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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.

Keywords: Crustacea; Cytopathic effects; Cell viability; $TCID_{50}$; Cell culture; Hyalinocytes; Semigranulocytes; Granulocytes; Virus

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

100 µl of inoculum were inoculated into 1 day old established hemocyte cultures in 24-well tissue culture plates (Falcon®, Becton Dickinson Labware), the plates were incubated at 22–24 °C for 4 days, and cultures were supplemented with new media at 2-day intervals. The inoculated primary cultures were examined daily with an inverted microscope, and images were taken daily for assessment of cytopathic effects (CPE) and calculation of 50% tissue culture infectious dose ($TCID_{50}$) as in Reed and Muench (1938).

2.6. Diagnosis of in vitro infection of PaV1 by in situ hybridization (ISH)

For diagnosis of *in vitro* infections of PaV1, hemocytes were cultured and inoculated with 1:10 diluted inoculum in chamber slides (Lab-Tek®). On days 1, 2, 3 and 4 post-inoculation, hemocytes cultured in chambers were fixed in 10% neutral buffered formalin for 10 min at room temperature, and then gently rinsed briefly with phosphate buffered saline (PBS) (8.0 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4; pH 7.4). Infections were then prepared for *in situ* hybridization (ISH). Briefly, slides were permeabilized with 0.5% (v/v) Triton X-100 (in PBS) for 10 min, rinsed with PBS (5 min × 3), then digested with Proteinase K (100 µg ml^{-1} in PBS) for 15 min at 37 °C, followed by a 5-min wash in 0.2% (w/v) glycine PBS solution to stop proteolysis, and incubated in 2× SSC (17.53 g L^{-1} NaCl, 8.82 g L^{-1} citric acid; pH 7.0) for 10 min at room temperature. Slides were incubated in pre-hybridization buffer (4× SSC, 50% (v/v) formamide, 0.5 mg ml^{-1} Salmon sperm DNA, and 1% (v/v) fetal bovine serum) at 42 °C for 45 min. After incubation, excess pre-hybridization buffer was carefully drained off, the area with tissue was outlined with a Frame-seal incubation chamber (MJ Research Inc.), then aliquots of 50-µl of hybridization solution (50% de-ionised formamide; 4× SSC; 0.5% (w/v) SDS; 25 µg ml^{-1} FITC-labeled DNA probe) (probe synthesis as in Li et al. 2006) were added within the area, sealed with plastic cover slip. Each slide was then placed into a thermal cycler for 3 min at 72 °C, and cooled on ice for 2 min. Slides were incubated in a humid chamber overnight at 42 °C. The slides were then washed in 2× SSC (2× 5 min), 1× SSC (2 × 5 min), PBS (10 min) and maleic acid buffer (MAB: 100 mM maleic acid, 150 mM NaCl; pH 7.5) for 10 min. The slides were then incubated in blocking solution (1% (w/v) Blocking reagents in MAB, Roche). Anti-FITC alkaline phosphatase conjugate antibody (Sigma–Aldrich) was diluted 1:2000 in blocking solution and sections were incubated with the diluted antibody for 3 h at room temperature with gentle agitation. Unbound antibody was removed with two 5 min washes in buffer I (100 mM Tris, 150 mM NaCl, pH 7.5) and two 5 min washes in Buffer II (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5). The slides were then incubated with BCIP/NBT liquid substrate solution (Sigma-Aldrich) in dark for 2 h. The color reaction was stopped by washing in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and dH_2O for 5 min, respectively. The slides were mounted with aqueous mounting medium (90% glycerol, 0.1 m Tris–HCl, pH 8.0 and 2.3% DABCO) and glass coverslips applied. Clear fingernail polish was applied to the edges of the cover slips to prevent evaporation. Slides were examined using an Olympus BX51 microscope, and images were captured with a Nikon DXM 1200 digital camera.

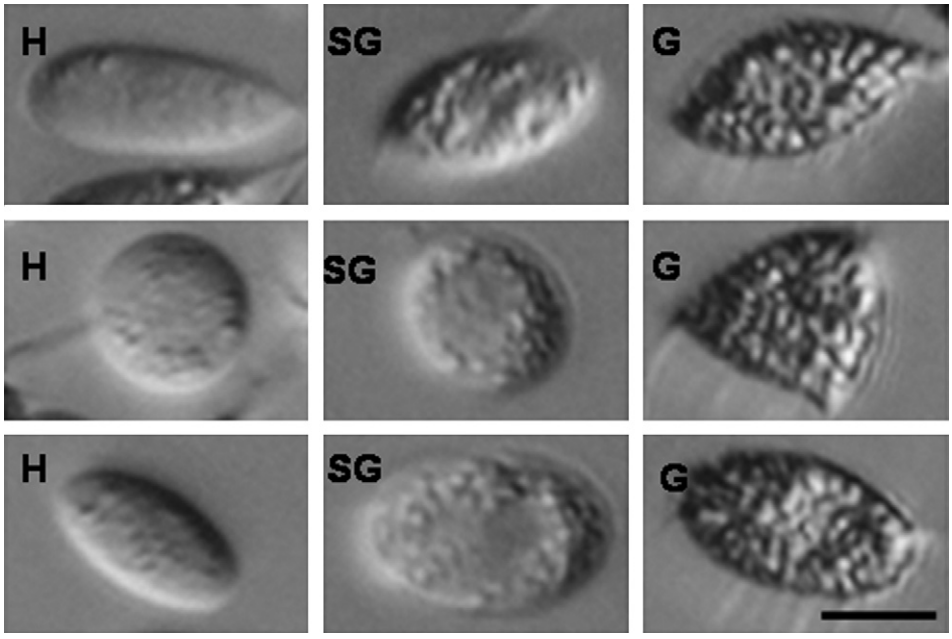

Fig. 1. Light microscopy of hemocytes from the spiny lobster, *Panulirus argus*: Hyalinocytes (H), semigranulocytes (SG), and granulocytes (G). Scale bar = 10 µm.

3. Results

3.1. Hemocytes of the spiny lobster P. argus

Three major hemocyte types were identified in the Caribbean spiny lobster: hyalinocytes, semigranulocytes, and granulocytes (Fig. 1). Hyalinocytes contained no or a few small granules, and were often found as a spindle or round shapes, ranging in size from 12 to 18 μm. Semigranulocytes contained many small and a few larger (>1 μm) granules, were morphologically variable from spindle, ovoid or round in shape, and ranged in size from 14 to 23 μm. Granulocytes contained many large (>1 μm) and a few small granules, were also variable in shape, and ranged from 17 to 28 μm.

The number of circulating hemocytes (total hemocyte count, THC) in the spiny lobster was around 8.0×10^6 cells ml^{-1}(range 6.68×10^6 – 9.58×10^6, $n = 16$). Hyalinocytes (H) represented approximately 15% ($15.2 \pm 2.0\%$, $n = 16$) of the total circulating hemocytes, semigranulocytes accounting for 58% ($57.9 \pm 3.7\%$, $n = 16$), and granulocytes, 27% ($26.9 \pm 4.2\%$, $n = 16$).

3.2. Optimization of culture media

The lobster hemocytes survived but did not proliferate in each of the four culture media tested, and no mitosis was observed in any of the hemocyte cultures. After 1 day, hemocytes cultured in ML-15, L-15, Grace's media and RPMI-1640 maintained high viability, with >90% of the cells remaining alive (Fig. 2A). However, over the next three days, the viability of cells cultured in RPMI-1640 decreased to 25%, which was significantly lower than cells in the other media (Fig. 2 A). By day 8, most cells cultured in RPMI-1640 had died, whereas mean cell viability was $79.5 \pm 4.7\%$ in ML-15 medium, $68.4 \pm 7.2\%$ in Grace's medium, $45.8 \pm 3.8\%$ in L-15 medium. By day 12, cell viability decreased to $43.0 \pm 2.2\%$ in ML-15 medium, $32.4 \pm 4.1\%$ in Grace's medium, and $26.4.0 \pm 2.9\%$ in L-15 medium (Fig. 2 A).

Fetal bovine serum, a standard supplement in many cell culture systems, was not an effective additive and hampered cell viability in this study. Hemocytes cultured in the ML-15 medium supplemented with 5%, 10%, and 15% FBS, respectively, were consistently degraded, with viabilities of only 3–4% after 11 days in culture (Fig. 2B). Hemocytes cultured in media without supplementation with FBS retained much higher viability (Fig. 2A and B).

3.3. Primary cultures of separated hemocytes

In previous mixed hemocytes cultures, within 2–7 days, most of the granulocytes dehisced, releasing their contents into the culture media, potentially impacting the survival of other the cell types (Fig. 3). In order to improve cell viabilities of cultured hemocytes, lobster hemocytes were separated into three distinct fractions using Percoll discontinuous gradients and cultured separately.

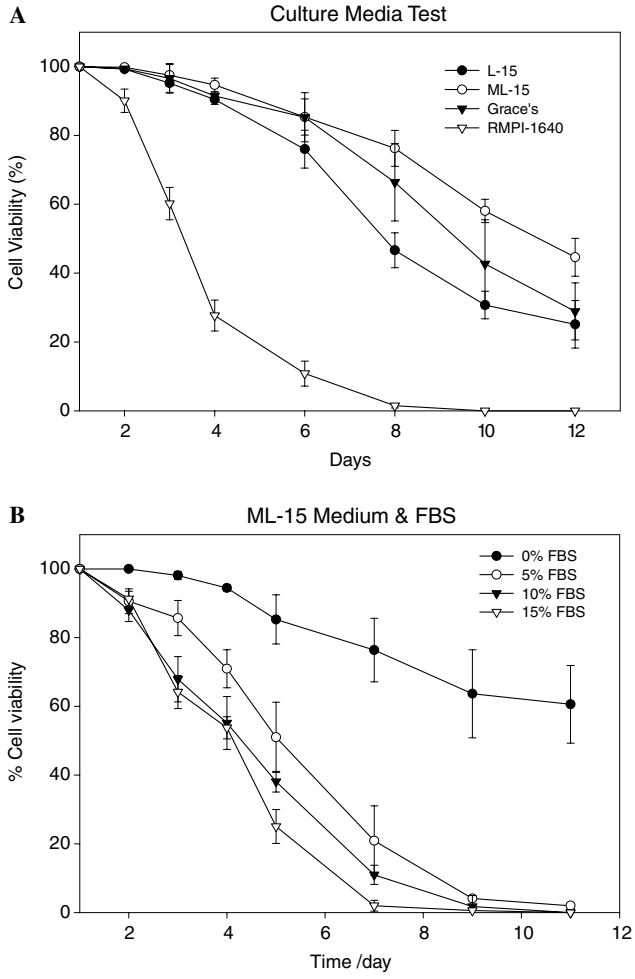

Fig. 2. (A) Viability of unseparated hemocytes of *P. argus* cultured in L-15, ML-15, Grace's Insect medium, and RPMI-1640 medium. (B) Viability of unseparated hemocytes of *P. argus* cultured in ML-15 medium supplemented with 0%, 5%, 10%, and 15% FBS. Values are means ± STD ($n = 12$).

In the column of the Percoll step gradients, the fraction enriched with hyalinocytes was located between density gradients of 1.062 and 1.067 g ml^{-1}; in this fraction (H), hyalinocytes comprised more than 80% of the cells with semigranulocytes making up the rest. The fraction enriched with semigranulocytes was located between density gradients of 1.067 and 1.072 g ml^{-1}. This semigranulocytes (SG) fraction was comprised of approximately 90% semigranulocytes, with 7% of hyalinocytes and 3% of granulocytes. The granulocytes (G) fraction accumulated in the interface between 1.082 and 1.086 g ml^{-1} gradients; over 94% of the cells in this fraction were granulocytes, with only a small proportion (<6%) of semigranulocytes and no hyalinocytes (Fig. 4).

From the culture of unseparated hemocytes, cells maintained in ML-15 medium survived better than those other media; therefore, ML-15 medium was selected as the medium for maintaining the separated cell types. Viability improved dramatically in hyalinocytes and semigranulocytes grown in the absence of granulocytes. Hyalinocytes and semigranulocytes survived up to 18 days with viability

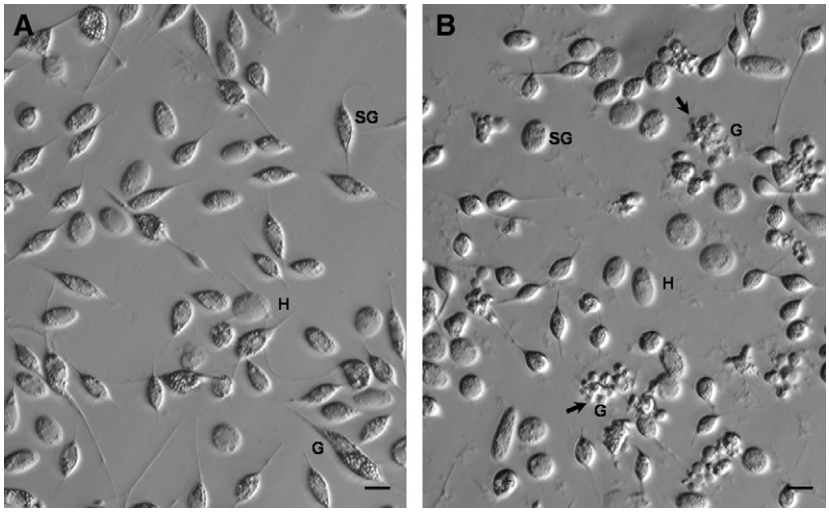

Fig. 3. Light microscopy of unseparated hemocytes of the spiny lobster *P. argus* at 1st (A) and 5th (B) day culture. Note those dehisced granulocyte (arrowheads). H: hyalinocytes; SG: semigranulocytes; G: granulocytes. Scale bars = 10 μm.

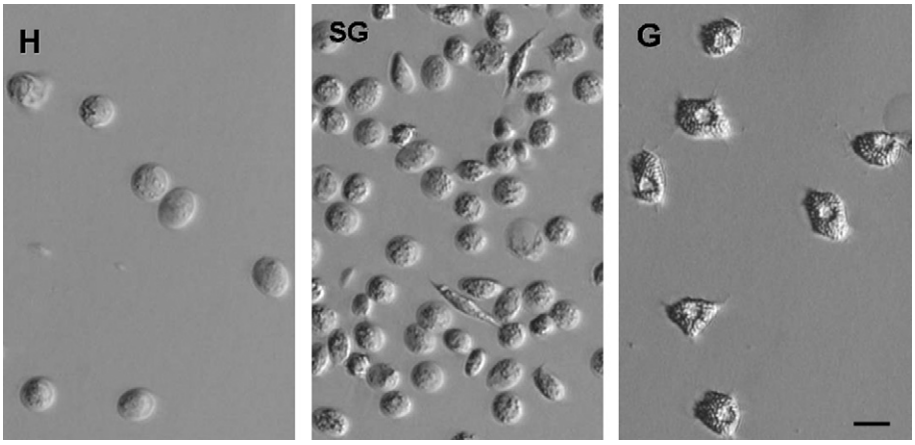

Fig. 4. Light microscopy of hemocyte fractions from Percoll discontinuous gradient separation: H: hyalinocytes, SG: semi-granulocytes, and G: granulocytes. Scale bars = 10 μm.

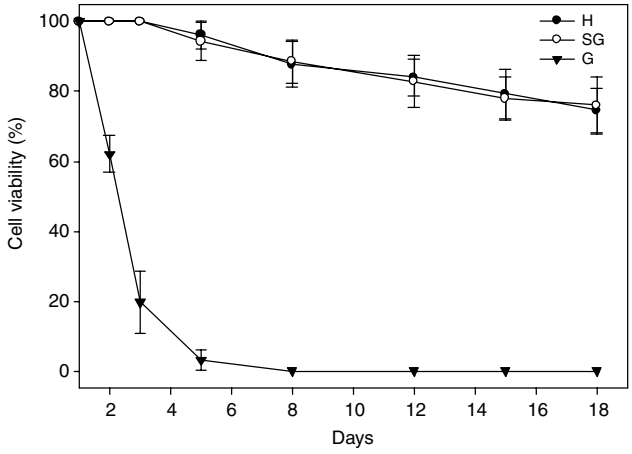

Fig. 5. Cultures of separated hemocytes grown in ML-15 media. H: hyalinocytes; SG: semigranulocytes; G: granulocytes. Values are means ± STD ($n = 5$).

of 77.6% (±6.4%) and 79.1 (±7.1%), respectively. Separated granulocytes died quickly, within 4 or 5 days (Fig. 5).

3.4. In vitro propagation of PaV1

Hemocytes from cultures inoculated with the virus showed cytopathic effects (CPE) after 12-h post-inoculation with 1:1 diluted PaVl inoculum. Initially, the affected cells changed from round or oval shapes to an irregular shape (Fig. 6A). As the infection progressed, the infected cells gradually shrank and became surrounded by exudates or cell debris (Fig. 6B). After 72 h, cell death was obvious, and inoculated cultures were comprised of few live cells and much cell debris ((Fig. 6C).

The infection of PaV1 in cultured hemocytes was confirmed by *in situ* hybridization using a specific 110-bp DNA probe of Li et al. (2006). The unambiguous dark staining of PaV1 infected cells was observed after 24 h post-inoculation. No PaV1 positive signals were detected in cell cultures

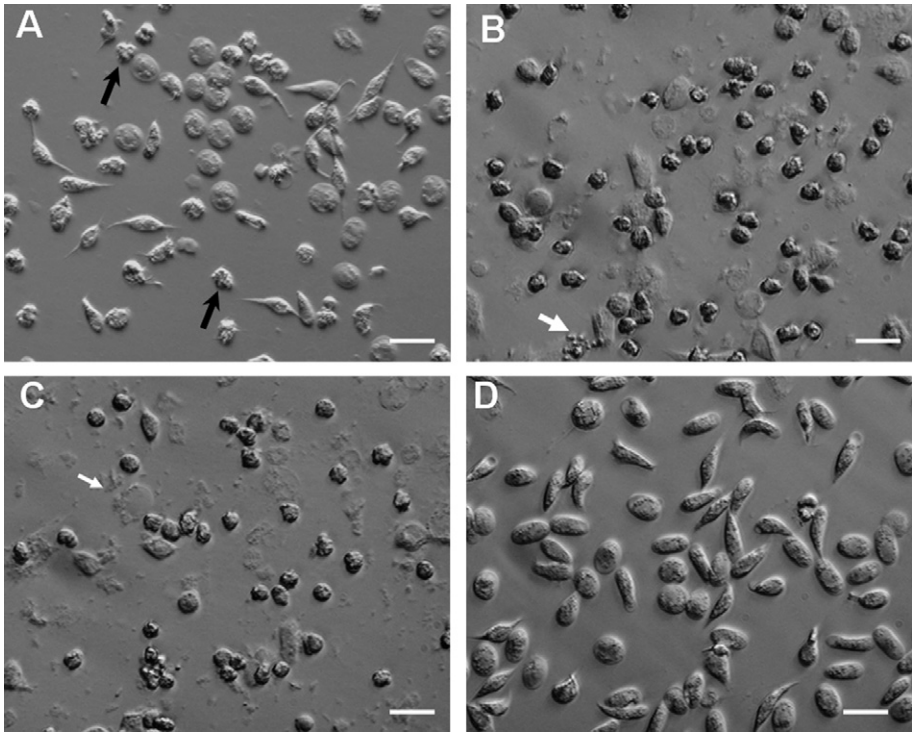

Fig. 6. Cytopathic effects of cultured hemocytes infected with PaV1 inoculum (1:10 diluted). (A) 12 h post-inoculation with PaV1 filtrates, (B) 48 h post-inoculation, (C) 72 h post-inoculation. Note the morphological change (black arrow) and degradation (white arrow) of hemocytes when infected with PaV1. (D) Control culture at 72 h post-inoculated with hemolymph filtrates from a healthy lobster. Scale bars = 20 μm.

inoculated with hemolymph filtrates from a healthy lobster (Fig. 7).

By 48 h post-inoculation, 23.45% (±10) of the cells exposed to the 1:1-diluted inoculum had survived; over 50% of cells exposed to greater dilutions of the inocula had survived, whereas more than 93% of cells in the control groups had survived. By 72 h post-inoculation, most cells that had been exposed to the virus had lysed in the 1:1 and 1:10 dilution groups; whereas most of the cells in control groups remained alive (~90%) (Fig. 8). The amount of infectious virus in the undiluted inoculum was 4×10^3 $TCID_{50}$ ml^{-1} when calculated with the end point dilution assay (Reed and Muench, 1938) based on percentage of cells surviving at the end of the assay.

4. Discussion

We have developed the primary culture of hemocytes from the Caribbean spiny lobster *P. argus* for *in vitro* studies of *P. argus* Virus 1 (PaV1). Primary cultures of

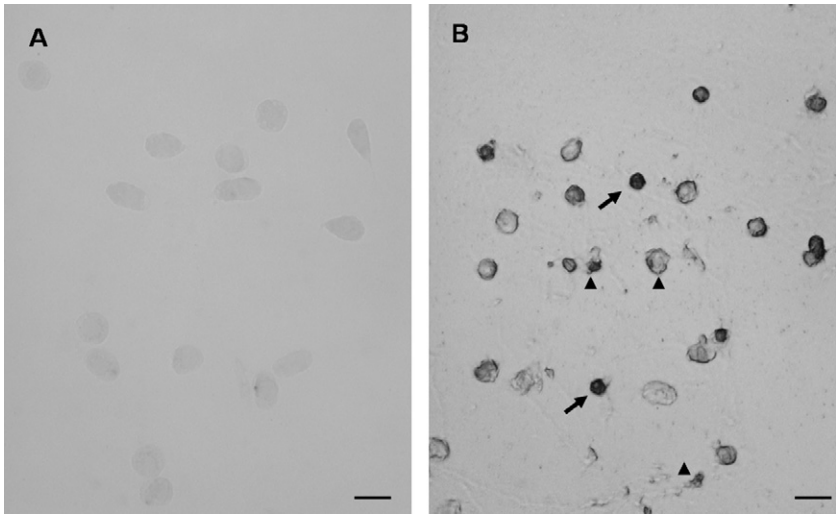

Fig. 7. Detection of PaV1 in primary cultures of separated hemocytes (hyalinocytes and semigranulocytes) of *P. argus* by *in situ* hybridization. A: 24 h post-inoculation with hemolymph fitrates from a healthy lobster, B: 24 h post-inoculation with PaV1 filtrates; note the dark staining of infected cells (black arrows), and the debris of lysed cells (arrow heads). Transmitted light microscopy. Scale bars = 20 μm.

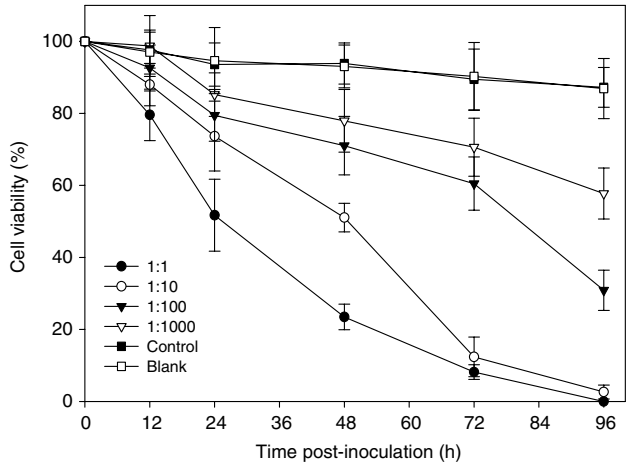

Fig. 8. Survival of cultured hemocytes of *P. argus* inoculated with serially diluted viral inoculum. Survival was defined as (numbers of survived cells / initial amount of cells) × 100% of each well. Values are means ± STD ($n = 12$).

hyalinocytes and semigranulocytes were susceptible to PaV1, showing obvious cytopathic effects (CPE) within hours of exposure to a high dose of PaV1. Even though the cultured hyalinocytes and semigranulocytes were susceptible to PaV1, we could not develop a plaque assay for quantitative study of the virus, because lobster hemocytes do not undergo mitosis and no confluent cell layer could be formed; i.e., the cells formed dispersed monolayers. However, a CPE assay using an estimate of the 50% tissue culture infectious dose ($TCID_{50}$) method provided an alternative to determine viral titer (see Darling et al., 1998). Such assays have been successfully applied to quantify the infectious titer of several other crustacean viruses including yellow head baculovirus (Assavalapsakul et al., 2003; Lu et al., 1995) and non-occluded baculo-like virus (Tapay et al., 1997). The *in vitro* quantal assay based on CPE will facilitate better understanding of infection dynamics of PaV1 in the spiny lobster. However, because hemocytes do not multiply *in vitro*, the application is limited in its ability to grow large quantities of the virus for *in vitro* studies.

We demonstrated that the PaV1 virus infected *in vitro* cultured hyalinocytes and semigranulocytes using a specific DNA probe. The rapid infection and mortality of the hemocytes is interesting given that infections in juvenile lobsters last from 30–80 d (Shields and Behringer, 2004). However, the dynamics within the host will no doubt be different due to immune defenses, stimulation of hematopoietic tissues, and other factors. In the lobster, the virus also infects the cells of the spongy connective tissues, and may cause different effects in these, however this tissue cannot as yet be successfully cultured.

Lymphoid and ovary tissues are often the targets for pathogenic viruses in shrimps; therefore, these cells are frequently used in *in vitro* studies of viral pathogenesis (Assavalapsakul et al., 2003; Chen and Wang, 1999; Lu et al., 1995; Maeda et al., 2004; Tapay et al., 1997; Wang et al., 2000). Comparatively limited efforts have been made to develop *in vitro* cultures of hemocytes from other crustacean species. Itami et al. (1999) cultured large granular hemocytes from *P. japonicus* for up to 10 days and inoculated them with penaeid rod-shaped DNA virus, however, no CPE was observed over the 10-d period of incubation. Walton and Smith (1999) separated and collected hyalinocytes from the crabs, *L. depurator* and *C. maenas*. They were able to maintain these cells for up to 14 days with more than 70% viability in an optimized L-15 medium. In our cultures, separated hyalinocytes and semigranulocytes survived up to 18 days with high viability (~80%), and maintained an even higher viability (>90%) in the earlier period of culture.

A variety of culture media have been tested for the primary culture of crustacean tissues, these include Leibovitz L-15, Grace's insect medium, RPMI-1640, Medium 199, and several specifically formulated media (Luedeman and Lightner, 1992; Nadala et al., 1993; Tong and Miao, 1996; Walton and Smith, 1999). Leibovitz L-15 and Grace's insect medium have been considered the best commercial medium for tissue culture of crustaceans (Luedeman and Lightner, 1992; Nadala et al., 1993; Walton and Smith, 1999). In our study, Grace's insect medium and double strength Leibovitz L-15 medium (2 × L-15) supported cell survival in the first 6 days of culture for unseparated lobster hemocytes; however, the Modified L-15 medium yielded better results for longer term viability (>80% by day 15). The higher concentration of glutamine and glucose in Grace's media and L-15 medium apparently benefited the survival of *in vitro* cultured hemocytes of the spiny lobster.

Fetal bovine serum (FBS) is often used as a supplement in crustacean and mollusk cell culture (Chen and Wang, 1999; Luedeman and Lightner, 1992; Sano, 1998; Walton and Smith, 1999). In this study, fetal bovine serum degraded the viability in whole hemocyte cultures even at a low concentration of 5%. Semigranulocytes and granulocytes are sensitive to foreign particles, particularly glucans, lipopolysaccharides, and bacteria, and often lyse to release prophenoloxidase and other components involved in cytotoxicity and melanization pathways (Söderhäll and Cerenius, 1992). FBS presumably caused the granulocytes to dehisce and lyse, causing further deterioration of remaining cells. Other supplements, such as cell-free plasma or filtrates from homogenized tissues, that have been used to support microbial pathogens of crustaceans (Toullec, 1999), were not tested in our study due to their potential to induce cross reactions among individual lobsters.

Traditionally, crustacean hemocytes have been classified into three types of cells: hyalinocytes, semigranulocytes, and granulocytes, according to the number and size of granules they contain (Bauchau, 1980) and their biological function (Söderhäll and Smith, 1983). Hose et al. (1990) proposed a different classification based on morphology, cytochemistry, and studies of cell function, and suggested that hyalinocytes and granulocytes represent two distinct cell lineages, with granulocytes representing a continuum of differentiation from the less mature small-granule hemocytes to the large-

granule hemocytes. We found that granulocytes were distinctly different from the other two cell types in morphology, and that they were particularly sensitivity to the *in vitro* environment, surviving only a short period when compared with the other two subpopulations. In the initial period (2–3 days) of separated hemocyte cultures, some hyalinocytes became morphologically like semigranulocytes, whereas no transformation was observed between semigranulocytes and granulocytes. Although we retain the traditional classification of hemocyte types in the Caribbean spiny lobster, more work is needed to fully appreciate the functional and biochemical differences among cell types. Additional experiments with culturing techniques will facilitate further *in vitro* study of crustacean hemocytes.

Acknowledgments

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