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Identification of Proliferating Cells in Hard Clams

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The origin of hemocytes, the circulating "blood cells" of bivalve molluscs, including hard clams (*Mercenaria mercenaria*) has not been identified (1, 2). Proliferation of hemocytes, however, can be recognized through their increased numbers in diseased animals. Proliferating cell nuclear antigen (PCNA), or cyclin, is a protein produced during the late G1 and S phases of the cell cycle (3, 4). Using antibodies that recognize PCNA in mice, we attempted to identify the origin of hemocytes in the hard shell clams. Quahog parasite unknown (QPX) is a protist that causes severe inflammation and mortality in infected clams (5, 6). We attempted to induce hemocyte proliferation by exposing clams to QPX in a 10-l water column in which 12 ml of undiluted QPX culture (at a concentration of $7*10^6$ cells/ml) were added every 10 days; by injecting QPX between the membranous mantles and the right valves, 3 cm ventral anterior to the siphon and into the pericardial cavities (0.25 ml of undiluted QPX culture); and by injecting an inert particle (India ink, 1:10 dilution in sterile seawater [7, 8]) into the pericardial cavities. The controls consisted of two groups of clams. One group was injected with sterile seawater in the pericardial cavities; the other was untreated. Groups were sampled at 24 h, and at 1, 4, and 8 weeks after the start of the experiment. At sampling, the animals were shucked, fixed in 10% neutral buffered formalin (NBF) for 24 h, and embedded in paraffin. Sections were cut (4–6 μm), mounted onto positively charged slides (Fisherbrand, Superfrost/Plus and ProbeOn Plus slides) and stained either with hematoxylin and eosin (H&E) (9) or with anti-PCNA with a hematoxylin counterstain (Zymed, PCNA Staining Kit).

Clams injected with QPX in the pericardial cavities showed mild focal inflammation associated with viable and necrotic QPX organisms. At 2 months post-injection, viable QPX organisms were no longer identified. QPX organisms and associated inflammation were not observed in clams injected in the mantle cavity. After 2 months of water column exposure, only very rare infection by QPX organs with minimal inflammation was observed in mantle tissue. India ink injection caused a minimal inflammatory response. Pools of injected ink in the tissues and vascular spaces were either engulfed by individual hemocytes or surrounded and sequestered by hemocytes (encapsulation), forming thin-walled granulomas (6, 10). Numerous individual hemocytes containing India ink were eliminated from the clams by diapedesis over lumenal epithelial surfaces (Fig. 1A). Thick-walled granulomas (6, 10) were also identified in the gills, pericardial sacs, and other

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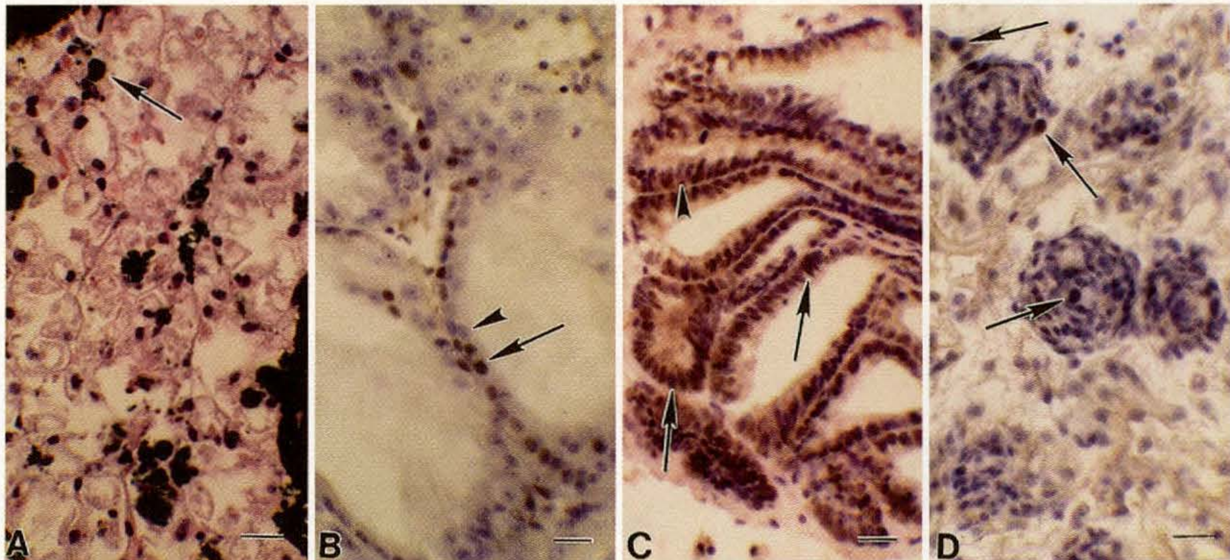

Figure 1. *Proliferating epithelial cells and hemocytes of* Mercenaria mercenaria *stained with anti-Mouse Proliferating Cell Nuclear Antigen (PCNA). (A) India ink is phagocytized and eliminated by diapedesis of India ink-filled hemocytes (arrow) into the renal tubular lumens (H&E, bar = 13 μm). (B) Nuclei of proliferating reserve cells of the digestive gland's tubular epithelia stained black (arrow) with anti-PCNA. PCNA negative nuclei stain blue (arrowhead) (hematoxylin counterstain, bar = 13 μm). (C) Strong PCNA nuclear staining is present in the proliferating nuclear epithelial cells at the base of the gills (arrows). PCNA negative nuclei stain blue (arrowhead) (hematoxylin counterstain, bar = 13 μm). (D) Nuclei of some hemocytes in thick-walled granulomas in the kidney tissue of clams are positive for PCNA stain (arrows) and present evidence for the proliferation of hemocytes directly at the inflammatory site (hematoxylin counterstain, bar = 13 μm).*

organs of saline-injected animals, indicating that the injection may not have been sterile.

Using the anti-mouse PCNA, areas of abundant PCNA staining (black-stained nuclei), indicating areas of marked cell proliferation, were identified in the reserve cells of the digestive gland (Fig. 1B), the proliferative epithelial cells of the gill base (2) (Fig. 1C), and in early proliferative phases of reproductive epithelium. As expected, cells of other tissues throughout the body also stained positive for PCNA (*i.e.,* epithelium of the intestine, foot and body wall, and gill plical epithelium), but in much lower numbers. Proliferating hemocytes were identified in the inflammatory cells forming the thick-walled granulomas (Fig. 1D) and rarely in adjacent non-inflammatory cells. In no other areas examined were proliferating hemocytes identified.

These results demonstrate that the epitopes associated with PCNA are conserved between the clam and the mouse, as shown by the positive staining of known proliferative cells in the clam body. Previous studies have shown that hemocytes appear to migrate to areas of infection in bivalves (2, 6). In diseased bivalves, hemocyte numbers appear to increase; the site of origin of these hemocytes has never been determined (2). This study provides evidence that the hemocytes of the hard clam proliferate directly at the inflammatory site, as opposed to a possible bone marrow-like area in the body of the clam, with subsequent migration of hemocytes to sites of infections, as seen in vertebrates.

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Conditions Affecting the Growth and Zoosporulation of the Protistan Parasite QPX in Culture

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Quahog Parasite Unknown (QPX) is a protistan disease of hard clams (*Mercenaria mercenaria*). The QPX organism has been classified in the phylum Labyrinthomorpha (1, 2). Disease resulting from QPX infection has been identified in New Brunswick and Prince Edward Island, Canada; Barnegat Bay, New Jersey; Chatham, Duxbury, and Provincetown, Massachusetts; and three locations in Virginia (1, 2). Mortality from QPX can be severe, with losses especially high in clams just under market size (about 2 years old). An important clinical sign of infection is the occurrence of QPX-infected inflammatory nodules in the mantle.

Whyte *et al.* (3) isolated QPX cells from infected clams; when placed in artificial seawater, these cells produced sporangia and zoospores. Kleinschuster and Smolowitz (2) recently described continuous *in vitro* culture of QPX. QPX was isolated from inflamed mantle nodules and cultured in modified MEM medium at pH 7.2 at 22°C. Mature cultures (after 5 to 10 days) showed thalli, immature sporangia, and mature sporangia containing endospores. Organisms in these stages ranged from 5 to 120 μm in diameter.

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Endospores released from mature sporangia became the new thalli. Cultured QPX organisms produced, and were embedded in, a thick mucoid material that could be removed intact from the remaining unused culture medium. When placed into sterile seawater, QPX produced motile zoospores within 4 days.

The effects of different environmental conditions on the occurrence of QPX and the resulting disease in the field are unknown. Determination of how environmental parameters affect cultured QPX may help in understanding the pathogenesis of the disease in the field. In this study, the environmental effects of temperature, pH, and salinity were investigated on QPX cells in culture.

Medium (pH 7.2 and salinity 40 ppt) was prepared using the standard methods (2). Modified medium (40 ppt) was prepared at pH 6.0, 7.0, and 8.0 by adjusting pH with 2 *M* HCl and 2 *M* NaOH. Modified medium (pH 7.2) was also prepared at 20, 28, and 34 ppt by proportionally reducing the salt content of the medium and monitoring the resulting solutions with a refractometer. All media were filter sterilized. To test the effects of pH and salinity on the proliferation of QPX in culture, 0.4 ml of two QPX subcultures was placed in a culture flask with 10 ml of each of the three pH variations or four (including standard) salinity variations. Fourteen cultures were created (seven of each subculture). These cultures were incubated for 10 days at 22°C. To test the effects of temperature on QPX growth in culture, the same procedure was followed