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Primary culture of hemocytes from the Caribbean spiny lobster, *Panulirus argus*, and their susceptibility to *Panulirus argus* Virus 1 (PaV1)

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Abstract

Primary cultures of hemocytes from the Caribbean spiny lobster *Panulirus argus* were developed for studies on the *in vitro* propagation of *Panulirus argus* Virus 1 (PaV1). A modified Leibovitz L-15 medium supported the best survival of hemocytes in *in vitro* primary cultures. However, degradation of the cultures occurred rapidly in the presence of granulocytes. A Percoll step gradient was used to separate hemocytes into three subpopulations enriched in hyalinocytes, semigranulocytes, and granulocytes, respectively. When cultured separately, hyalinocytes and semigranulocytes maintained higher viability (~80%) after 18 days incubation compared with granulocytes, which degraded over 2–3 days. Susceptibility of the cell types was investigated in challenge studies with PaV1. Hyalinocytes and semigranulocytes were susceptible to PaV1. Cytopathic effects (CPE) were observed as early as 12 h post-inoculation, and as the infection progressed, CPE became more apparent, with cell debris and cellular exudates present in inoculated cultures. Cell lysis was noticeable within 24 h of infection. The presence of virus within cells was further confirmed by *in situ* hybridization using a specific DNA probe. The probe gave a unique staining pattern to cells infected with PaV1 24-h post-inoculation. Cells in the control treatment were intact and negative to hybridization. This assay was further applied to the quantification of infectious virus in hemolymph using a 50% tissue culture infectious dose assay ($TCID_{50}$) based on CPE. These tools will now allow the quantification of PaV1 using established culture-based methods.
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1. Introduction

The Caribbean spiny lobster, *Panulirus argus*, is widely distributed throughout the Caribbean basin and along the Atlantic Coast ranging from Brazil to Georgia, USA. It supports one of the most valuable fisheries in the Caribbean. Recently, a pathogenic virus, *P. argus* Virus 1 (PaV1), was identified during field surveys of juvenile lobsters from the Florida Keys (Shields and Behringer, 2004). The virus infects the soft connective tissues, and two classes of hemocytes: hyalinocytes and semigranulocytes. The virus is highly pathogenic to juvenile spiny lobsters, which die within 30–80 days in experimentally induced infections (Shields and Behringer, 2004). Healthy lobsters are, however, able to detect diseased animals and avoid them (Behringer et al., 2006). Given its distribution throughout the Florida Keys and its relatively high prevalence in juvenile lobsters, PaV1 is thought to have significant potential to damage the fishery. Thus, it is critical to develop specific and sensitive diagnostic methods to better understand the pathogenesis of this viral pathogen.

Tissue culture is an important tool employed in the studies of viral pathogens of vertebrates, but it has not been fully developed for assessment of viral infection in invertebrates (Rinkevich, 1999; Toullec, 1999; Villena, 2003). At present there is no continuous culture of crustacean cell lines, however, primary culture of crustacean tissue has previously been developed for the diagnosis and *in vitro*

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proliferation of shrimp viruses (Chen and Wang, 1999; Fraser and Hall, 1999; Frerichs, 1996; Hsu et al., 1995; Nadala et al., 1993; Rinkevich, 1999; Tapay et al., 1997; Toullec et al., 1996). Because PaV1 infects hyalinocytes, semigranulocytes, and soft connective tissues (Shields and Behringer, 2004), these hemocytes and soft connective tissues represent targets for the *in vitro* study of the virus. Connective tissues have not been successfully obtained in culture. However, primary cultures of hemocytes have been obtained from the penaeid shrimp, *Penaeus japonicus* (Itami et al., 1999; Sano, 1998) and two species of crab, *Liocarcinus depurator* and *Carcinus maenas* (Walton and Smith, 1999). The present study aimed to develop a primary culture of the hemocytes from the spiny lobster, *P. argus*, and to assess the susceptibility of hemocytes to PaV1 in these cultures. The cell culture system with the virus was further developed into an *in vitro* assay for the quantification of virus in the hemolymph of infected lobsters.

2. Materials and methods

2.1. Experimental animals

Juvenile spiny lobsters, *P. argus*, were collected from the Florida Keys, and housed in clean aquaria (salinity = 35 ± 1‰, temperature = 24 ± 1 °C) equipped with biological filters (Whisper) filled with crushed coral. Lobsters were fed with squid three times per week. Water quality was monitored weekly and water changes were made to ensure that various water quality parameters remained within acceptable limits: ammonia (0–0.3 ppm), nitrite (0–0.6 ppm), pH (7.4–8.4).

2.2. Analysis of *P. argus* hemocytes

Hemolymph was drawn with a 27-ga syringe from the juncture between the basis and ischium of the fifth walking leg. Prior to bleeding, the sample area was wiped with 70% ethanol. In most cases, hemolymph was collected into a syringe containing an equal volume of anticoagulant (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA; pH 5.4; Söderhäll and Smith, 1983). Freshly collected hemocytes were examined with an Olympus BX51 microscope equipped with a U-UCD8 Universal condenser and Nomarski differential interference contrast filter. Hemocytes were categorized based on cell size, cell shape, and granularity (Söderhäll and Cerenius, 1992). Total hemocyte counts (THC) and differential hemocyte counts (DHC) were performed using a hemacytometer (Neubauer improved, Bright Line; two counts per lobster).

2.3. Optimization of culture media

Samples of collected hemolymph were centrifuged at 250*g* for 10 min at 4 °C (IEC Thermo Centra, with swinging bucket rotor), then resuspended in appropriate medium. Aliquots of 0.5 ml of the hemocyte suspension at densities of $\sim 1 \times 10^6$ ml^{-1} were seeded into 24-well culture plates containing an additional 1.0 ml of culture medium per well. Plates were incubated in a Chamber (Lab-Line®) at 22 – 24 °C. Media were refreshed on day 2 and thereafter at 2-day intervals. Cultured cells were observed with an Olympus IX50 inverted microscope equipped with a Hoffman modulation contrast condenser. Images were taken with a Nikon DXM 1200 digital camera at days 1, 3, 5, 7, 9, and 11, prior to media refreshment. Cell viability was confirmed by the Trypan Blue exclusion method (Mascotti et al., 2000). Several commercial media were assessed for hemocyte viability: Leibovitz L-15 medium (L-15), modified Leibovitz L-15 medium (ML-15: double strength components, supplemented with 0.6 g L^{-1} L-glutamine and 0.7 gL^{-1} glucose), Grace's insect medium, and RPMI-1640 medium. Each medium was adjusted to match the osmolarity of the hemolymph of the spiny lobster (1025 ± 6 mOsM kg^{-1}) by addition of NaCl. Penicillin (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}) were added to each culture medium to minimize potential bacterial contamination. In addition, some media were supplemented with 0%, 5%, 10%, or 15% charcoal–dextran-treated fetal bovine serum (FBS) for putative growth assessment. All media were sterilized by filtering through Nalgene® Disposable Filters (Pore size = 0.20 μm).

2.4. Primary culture of separated hemocytes

Hemocytes were separated by centrifugation using Percoll (Amersham Biosciences) in fine step-density gradients (1.110, 1.096, 1.091, 1.086, 1.082, 1.077, 1.072, 1.067, and 1.062 g ml^{-1}, respectively) made with lobster physiological buffer (0.4 M NaCl, 0.01 M KCl, 0.01 M Na_2HPO_4, 0.01 M KH_2PO_4, $NaHCO_3$; pH 7.8). Briefly, 1 ml of each density solution was carefully layered into the same 15-ml centrifuge tube. Aliquots of 3 ml of the hemolymph–anticoagulant suspensions were gently layered on the top of the stacked gradients, then centrifuged at 400*g* for 30 min at 4 °C. Cell fractions were carefully aspirated into anticoagulant using Pasteur pipettes, and washed twice with culture media. Cell fractions were centrifuged at 250*g* for 10 min at 4 °C to remove residual Percoll prior to resuspension in fresh culture media. Separated hemocytes were cultured in the optimized media and assessed for cell viability as above.

2.5. Virus inoculation of cell cultures

The virus inoculum was extracted from the hemolymph of a heavily diseased lobster. Briefly, diseased hemolymph was mixed with 4-fold volume of ML-15 medium and homogenized with a homogenizer (Pyrex®, Corning Inc.) at 4 °C for 10 min, the homogenized mixture was centrifuged at 3000*g* for 10 min at 4 °C, and the supernatant was filtered through 0.45-μm filter. Viral filtrates were serially diluted with ML-15 culture medium to 1:1, 1:10, 1:100 and 1:1000 and used as inoculum. Hemolymph from a healthy lobster was processed similarly and used as a control. Aliquots of

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