ANTIGENIC AND ULTRASTRUCTURAL CHARACTERISTICS OF EPITHELIOCYSTIS INFECTION IN CULTURED WHITE STURGEON

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Introduction

Epitheliocystis disease is a common condition of a putative infectious etiology that has been described in various teleosts (Paperna and Sabnai, 1980; Wolf, 1988; Turnbull, 1993; Fryer and Lannan, 1994). The descriptive term has been derived from the appearance of epithelial lesions manifested secondary to infection. Microscopically, progressive enlargement of infected epithelial cells subsequently results in the formation of spherical cysts that are circumscribed by an eosinophilic, hyaline capsule. Infected cells contain coccoid or coccobacillary organisms that complete a pleomorphic life cycle with specific morphological characteristics dependent on the stage of intracellular development. Development of the organism is similar to the chlamydiae (Ward, 1988) and proceeds from small, rigid, infectious forms to larger, pleomorphic, non-infectious forms that divide by fission to produce a new generation of infectious daughter cells. Taxonomic classification of the epitheliocystis agent has been speculative due to the inability to isolate the organism *in vitro*. Clinical disease has been attributed to respiratory distress secondary to gill epithelial hyperplasia and excessive mucous production although this association remains empirical.The present report expands the species catalogue of infection and presents antigenic and ultrastructural similarities between the white sturgeon (*Acipenser transmontanus*) epitheliocystis agent and the chlamydiae.

Materials and Methods

The white sturgeon examined were subyearlings (11 months, 250-300 g) from a population maintained at a private culture facility in southern Idaho. The population was divided into seven 1.0 m^3 aquaria with 50-75 fish per enclosure that were supplied with ambient, 13.5 C, non-recirculating, spring water at 45.4 liters per minute. Fish were fed a commercial soft-moist pellet at 2% body weight per day with a feed conversion of 1:1.7-1:1.9. Diagnostic efforts were initiated due to a 4-8% mortality (3-4 fish per enclosure) that was considered abnormal for this age-class under these culture conditions. Moribund fish were randomly selected from the affected population and fixed in 10% neutral-buffered formalin. Tissue sections were cut to 5 µm and stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS), Brown and Brenn (BB), Macchiavello and Gimenez reagents. Selected areas of gill tissue were procured from the paraffin blocks for transmission electron microscopic examination. A standard peroxidase-antiperoxidase (PAP) immunohistochemical staining procedure using a primary monoclonal antibody specific for the chlamydial genus-specific lipopolysaccharide (LPS) antigen was used to demonstrate the expression of chlamydial antigen in infected epithelial cells of the gills.

Results

Cellular lesions typical of epitheliocystis infection were observed in the gills. Infected branchial epithelial cells contained the coccoid to coccobacillary epitheliocystis organisms that appeared as cytoplasmic inclusions composed of a fine, homogeneous, dense, basophilic, granular material (Figure 1). The infected cells were variably enlarged with spherical to oval profiles and randomly distributed throughout the branchial epithelium. The inclusions were circumscribed by a variable amount of host cell cytoplasm that consequently became less abundant with progressive enlargement of the cytoplasmic inclusions. The nuclei of infected cells were initially enlarged and eccentrically located with a single prominent nucleolus or multiple nucleoli but became attenuated and progressively less prominent subsequent to the progressive enlargement of the cytoplasmic inclusions.The cytoplasmic inclusions stained positive with Macchiavello but negative with Brown and Brenn, PAS and Gimenez stains. Expression of chlamydial antigen was demonstrated within the cytoplasmic inclusions using a standard peroxidase-antiperoxidase immunohistochemical technique and a primary murine monoclonal antibody specific for the chlamydial genus-specific antigen (Figure 1). Host reaction was absent or limited to a mild epithelial hyperplasia of the gills although the latter could not be definitively attributed to the infection.

Three stages of coordinated intracellular development were recognized by electron microscopy. Regardless of the developmental stage, the organisms were delimited by a cell membrane that was composed of two electron-dense zones separated by an electron-lucent layer. A variable number of discrete cytoplasmic condensations that were not membrane-bound and of variable density dependent on the stage of development were also a common feature.The reticulate bodies were oval to spherical and 0.4-0.8 x 0.5-1.4 µm but often exhibited a pleomorphic and convoluted appearance due to variable membrane invaginations and evaginations suggestive of uneven fission and budding. A few enlarged reticulate bodies with a maximum size of 2.2 x 3.7 µm had an exaggerated pleomorphic and convoluted appearance and contained multiple cytoplasmic condensations. The pleomorphic appearance of the enlarged reticulate bodies was due to multiple membrane evaginations consistent with the process of budding. Separate host cells contained intermediate bodies that were spherical to oval and 0.2-0.4 x 0.3-0.6 µm although often observed in the process of apparent uneven division. These cells contained a single, compact, cytoplasmic condensation that were completely circumscribed by an electron-lucent zone or halo. The presence of a cap or plaque composed of hexagonally arrayed, fibrillar, surface projections was initially recognized at this stage. Tangential sections demonstrated that the cap was composed of up to 30 surface projections. An homogeneous population of elementary bodies that were oval and 0.3-0.4 µm also occurred separately in individual host cells (Figure 2). This developmental stage had a single, dense, compact, eccentrically located, cytoplasmic condensation and a single cytoplasmic vacuole that was not membrane-bound. The hexagonally arrayed fibrils were more distinct at this stage and occurred opposite to the eccentrically located, cytoplasmic condensation. The cell membrane associated with the fibrils constituted approximately 25% of the cell circumference and displayed a prominent electron-density relative to the remainder of the cell membrane.

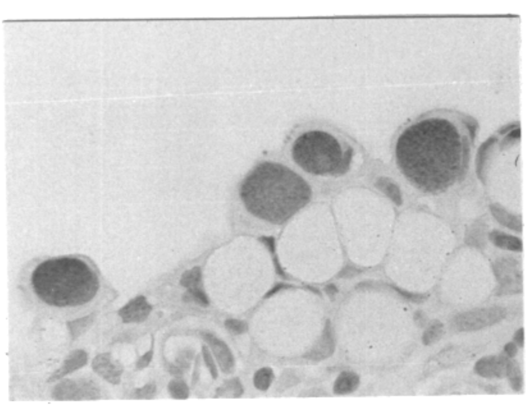

Figure 1. Infected branchial epithelial cells positive for chlamydial LPS antigen.

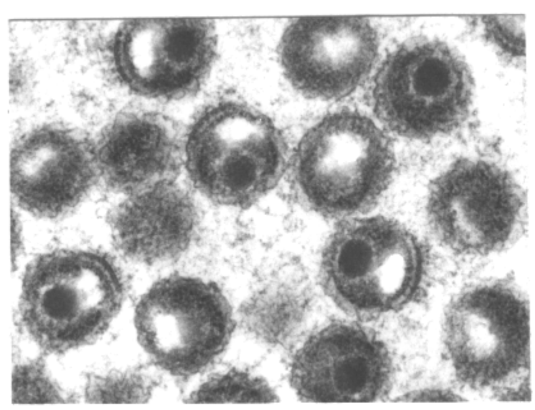

Figure 2. Epitheliocystis elementary bodies with characteristic nucleoid and hexagonally arrayed fibrils.

Discussion

Epitheliocystis disease is a common condition that has been observed in various teleosts as an incidental finding (Paperna and Sabnai, 1980; Wolf, 1988; Turnbull, 1993). This incidental form of the disease has been characterized as a non-lethal or chronic condition due to the apparent mild infection and associated host response that is typically absent or limited to a mild epithelial hyperplasia. A severe form of the disease characterized as an hyperinfection may result in a diffuse, severe, proliferative, epithelial hyperplasia often with branchial lamellar fusion and excessive mucous production (Paperna, 1977; Rourke et al., 1984; Bradley et al., 1988).

Previous attempts to classify the epitheliocystis agent have been based on *in situ* ultrastructural characteristics of the organism due to the inability to culture and isolate the organism *in vitro*. Development of the organism is similar to the chlamydiae that proceed from small, rigid, infectious forms to larger, pleomorphic, non-infectious forms which divide by fission to produce a new generation of infectious daughter cells (Ward, 1988). The morphological characteristics observed in the present report were consistent with the features that occur during these various stages of chlamydial development.

Five morphological stages of epitheliocystis development have been described in various species and referred to as the initial or reticulate bodies, elongated cells, round cells, small cells and head and tail cells (Turnbull, 1993). The initial or reticulate body designation has been used to describe the 0.7 to 1.25 μm, irregularly shaped, epitheliocystis cells due to the morphological similarities of these cells with the 0.5-1.3 μm reticulate bodies characteristic of early chlamydial development (Rourke et al., 1984; Bradley et al., 1988; Desser et al., 1988). The reticulate bodies observed in this report were similar to these previous descriptions. Furthermore, the pleomorphic and convoluted appearance that was often highly exaggerated and suggestive of division has previously been described for epitheliocystis in other host species (Desser et al., 1988) and for the chlamydiae (Ward, 1988). Two forms of elongated cells have been described for the epitheliocystis agent (Paperna et al., 1981). The primary long cells (PLC) have a reported maximum size of 0.3-0.6 x 7.5 μm and contain an electron-lucent matrix with peripheral ribosomes and loose, fibrillar, cytoplasmic condensations. The intermediate long cells (ILC) are produced from the PLC by binary fission and budding but are usually smaller with a reported range of 0.3-0.6 x 1.0-2.0 μm and contain more dense cytoplasmic condensations relative to the PLC. These elongated cells may represent morphological variants of the reticulate body in these host species although their structure and mode of division has been reported to resemble the rickettsial giant or initial body (Paperna et al., 1981).

Epitheliocystis round cells have been observed in various host species and referred to as intermediate bodies due to the shared ultrastructural similarities with the intermediate developmental stage of the chlamydiae that occurs in transition between the reticulate body and elementary body forms (Paperna and Sabnai, 1980; Wolf, 1988; Turnbull, 1993). These round cells have a reported size range of 0.3-1 μm and are similar to the 0.2-0.4 x 0.3-0.6 μm spherical to oval, intermediate bodies observed in this study. The final and infective stage of epitheliocystis development has been assumed to be the designated small cell due to the similarity with the chlamydial elementary bodies (Turnbull, 1993). These small cells are less than one micrometer in diameter and contain an electron-lucent vacuole, a dense cytoplasm and a dense, compact, cytoplasmic condensation that is surrounded by an electron-lucent zone or halo similar to the 0.3-0.4 μm oval elementary bodies observed in this report. This dense, compact, cytoplasmic condensation or nucleoid has been considered a hallmark of the chlamydial elementary body (Costerton et al., 1976). The cap of hexagonally arrayed, fibrillar, surface projections that occurred opposite to the location of the dense, compact, matrix condensation as described in this report has also been observed in the later stages of epitheliocystis development in the brown bullhead (*Ictalurus nebulosus*; Desser et al., 1988). These surface projections are similar to the unique structures described for the chlamydial elementary bodies that are considered requisite for inclusion in this group (Gregory et al., 1979). Although the function of these surface projections remains speculative, their hollow and cylindrical structure may be indicative of a transmembrane pore that connects the interior of the cell to the external environment or that is involved in interchlamydial communication (Louis et al., 1980; Matsumoto et al., 1982). The presence of these surface projections in the later stages of development also suggests a role in the attachment to host epithelial cells prior to infection.

Head and tail cells have been previously observed in only two species of salmonids (Rourke et al., 1984; Bradley et al., 1988). These cells reportedly exhibit the common epitheliocystis features and are comprised of an elliptical, 0.3-0.4 µm head region that contains a dense cytoplasmic condensation and a tail region up to 0.3 µm in length with a terminal 0.125 µm expansion. These cells reportedly resemble a degenerative form of the rickettsial organism, *Coxiella burnetti* (Weiss and Moulder, 1984), and may represent failed or delayed division, an artifact or a form unique to these host species (Turnbull, 1993). However, cells with this characteristic morphology were not observed in this study.

Attempts to classify the epitheliocystis organism based on ultrastructural morphology have been unsuccessful due to the limitations inherent in these methods that include sampling error and the inability to accurately assess the variability in epitheliocystis development that may occur with strain differences or with differences in the host species influence. However, inclusion in the Chlamydiales has been suggested since the various developmental stages share ultrastructural similarities with members of this group (Paperna et al., 1981; Desser et al., 1988). Placement in the Rickettsiales has also been proposed based on the similarities in morphology and mode of division of the epitheliocystis PLC with the rickettsial initial or giant body (Paperna et al., 1981). This argument has been supported by the failure to detect epitheliocystis expression of the chlamydial genus-specific LPS antigen in previous investigations (Bradley et al., 1988; Turnbull, 1993). However, positive demonstration of chlamydial antigen in the present study provides further evidence that the epitheliocystis organism is related to members of the Chlamydiales.

The source of infection in the present report was not determined although transmission via fomites (Paperna, 1977) or through the water supply was considered the most likely explanation. The high-density culture of these sturgeon would further promote the transmission and exacerbation of infection within the population. The sturgeon examined in this study were randomly selected and may not have been representative of the severity of infection responsible for the low-level mortality in this population. Therefore, epitheliocystis infection cannot be ruled-out as the primary cause of mortality in this population especially since other infectious disease agents were not identified nor were water quality or other environmental parameters considered a limiting factor.

In conclusion, the ultrastructural characteristics of the epitheliocystis organism in these white sturgeon was similar to previous descriptions in other host species and expands the species catalogue of epitheliocystis infection. Ultrastructural characteristics similar to the chlamydiae in association with the immunohistochemical demonstration of chlamydial antigen within the cytoplasmic inclusions provides further evidence that the epitheliocystis agent is related to members of the Chlamydiales. Although the infection was considered mild to moderate and could not be definitively attributed to the mortality in this population, the institution of surveillance methods to detect and subsequently prevent the transmission of epitheliocystis infection in cultured populations should be considered. Likewise, the practicality and viability of alternative husbandry methods that reduce or eliminate the potential adverse impact of epitheliocystis infection especially in high-density operations need to be evaluated.

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