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Short communication

Attempts at producing a hybridised *Penaeus mondon* cell line by cellular fusion

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ABSTRACT

The lack of a standardised system for the isolation, identification and purification of prawn viruses, is a major obstacle to the control of viruses in penaeid aquaculture. To date, spontaneous and induced transformation of somatic penaeid cells has failed. Hybrid cells with the aim of supporting the growth of penaeid viruses were created using polyethylene glycol (PEG)-mediated fusion with two immortal cell lines, Epithelioma papulosum cyprinid (EPC) and *Spodoptera frugiperda* pupal ovarian cells (Sf9), fused with *Penaeus monodon* haemocytes. The immortal cell lines were biochemically blocked with actinomycin D and puromycin before fusion occurred. A total of 78 hybrid clones were created. The methods used to confirm the presence of *P. monodon* genes and proteins in the hybrid cells did not detect crustacean components, nor was any viral amplification detected by real-time PCR after hybrid cells were inoculated with two *P. monodon* parvoviruses, *Penaeus merguiensis* densovirus and infectious hypodermal and haematopoietic necrosis virus. These results suggest although the creation of the hybrid cells appeared successful, the cell lines lacked key crustacean cell components required for their use as an *in vitro* system for virus replication.

1. Introduction

The lack of a standardised system for the isolation, identification and purification of prawn viruses is a major obstacle to the control of viruses in penaeid aquaculture. In addition to the known viral pathogens, previously unknown or newly emerging viruses are posing further threats to this industry. In an attempt to try and keep up with both established and emerging penaeid viruses, the use of cell cultures is vital, not only to aid identification as a sensitive diagnostic tool, but also for the analysis of interactions between viruses and their host cells, in an effort to identify some of the mechanisms involved in the steps of viral infection [1].

Several investigators have demonstrated that prawn primary cell cultures can support in vitro growth of monodon baculovirus [2], yellowhead virus [3–7], whitespot syndrome virus (WSSV) [4,8–10] penaeid rod-shaped baculovirus (PRBV – synonym of WSSV) [11] and systemic ectodermal and mesodermal baculovirus (synonym of WSSV) [12]. However, primary cell cultures are tedious to develop, contain a heterogeneous mix of cells, have a limited life span and are prone to contamination. Immortal cell lines have several advantages over primary cell cultures as a research and diagnostic tool such as their ability to be continuously cultured in vitro, eliminating the need to return to live animals as a source of cells; the cell lines are often homogeneous with respect to genotype and phenotype and therefore exhibit stable properties; and can be mass cultured within a short period, which may be essential in the face of a serious and extensive disease outbreak [13]. Currently no verified crustacean cell line exists despite numerous attempts.

Considering spontaneous and induced transformation of somatic penaeid cells has failed to date, creation of hybrid cells capable of supporting the growth of penaeid viruses was attempted by fusing cells from an immortal cell line with prawn cells. In the development of modern biology, the creation of cell hybrids by artificially-induced cell fusion has been a vital tool for the creation of monoclonal antibody-producing hybridoma lines in which fusion is induced between an antibody producing B lymphocyte and an immortalised myeloma cell [14–17].

The aim of this study was to first create *Penaeus monodon* cell hybrids, and then assess their viability and devise methods to confirm the presence of crustacean cellular components. Cells were also tested for their ability to support two penaeid parvoviruses, using real-time PCR for assessment of viral replication.

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2. Materials and methods

2.1. Source of animals

P. monodon were collected from wild stocks off the coast of northern Queensland, Australia, or from a commercial farm located at Innisfail, Queensland, Australia. Following collection, *P. monodon* were maintained at 28 ± 3 °C at James Cook University in 1 t Reln© recirculating tanks with biological filtration.

2.2. Cell lines

Two immortal cell partners, Epithelioma papulosum cyprinid (EPC) and *Spodoptera frugiperda* pupal ovarian cells (Sf9) were investigated in cell fusion experiments. Cells were maintained in 25 cm^2 flasks containing 10 ml of Leibovitz 15 (L-15) (Gibco, Brisbane, Australia) or tissue culture medium 100 (TC-100) (Gibco, Brisbane, Australia) for the maintenance of the EPC and Sf9 cells respectively, and supplemented with 10% foetal bovine serum (FBS) (Trace Biosciences, Castle Hill, Australia). The cells were observed daily with an Olympus IMT-2 inverted phase-contrast microscope, and once a monolayer formed, cells were trypsinised, split and re-seeded.

2.3. Prawn haemocytes

A total of 300 μl of haemolymph from each *P. monodon* was extracted using a 25-gauge needle inserted into the dorsal thoracic chamber. A 50% dilution with Alsever's anticoagulant (27 mM $Na_3C_6H_5O_7$, 336 mM NaCl, 226 mM glucose, 9 mM EDTA) was used to avoid clotting. After brief centrifugation (500 × *g* for 5 min), the haemocytes were resuspended in L-15 medium with 10% FBS supplemented with antibiotics (penicillin 200 IU/ml, streptomycin 200 μg/ml, kanamycin 80 μg/ml, polymyxin B 5 μg/ml, and fungizone® 2.5 μg/ml). Haemocytes from one animal were used for each fusion.

2.4. Fusions of immortal cell lines and prawn haemocytes

EPC and Sf9 cells at 4×10^6 cells per flask were suspended in 20 ml culture medium without FBS. The cells were treated with actinomycin D (0.25 μg/ml) and puromycin (2×10^{-4} M) [17]. Culture flasks were incubated at 28 °C for 3 h and rocked gently every 30 min to mix the cell suspension. To determine the amount of viable cells, equal volumes of a cell suspension and 0.2% trypan blue solution were mixed, loaded into the counting chamber of a haemocytometer, and examined using an Olympus IMT-2 inverted phase-contrast microscope. The viable cells, which were not stained blue, were counted and converted into viable cells/ml. The three flasks of biochemically-blocked cells were combined to provide a total of 1.20×10^6 viable cells. The cells were washed twice with culture medium without FBS and pelleted in a 10 ml tube. Freshly harvested prawn haemocytes were added to the pelleted biochemically-blocked cells in a 1:1 ratio. The tube was made up to 10 ml with culture medium and then centrifuged. The supernatant was carefully removed and pre-warmed PEG-4000 (Koch-Light) (50%) was added dropwise over a 45 s with constant swirling in a 27 °C waterbath. The cells were left for 60 s and then 10 ml of culture medium was added dropwise to dilute out the PEG solution. The cells were gently inverted to mix the PEG and left undisturbed for a further 5 min. The cells were pelleted, then resuspended in 10 ml of culture medium with 20% FBS. To obtain single cell colonies, the cells suspension was seeded out over a 96-well plate at 200 μl per well. Columns 2 through to 7 were used, with columns 1 and 8 containing culture medium only to help protect the cell-containing wells from drying out.

The plate was sealed inside a plastic container and incubated at 28 °C. Cells were examined on days 1 and 6 post-fusion and 100 μl of medium exchanged. On day 14 post-fusion, cells of viable appearance were transferred to 24-well plates. Once confluent in the 24-well plates (generally from 7 d post-transfer), the cells were transferred to 25 cm^2 flasks and supported in culture medium supplemented with 10% FBS. When confluent in the flasks, cells were trypsinised and stored in liquid nitrogen, and later tested for crustacean components.

2.5. Tests for crustacean components in surviving cells

To verify that crustacean DNA was incorporated into the fused cells, two PCRs were conducted using arthropod specific 18S rRNA primers 143F/143R [18] and primers designed on the *P. monodon* haemocyanin gene (GenBank accession no. AF431737) [19]. Once a confluent monolayer of cells had formed, cells were trypsinised and pelleted using slow centrifugation (500 × *g* for 5 min). DNA was extracted from the cells using a High Pure PCR Template Preparation Kit (Roche Sciences, Brisbane, Australia).

For PCR analysis, 1 μl of the DNA template (50–150 ng) was added to 49 μl of PCR reaction mixture consisting of MBI fermentas PCR buffer (750 mM Tris–HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween20) 200 μM dNTPs, 1 μM of each primer, 1.5 mM $MgCl_2$ and 1U *Taq* polymerase (MBI Fermentas, Hanover, USA). The arthropod 18S rRNA primer sequences and methodology are described elsewhere [18]. For the haemocyanin PCR, primer sequences were H-299, 5′ -TCG-ACA-GGG-CTG-GTA-ATC-ATA-3′ and H-594, 5′-CCG-GCA-AAT-GTT-AGT-TCT-3′ with an expected amplicon of 295 bp. The optimised PCR profile consisted of an initial 3 min denaturing at 95 °C followed by 40 cycles of 95 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min and a final 5 min extension at 72 °C.

To analyse the PCR products, 10 μl of the PCR reaction mixture was used in electrophoresis on 1.5% agarose gels containing 1 mg/ml ethidium bromide and visualised under ultraviolet trans-illumination. Amplicons selected for DNA sequencing were purified from the agarose gel using QIAquick Gel Extraction Kit (Qiagen, Melbourne, Australia) and sent to Macrogen Corporation (Seoul, South Korea). Analysed sequences were then compared to available databases using the Basic Local Alignment Search Tool (BLAST) to determine approximate phylogenetic affiliations.

A monoclonal antibody (MAb) developed by Ainsworth [20], was used to assess the presence of crustacean proteins in hybrid cell populations. After assessing contamination and responsiveness of the antibodies, antibody 1.9B2-5 was selected for immunostaining of the hybrid cell lines which was categorised as a type I antibody with general haemocytic specificity, staining approximately 78% of *P. monodon* haemocytes [20].

Polyclonal antibodies (PAb) were also used to assess crustacean proteins in hybrid cell populations. In contrast to monoclonal antibodies, which are derived from a single cell line, a PAb contains a mixture of immunoglobulin molecules secreted against many antigens. The polyclonal antibodies used in the present study were initially developed in chickens against gill-associated virus (GAV) of *P. monodon* [21]. This particular PAb was found to lack reactivity against GAV and was instead concluded to have a presumptive prawn cell origin which therefore may contain an array of non-specific proteins that react with *P. monodon* cells, making it an ideal candidate to use against the developed hybrid cells.

For immunohistochemisty (IHC) cells were spun onto positive charged microscope slides and fixed in acetone for 20 min. Endogenous peroxidase activity was blocked by adding 0.3%