OVERSEAS DEVELOPMENT ADMINISTRATION

NRED AQUACULTURE RESEARCH PROGRAMME

Cell Culture Systems for the Isolation of Crustacean Viruses

ODA RESEARCH PROJECT R5525

FINAL REPORT

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by

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OBJECTIVES OF THE PROJECT

1 Define the biological and environmental factors mediating the in vitro growth of cells derived from shrimp/prawns.

2 Establish, if possible, laboratory cell line cultures from economically important tropical shrimp/prawns.

3 Assess the value of crustacean cell line cultures for the isolation and growth of shrimp/prawn viruses.

Modified April 1994 to:

Investigate the use of fish and insect cell line cultures for the recovery of crustacean viruses.

WORK CARRIED OUT IN THIS PERIOD

The crustacean cell culture and virus isolation studies carried out in the presently reported project have extended the work initiated under the 1993/94 NRED Development of Aquatic Crustacean Cell Cultures Project R4785.

As it had not previously proved possible to establish a continuous cell line culture from crustacean tissues, investigations were continued on defining the electrolyte and nutritional factors mediating the survival and growth of dissociated cells in culture. Material derived from larval tissue of Macrobrachium rosenbergii was used preferentially as this freshwater prawn species is readily available through the Institute of Aquaculture, eggs can be harvested without sacrificing the donor animal and relatively homogenous cell suspensions can be obtained by mechanical disruption of embryonic material. With the generous collaboration of Dr Richard Smullen, Institute of Aquaculture Prawn Unit, a batch of eggs from the marine shrimp Penaeus indicus was also obtained from southern Africa from similar study.

Six commercially available media for the culture of mammalian cells and four formulations used for the culture of insect cells, selected on the basis of specific differences in available carbon sources, trace elements and vitamins, were subjectively assessed for ability to promote attachment of cells to the culture vessel surface, maintain cell viability and support cell growth and multiplication. The effect of salt concentration, the level of serum supplementation and the use of heat-inactivated serum on culture development was also evaluated (Table 1).

The possibility of inducing cell attachment and multiplication by serial passaging of non-proliferating cultures was investigated.

With the co-operation of Dr Matthew Briggs, Institute of Aquaculture Prawn Unit, clinically normal juvenile Penaeus monodon originating from south east Asia and shown histologically to be lightly infected with monodon baculovirus (MBV), hepatopancreatic parvo-like virus (HPV), Type C baculovirus (BMN) and lymphoid organ parvo-like virus (LOV) infections, were examined for viral infections by cell culture methodology. Homogenised tissue extracts

of pooled internal organs from groups of prawns were either purified by ultracentrifugation, treated with Arklone-P to remove excess non-viral protein, treated with 10% w/v polyethylene glycol 6000 to precipitate virus particles or left untreated. Extracts were inoculated into 10 selected fish cell lines and 1 insect (mosquito larva) cell line and examined by standard procedures.

Table 1 Media and supplements used for crustacean cell culture

Medium	Serum	Salt
Mammalian		
Eagle's MEM Alpha MEM Leibovitz L-15 McCoy's 5A Medium 199 RPMI 1640	Foetal bovine serum (FBS) 5%, 10% or 20% Untreated or heat-inactivated (56^0/30 min)	Diluted or salt (NaCl) supplemented to provide osmolalities 210, 290, 450 or 700 mOsmol/litre
Insect		
Grace's TC - 100 Mitsuhashi & Maramorosch IPL - 41		

RESULTS OBTAINED BY THE PROJECT

A standardized method was developed to reliably provide good yields of homogenous, well-dissociated, uncontaminated, single cell suspensions of viable cells from Macrobrachium and Penaeus eggs. The main problem of bacterial and fungal contamination was resolved by incorporating a mixture of buffered iodophor disinfectant (1:10) and malachite green (0.001%) at greater than normally recommended concentrations at all but the final stages of tissue processing.

Based on the accumulated subjective observations from a large number of complementary studies, all the basic synthetic media examined appeared compatible with maintaining a fair degree of viability of dissociated crustacean ce..s. Amongst the mammalian cell culture formulations, Medium 199 and McCoy's 5A appeared more favourable for cell survival then Eagle's MEM, Alpha MEM, L-15 or RPMI - 1640, but scrutiny of the media compositions could not pin-point any factor to account for this effect. All four insect culture media appeared very similar and evinced a greater degree of primary cell adherence to the culture flask than the mammalian media, although cell vitality seemed superior in the two favoured mammalian formulations. This feature of enhanced adhesion in insect media was lost on subsequent passage of material and for all media the first transfer of primary cultures resulted in severe degeneration and reduction of viable cells together with virtually complete loss of surface attachment.

The addition of salt (NaCl) to the culture media to raise osmolality form the standard mammalian requirement of 290 to 450 m Osmol/litre appeared clearly beneficial in

maintaining integrity of Macrobrachium cells, even though the osmolality of the haemolymph of this freshwater prawn would suggest a decrease rather than increase in salt content to be appropriate.

Repeated observations on cell culture performance with varying concentrations and types of foetal bovine serum supplementation indicated that untreated serum normally used for tissue culture may be toxic to crustacean cells. Heat-treated serum, on the other hand, appeared positively beneficial when included at the 20% level.

Attempts to induce cell growth and multiplication by continued transfer of material from one flask to another proved unsuccessful in respect of cell proliferation, although the cell populations remaining after the initial 1-2 passages withstood a further 5-8 passages with only a gradual loss of viable cells at each transfer. Cells maintained in Medium 199 survived longer then in the other media.

Studies on the culture of Penaeus tissues have been much less extensive than with Macrobrachium cells due mainly to limited availability of material. Attempted culture of cells of P.indicus, however, gave very similar results to the freshwater prawn and suggested that the factors mitigating against the establishment of cell lines are probably common to both categories of crustacean.

Homogenised internal organ tissue extracts from groups of clinically normal Penaeus monodon prawns histologically determined to be carriers of mixed hapatopancreatic parvo-like virus (HPV), monodon-type baculovirus (MBV), Type C baculovirus (BMN) and lymphoid organ parvo-like virus (LOV) infections were examined in fish cell cultures. Concentrated and untreated extracts proved highly toxic for FHM (minnow), EPC (carp), MUL (mullet), SSN-3 (snakehead) and FAG (gourami) cells. Diluted extracts and those chemically treated to partially purify any virus component were markedly less toxic, but no transmissible agents were recovered on passage of material through these five cell culture systems.

A further sample of 60 juvenile Peneus monodon lightly infected with monodon baculovirus (MBV) was examined for viral infection using heterologous cell cultures. A second batch of 13 shrimps from the same population was similarly examined six weeks later after stress induction by allowing a build-up of ammonia in the rearing tank. No cytopathic viral agents were recovered following inoculation, incubation and passage of both purified and untreated tissue extracts in AAL(mosquito larva), SSN-1 (snakehead), GCP (grass carp), ONP (tilapia) RSN-2 (red snakehead), PSP-1 (puntius), EPC and FHM cells.

IMPLICATIONS OF RESULTS FOR ACHIEVING OBJECTIVES

The successful establishment of crustacean cell lines for virological studies has been sought and eluded scientists for a very long time. The present study has extended over almost three years and has disappointingly yielded no greater success than reported by other workers.

Although the preparation of primary cell cultures directly from crustacean tissue has proved reasonably straightforward, and maintenance of viable cells over an extended period of time has been achieved, the transformation of cells derived from embryonic prawn/shrimp material to an established in vitro cell line culture has not materialised. Dissociated prawn cells from

normal donor tissue are probably anchorage - dependent (as with normal cells from vertebrate tissues), and the failure of the crustacean cells to adhere to the culture flask surface may be the most important single factor in this lack of success.

It should be noted, however, that even if the methodology for establishing crustacean cell lines can be developed, the rate of growth of the cultured cells may prove impractically slow for routine virological requirements. Furthermore, as almost all potential donor stocks of prawns/shrimps would appear to be carriers of a range of crustacean viruses, cell lines derived from these animals may be of limited value for virus studies. Either the cells will be resistant to infection with viruses of interest or they may be persistently infected from the donor animal.

The value of heterologous insect or fish cell lines for the isolation of crustacean viruses remains equivocal. Other workers have reportedly isolated a rhabdovirus and a parvo-virus from penaeid shrimps but this was not repeated in the present study. The examination of different Penaus species carrying a different range of viruses may be the simple reason for this discrepancy. The failure to cultivate any baculoviruses in non-crustacean cultures, however, would suggest that the use of heterologous cell lines may be limited to a few crustacean viruses of lesser economic significance.

PRIORITY TASKS FOR FOLLOW-UP

It is recommended that further studies be undertaken into the phenomeneon of non-attachment of crustacean cells to the solid surface substrate of culture vessels. The presently reported strategic research project was designed to investigate the possible application of conventional methods for mammalian and insect cell culture to the establishment of crustacean cells. Elucidation of the reasons for the failure of attachment of dissociated cells was outside the remit of the project and would require a more basic research study into the composition and properties of crustacean cell membranes by cell biologists/biochemists.

It is recommended that the possibility of isolating crustacean baculoviruses in insect cell line cultures be further examined. Baculoviruses are recognised parasites of invertebrate species and have been isolated in mosquito and moth larvae cell lines from infected hosts. At least 6 baculoviruses are recognised pathogens of commercially important shrimp/prawn species, but none have so far been isolated in tissue culture. In the presently reported project, one mosquito larva cell line failed to detect a persistent infection of two baculoviruses in clinically healthy penaeid shrimps, but the use of a wider range of insect cells and baculoviruses in the context of disease situations has not been evaluated.

As wild penaeid shrimps would appear to be endemically and persistently infected with a variable mix of potentially pathogenic viruses, the establishment of cultured virus-free stocks could provide invaluable sources of material for pathogenicity and immunology studies, development of diagnostic procedures and tissue culture and recombinant DNA technology utilizing baculovirus vector systems.

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