interesting and perhaps relevant that thymic medullary myoid cells have been described frequently in amphibians and reptiles (Cooper, 1973). By means of electron microscopy, what appears analogous to epithelial reticular Hassall's corpuscles of mammals, by light microscopy, are, in fact, striated muscle cells. The thymus in amphibians and reptiles is apparently not phagocytic, but representatives of both groups possess other anterior lymphoid organs capable of phagocytosis and antibody synthesis. Given its position in phylogeny, perhaps the hagfish possesses thymus-muscle-phagocytic complex all in one site, inseparable into discrete organs. This is in contrast to the trend in immunoevolution for separation of distinct function into distinct organs.

White cells from the peripheral blood of the larval lamprey, ammocoetes, can be stimulated to blastogenesis in culture with phytohemagglutinin (PHA) suggesting the presence of T-cells (Cooper, 1969). Our preliminary studies of hagfish white cells from blood show that they do proliferate in response to PHA stimulation. Perhaps this line of investigation can be one functional approach.

In ontogenesis of the frog (Rana pipiens) immune system, the thymus is the first lymphoid organ to appear; its cells are derived from elements of the thymic primordium rather than blood-borne stem cells (Turpen et al., 1973; 1975). Furthermore, lymphocytes from the thymus then proceed to populate the developing spleen, kidneys, and eventually the bone marrow. It is possible that the hagfish thymus is a strictly embryonic organ which likewise proceeds to populate other sites, particularly the kidneys, and then, unlike in the frog, virtually disappears. If this is the case, the velar system could be the thymic remnant and may be only, minimally, if at all, functional in adult fish. The kidney (pro nephros) of the hagfish is known to be hemopoietic and phagocytic (Holmgren, 1952). However, its functions are cyclic. A final answer to the question of whether or not the hagfish has a thymus will require embryonic material, but the nature of the velar system indicates a strong possibility that the thymus does exist at this level of phylogeny.

REFERENCES


