Skin lectin and the lymphoid tissues in the leptocephalus larvae of the Japanese eel Anguilla japonica

Yuzuru SUZUKI1* AND Tsuguo OTAKE2

1Fisheries Laboratory, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Maisaka, Shizuoka 431-0211 and 2Faculty of Bioresources, Mie University, Tsu City, Mie 514-8507, Japan

SUMMARY: Development of skin lectin and the lymphoid tissues were studied in the leptocephalus larvae of the Japanese eel Anguilla japonica. The specimens ranging in total length from 11 to 58 mm were captured at 12–19°N, 131–137°E and at 21–23°N, 123–128°E in the Pacific Ocean. The skin of leptocephali contained extremely active lectin which agglutinated rabbit red blood cells the same as adults. Club cells, known as lectin secreting cells, were also recognized in the epidermis of leptocephali, although the shape was not an elongated club form but oval. The cells were confirmed to contain lectin in the secretory vacuole by an immunofluorescence technique. The lectin in the cells was also recognized in the 8 days post-hatch preleptocephalous larvae, which was obtained from an artificially spawned eel, suggesting the importance of the lectin in the early larval development. In contrast, lymphoid tissues concerning immune functions showed delayed development with the exception of the thymus. No blood cells were seen in the kidney and spleen, even in a large specimen. Only a few undifferentiated leukocyte-like cells could be observed; some of them showed phagocytic figures. The thymus along with many thymocytes could be seen in the smallest leptocephalus of total length (TL) 11 mm. T cells may have a function in defense mechanisms during the leptocephalus stage, although other immune cells were still underdeveloped.

KEY WORDS: Anguilla japonica, club cell, eel, kidney, leptocephalus, lymphoid tissue, ontogeny, skin lectin, thymus.

INTRODUCTION

Anguilliform fish grow to adult stage via the larval stage called leptocephalus, during which they possess a uniquely transparent leaf-like appearance. The fish thus undergo two metamorphoses, from the hatch-out larval stage (preleptocephalus) to the leptocephalus stage, and from the leptocephalus to the elver stage. Such a form of metamorphosis is not typically observed among vertebrates, except for amphibians. Given that anguilliform fish differ in this respect from other fishes, elucidating the development of physiological function is important. Data are available on body,1–3 metabolism,4 digestion,5,6 osmoregulation,7,8 nervous system,8,9 and endocrine function,10–12 but it is not yet known how anguilliform fish maintain their immunity against pathogens in the open ocean. With an interest in the defense mechanisms of the delicate-looking leptocephalus, we focused our study on skin function as it provides the outer barrier and also on lymphoid development.

In the case of Japanese eel Anguilla japonica, examining their early development had not previously been possible as the location of breeding sites had eluded researchers for many years. However, in 1991, expedition members of the research vessel Hakuo-maru of the Ocean Research Institute, the University of Tokyo were the first to find many small leptocephalus larvae in the open sea and to identify the spawning area at about 15°N, 140°E in the Pacific Ocean.13 It is considered that the leptocephalus larvae drift from this area to near Japan, via the Kuroshio current within 4 to 7 months. Once in the proximity of the coast, they metamorphose to the elver stage, and then ascend the rivers. In the 1991 University of Tokyo expedition, more than 900 specimens of eel leptocephalus were collected. In addition, we obtained more leptocephali from the cruises of RV Hakuo-maru in 1994 and RV Taisei-maru in 1996. We were therefore able to analyze the development of the eel
leptocephalus larvae at the early stages, although as yet no eggs or preleptocephalous larvae have been obtained under natural conditions.

Eel is one of the most important fish species cultured in Japan. Although much effort has been directed to its breeding under artificial rearing conditions and in the laboratory of one of the authors,14,15 such that fertilized eggs and preleptocephalous larvae can be obtained fairly constantly, there is still no success in cultivating the larval fish to adults (i.e., culturing it to the leptocephalus stage has not yet been possible). Information concerning systems of defense mechanisms at the early stages seems to be one prerequisite in successful aquaculture. Nevertheless we have only limited data, owing to the difficulty of collecting sufficient samples of eel larvae.

For the analysis of the outer and inner defense mechanisms of eel larvae using preleptocephali obtained via artificial spawning, and leptocephali collected by the expeditions, we examined two subjects in particular. First, skin morphology and lectin in the skin were considered. Skin mucus of adult eel is known to have high hemagglutinative activity.16 This hemagglutination is caused by a protein called lectin, which bind specific carbohydrate. The eel skin mucus lectin is known to be secreted from the club cells in the skin.17 Although the function of the lectin is not necessarily clear, it is believed to be one important factor in the self-defense mechanisms in fish.18,19 We therefore examined whether the eel larvae possess lectin activity in the skin mucus, and looked for the presence of lectin in the club cells. Second, we observed the early development of the lymphoid tissues, which are essential for internal defense mechanisms in fish.

**MATERIALS AND METHODS**

**Fish**

Leptocephali of an early developmental stage were caught at 12–16°N, 131°E during the KH91-4 and KH94-2 cruises of RV Hakuho-maru of the Ocean Research Institute, the University of Tokyo. Leptocephali of late stages, longer than 50 mm in body length, were caught at 21–23°N, 123–128°E on the KT96-19 cruise of RV Tansei-maru of the institute. All the specimens were composed of four age groups as summarized in Table 1. The ages of the specimens were determined via otolith analysis by Tsukamoto and Umezawa20 and Arai et al.21 For comparison in light microscopic studies, we used preleptocephali larvae obtained from females matured artificially (Sato et al. 2000) by a new method.14,15,22 Since all the larvae died within 2 weeks, we could use only several individuals for each day group of 0 to 11 days post hatching from four mother fish. Individuals of the day 0 group were 2.8 mm in total length (TL), and grew to 5.4–5.8 mm on day 11. Also used were Elver eels caught at Lake Hamana, Shizuoka Prefecture, and adult eels cultured near Lake Hamana obtained from a local fish market.

**Skin lectin assay**

Lectin activity was assayed by hemagglutination titer against rabbit red blood cells, using microtitration techniques.16 The fluid on the body surface containing mucus (25 μL) were squeezed from the skin of freshly caught leptocephalus with a micro diluter. A serial twofold dilution of the fluid was then made on a microtiter plate with phosphate-buffered saline (PBS) pH 7.2. To each well was added 25 μL of 2.5% (v/v) rabbit red blood cells. After allowing for a 1 h incubation at room temperature, lectin titer was determined as the weakest dilution showing hemagglutination. In groups 2 and 3, the volumes of some samples were less than 25 μL, since the specimens were too small to collect enough mucus. In addition, we did not measure the exact volume, since we could not use a micro-balance on the ship. Because of impreciseness of the fluid volume and of the differences among the red blood cells, the lectin activities were expressed as relative value: ~, not detected; ±, trace; +, log₂(titer) ≤3; ++, 7 ≤log₂(titer) ≤10; ++++, log₂(titer) ≥15.

**Histological techniques**

Live preleptocephalous larvae were anesthetized with MS222 and their early development of blood cells was observed under Nomalski’s differential phase-contrast (Olympus BH50-Dic, Tokyo, Japan).

Leptocephali for light microscopy were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.2),

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with the exception of the two smallest individuals that were fixed in 10% formalin. Preleptocephalous larvae were fixed in 10% formalin. Specimens were embedded in paraffin and the sections were stained with hematoxylin and eosin (HE). We also examined the elver eels, and the skin and lymphoid tissues of adult eel in the same manner. The skin specimens were also used for immunocytochemistry.

For electron microscopy, leptocephali were fixed in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4, and postfixed in 2% OsO4. Specimens were embedded in Epon 812 (Oken Co., Tokyo, Japan). Ultrathin sections were stained with lead citrate and uranyl acetate, and observed with a JEOL 100S electron microscope (Tokyo, Japan).

Immunocytochemistry

Rabbit antiserum against eel skin lectin prepared as previously reported was used. Fluorescein isothiocyanate (FITC)-labeled antirabbit IgG goat IgG (Vector) was used as the second antibody.

Whole-mount staining was adopted for immunocytochemistry of lectin in the preleptocephalous and leptocephalus larvae. In the latter, we peeled off the skin of the head with forceps under a dissection microscope and used it for immunostaining without further treatment. Whole preleptocephali and the head skins of leptocephalus were washed three times for 1 h with phosphate-buffered saline supplemented with Tween-20 (PBS-T; Wako, Tokyo, Japan). They were then reacted with the anti-lectin rabbit serum diluted 1:200 with PBS-T for 2 h at 20°C or overnight at 4°C. After washing with PBS-T three times, they were reacted for 1 h with the FITC-labeled antirabbit IgG goat IgG diluted 1:50 with PBS-T containing 500 ng/mL of propidium iodide (PI; Molecular Probes Inc., Engene, OR, USA) and 10% normal goat serum. After washing with PBS-T three times, they were mounted with Vectashield (Vector) mounting medium. For the skin of elver and adult eel, paraaffin sections were reacted with the anti-lectin rabbit serum and with FITC-labeled antirabbit IgG goat IgG, in the same manner. These specimens were observed under a confocal laser scan microscope (CLSM-diaplan; Leica, Heidelberg, Germany).

RESULTS

Lectin activity

Hemagglutinici activities with the lectin are summarized in Table 2. No activity was recognized in five individuals, while the other individuals showed lectin activity. All the leptocephali of late developmental stage with 50–58 mm TL showed high activity. In particular, two live specimens showed extremely high activity of lectin (data not shown), while all others were already dead when caught.

Histological observations of the skin

In many specimens of leptocephali, trunk epithelial tissue was almost completely absent. However, epithelial tissue was found in fairly good condition in the head.

The epithelium was composed of one layer of club cells, and some epithelial cells in fish of all size groups (Fig. 1). No mucous cell could be found. The club cells of the leptocephalus were a large oval shape, differing from that of adult anguilliform fish, which is normally of an elongated clavate shape. Nevertheless, the club cells were identified as club cells, since they each possessed a secretory vacuole in the center. Club cells of a large oval shape were also found in the epidermis of preleptocephalous larvae from artificially spawned eggs, at 8 days post hatching. In the elver eel, the club cells were also oval. Mucous cells had already appeared at the elver eel stage.

In one specimen of leptocephalus of TL 33 mm, we found epithelial tissue on the surface of the body trunk (Fig. 2). The epithelial tissue was composed of three layers of cells (i.e. superficial epithelial cells, basement epithelial cells, and club and epithelial cells sandwiched
between the superficial and basement epithelial layers). The superficial epithelial cells were characterized by numerous vesicles harboring various inclusions. Basement cells were characterized by bundles of well-developed tonofilaments. No desmosomal junction between the adjacent cells was recognizable. Club cells in the leptocephalus were essentially identical to those in the adult eel.\textsuperscript{17,23,24} Cells each possessed a secretory vacuole just adjacent to the nucleus which contained electron-dense materials. The remainder of the cell was filled with helical filaments, although helical structures were not necessarily clear in the leptocephalus.

**Immunocytochemistry**

In the adult eel, lectin was clearly recognized in the secretory vacuole of club cells, which occupied the major portion of the epidermis, as reported previously.\textsuperscript{17} In elver eel too, lectin was detected in club cell, although the shape of the cell was not elongated as in an adult, but oval.

In the leptocephalus, lectin was clearly recognized in the secretory vacuole of the club cells of the head skin (Fig. 3a). The density of the club cells which were positive for lectin was as high as in the adult or elver eel.

In preleptocephalus, lectin was also detected in the club cells of the skin (Fig. 3b). The first appearance of the lectin was in 8-day post-hatch larvae, although the number was as yet small. The lectin was seen in the secretory vacuole as shown in leptocephali. In some individuals, however, a positive reaction was seen in the whole cytoplasm with no vacuole.

**Histology of lymphoid tissues**

Blood cells appeared in the blood vessels of preleptocephalous larvae 6 days after hatching, although the
number were few. In some specimens, cells were fairly abundant in the liver.

In the blood vessels of the leptocephali, no red blood cells and a few blood cells of several types were observed. Moreover, no hemopoietic tissue was recognized in the kidney in any of the leptocephali specimens (Fig. 4a). On the other hand, elver eels had well-developed kidney hemopoietic tissue.

In some specimens, many unidentifiable blood cells were observed in the connective tissue around the gut, especially at the anus (Fig. 4b). Detailed observations could not be undertaken by light microscopy.

The differentiation of the thymus occurred at the leptocephalus stages (Fig. 4c). The thymus along with many thymocytes was seen even in the smallest specimens with TL of 11 mm. The thymus was not observable in the 6- and 11-day-old preleptocephalus larvae. In elver eel, the thymus was properly developed.

Leptocephali of TL 20 mm were observed to possess a spleen, but no lymphocytes were seen even in the largest specimens of about 30 mm larvae. The spleen was not seen in 11 and 12 mm leptocephali, nor in the preleptocephalous larvae. On the other hand, at the elver eel stage, the spleen was well developed.

By electron microscopy, we could not observe the thymus and the spleen, since no adequate sample was obtained. On the other hand, we could observe blood cells around the gut (Fig. 5). There were phagocytes with many phagosomes in the cytoplasm, although many of the cells could not be identified.

**DISCUSSION**

The results described above clearly show that the larvae of eel have high lectin activity in the skin, and moreover, club cells having lectin appeared at an extremely early stage of larval development, in preleptocephalus 8 days after hatching. On the contrary, the lymphoid and hemopoietic tissues of the larvae were extremely under-developed, except for the thymus. Lectin is believed to be an important factor in the self-defense mechanisms of fish. Therefore, lectin may have much importance in the early life stage of eel.

Lectin activities were noted in only five of ten individuals of 18 to 33 mm TL. Furthermore, only one individual showed an extremely high titer. As observed by light and electron microscopy, almost all epithelial tissues were already absent when the fish were caught. The epithelial cell layers of the larvae are extremely thin and delicate with no or few desmosomal junctions. The tissue may have been rubbed off during the sampling process.

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**Fig. 4** Kidney (a), free leukocyte-like cells (arrowhead) in the connective tissue around the gut (b) and thymus (c) of leptocephalus. No hemocytes are visible in the tissue. G, glomerulus; B, Bowman’s capsule; GC, gill chamber; O, operculum. Bar = 50 μm.

**Fig. 5** Ultrastructure of some leukocyte-like cells in the gut connective tissue. Phagocytes having phagosomes (arrowhead) in the cytoplasm are visible. Bar = 5 μm.
process due to handling and contact with many other planktonic animals caught together with the larvae in the sampling net. Only a few samples were obtained in good condition. This may be one reason for the observation of low lectin titers in many leptocephali of early developmental stage. Rather, we consider that the high titer shown in one specimen is normal, and the lectin is essential to the development of early defense mechanisms. This was also supported by the fact that all the specimens of more developed leptocephali, 50 to 58 mm in TL, showed high lectin activities especially in live specimens.

We attempted to take mucus from the skin surface when we measured the hemagglutinin titer, but we had possibly collected epithelial tissues with sea water from the skin surface instead of the mucus. This is because we could not observe the mucous cells in the leptocephalus epithelium. Absence of mucous cells will be common in eel leptocephali as observed also in Ariasona balearicum. Further observations using samples in good condition are needed to elucidate whether leptocephali actually possess mucus. However, it can be stated that the skin of the leptocephali contains extremely active lectin.

Club cells which secrete lectin in adult eel could be seen in leptocephalus larvae and also in preleptocephalus larvae obtained by artificial spawning as early as 8 days after hatching. The cells were oval in shape. This is quite different from the elongated clavate shape of the cells in adult fish. Nevertheless, the cells were recognizable as club cells by the presence of a secretory vacuole just adjacent to the nucleus, which is characteristic of club cells in anguilliform fish. The lectin was detectable in the vacuole by an immunocytochemical procedure, and therefore the cells were functionally mature as lectin secreting cells. On the other hand, cells in some individuals have the lectin in the whole cytoplasm with no vacuole. It is not yet clear whether this is normal.

Tissues relating to immune response in leptocephali showed delayed development. Leptocephali had only a few blood cells in the circulation and, moreover, we could not observe hemopoietic tissues around the kidney. Regarding lymphocytes, no cells were found in any tissues other than in the thymus. In addition, the leptocephali had no spleen, which is the organ most important in antigen trapping and humoral immune responses in adult fish. Elver eel had a well-developed spleen. Therefore, the spleen may be an organ which appears in the late leptocephalus stage, or during metamorphosis to elver. Relatively slow development of the spleen is commonly observed in many fish species, while it is more uncommon to note delayed development of hemopoietic tissues around the kidney. These observations show that the humoral immune response has less significance in leptocephali, although the thymus appeared in the early developmental stages, as discussed below.

Blood cells were observed in the hepatic sinusoid of preleptocephali. In mammals, the liver is known as the main hemopoietic site of the fetus. We, however, found no signs of hemopoiesis by histological observations of preleptocephali.

In some specimens, many free leukocyte-like cells could be observed in the connective tissue of the gut, especially around the rectum. Some of the cells showed phagocytic features. Rombout et al. reported that the macrophages in the second gut segment of carp function to process intact protein, which is ingested via intestinal epithelium, and is also functioning to present the antigen to lymphocytes. Therefore, the phagocytes around the gut may have some immunological meaning in regard to proteins taken up by the epithelium. However, the leptocephalus does not have enough immunological ability in the early developmental stages given its few lymphocytes, especially B cells. This was indicated from the significantly delayed development of hemopoietic tissues in the kidney. It is thus difficult to consider the immunological meaning of the free leukocyte-like cells around the gut observed in leptocephali.

Uptake and intracellular digestion of proteins by the epithelium is commonly observed in larval fish, including leptocephali. This phenomenon may be of help to the lower digestive ability in larval intestine. Therefore, it is possible that the phagocytes contribute to the digestion of absorbed proteins, rather than to the immune responses. From this point of view, the leukocyte-like cells around the gut are not relevant to defense mechanisms but to digestion.

The thymus appears and becomes functional in the early stages of leptocephalus development, as observed in many fish species. Many thymocytes were seen even in 20 mm larvae. T cells, derived from thymocyte, function to identify non-self from self, in the process of initiation of immune responses. Self recognition by T cells may, therefore, be of importance in the immune responses in the leptocephalus stages in eel. On the other hand, B cells are absent in this stage, although they should be essential for humoral immune responses. In addition, the spleen, the site of immune response, is extremely underdeveloped. Thus the existence of T cells do not necessarily imply the existence of humoral immune systems. T cells in the leptocephalus larvae may have some importance in conferring cellular immunity, although no direct evidence has been obtained concerning the functions of the T cells.

Despite much effort in searching spawning grounds of eel, we have not yet succeeded in obtaining eggs and the preleptocephalus larvae of natural origin. Information from wild specimens of undesdeveloped eel is thus required. We hope the spawning habitat of eel will be clarified in the near future. In addition, we have not yet obtained information on further developments in the latter half of the leptocephali life cycle which is the duration before metamorphosis to the elver stage. The
leptocephalus of eel are known to grow to 55–60 mm in about 4–7 months. 20 We need to analyze specimens of metamorphosing eels, which are known to occur at about 100 to 160 days after hatching. 21

In summary, eel larvae have extremely active lectin in the skin with underdeveloped immune systems in the internal body. The skin lectin may compensate for the delayed development of immune function. Is this related to their habitat, growing up in the clean, open sea? Knowledge about the early development of larvae of marine fish species is still too limited to answer this question.

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