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Primary Cell Cultures from Tissues of Penaeid Shrimps and Their Susceptibilities to Monodon-type Baculovirus (MBV)

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Monolayer cultures were established from ovary, heart, lymphoid tissue and peripheral hemocytes of penaeid shrimps including *Penaeus monodon, P. japonicus* and *P. penicillatus.* The most favorable conditions for the culture of penaeid shrimp tissue cells *in vitro* was in CMRL and L-15 tissue culture media when used within a range of osmolarity 620-760 mmol/kg. The optimal maintenance temperature was 20℃ for tissues of *P. japonicus* and 28 ℃ for tissues of *P. monodon* and *P. penicillatus.* Among the four tissues tested, lymphoid tissue, or "Oka organ", was superior to the other tissues for the formation of confluent cell sheet. Cell cultures from lymphoid tissue and ovary have been subcultured up to three times. When peripheral hemocytes and heart were cultured, a maximum survival of 4 days was obtained. In contrast, cell cultures derived from ovary and lymphoid tissue were maintained alive for at least 20 days in appropriate culture systems. Neither confluent cell sheet nor adherence of cells was obtained in cultivation of hepatopancreas using the present culture systems. The results obtained from the present study also revealed that ovary extract, muscle extract and lobster hemolymph enhanced the survival of the cultured cells of penaeid shrimp *in vitro.* Cell cultures of lymphoid tissue from *P. monodon* and *P. penicillatus* were demostrated to be susceptible to infection by *P. monodon* baculovirus (MBV) at an incubation temperature of 28 ± 1 ℃. Significant cytopathic effect (CPE) was observed in a few areas of a confluent sheet of LT cells 2-3 days after incubation. With an electon microscope, virons were observed in the infected cells betweeen the inner and outer lamella of the nuclear membrane. Approximately 2 to 4 cells out of every 50 cultured lymphoid cells of *P. monodon* and *P. penicillatus* were found to be infected by MBV.

INTRODUCTION

With the rapid expansion of high density culturing of penaeid shrimp, the recognition of prawn infectious diseases, especially of viral etiology, has become especially important. Several viruses including *Baculovirus penaei* (BP) (Couch, 1974), *P. monodon* baculovirus (MBV) (Lightner and Redman, 1981; Lightner 1983; Lightner

et al., 1983a), baculoviral midgut gland necrosis virus (BMNV) (Sano *et al*., 1981) and Infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Lightner *et al*., 1983b) are known to be causative agents for a mass mortality of cultured penaeid prawns. Although several related papers have been published about viral diseases of cultured prawns, almost all of the studies were limited to histopathology observations using light and electron microscopy.

In order to produce a large amount of prawn viruses for detailed study which may lead to a prevention of infections, a cell culture system for multiplication of prawn virus *in vitro* is urgently needed.

Previously, we have obtained a monolayer cell culture from tissues of *P. monodon* and *P. penicillatus* using a culture system consisting of Leibovitz's L-15 plus foetal calf serum, prawn muscle extract, and lobster hemolymph (Chen *et al*., 1988). The primary cell culture derived from lymphoid tissue, "Oka organ", of *P. monodon* was also demostrated to be susceptible to MBV (Chen and Kou, 1988; Oka, 1969).

In this paper we describe the results obtained in a series of experiments in which the *in vitro* culture of fragments of lymphoid tissue, ovary, heart and hapotopanereas and peripheral hemocytes from *P. monodon, P. penicillatus* and *P. japonicus* was evaluated in a variety of culture systems. Monolayer cell sheets derived from lymphoid tissues of these species were also evaluated for their susceptibility to MBV.

MATERIALS AND METHODS

Adult penaeid shrimp including *Penaeus monodon P. penicillatus* and *P. japonicus*, weighing approximately 30-100 g were used for the present study. Prior to the experiment, all shrimps were sterilized as described previously (Chen *et al*., 1986) using 5% sodium hypochlorite. Subsequently, tissues including lymphoid tissue, "Oka organ", located at the anterio-ventral surface of hepatopancreas, heart and hepatopancreas were removed and rinsed in double strength (2X) Leibovitz L-15 medium for 3-4 times. Ovaries at developing stage as categorized by Motoh (1981) were used for the present study. The tissues were then immersed in a solution consisting of 3,000 IU/ml penicillin and 3,000 μ g/m*l* streptomycin for 5-10 minutes, and minced into 1-2 mm cubes for each tissue. Subsequently, fragments derived from 0.05 mg- 0.1 mg of "Oka organ" or 1-2 g of ovary, heart or hepatopancreas were placed into each 25 cm^2 Falcon plastic flask containing media as described in Tables 1 and 2, respectively. Each culture experiment was performed at least three times.

For *in vitro* cultivation of hemocytes, hemolymph obtained from inferior abdominal artery of shrimp was used. Immediately after harvesting of hemolymph using a syringe with No. 23 needle, 0.1, 0.2, 0.3, 0.5, 0.6 and 0.9 m*l* of hemolymph was placed into 25 cm^2 Falcon plastic flasks containing cultured media as described in Table 3.

Lobster hemolymph and muscle extract of shrimp were prepared as described previously (Chen *et al*., 1986; 1988; and Chen and Kou 1988). Artemia extract was obtained by homogenizing 1 g of cysts of brine shrimp (*Artemia salina*) in normal saline. To prepare ovary extract, 5 g of ovary from adult *P. monodon* were homogenized in 50 m*l* normal saline. The extracts were centrifuged at 10,000 *g* for 30 minutes at 4℃. The supernatants were then filtered through 0.45 μ m Millipore membrane. The protein concentration for each extract was measured by using Bio-Rad protein assay kit. The pH values were adjusted to range of 6.8 to 7.2 for all the culture media.

All the cultures were observed under Olympus IM inverted microscope. Cells displaying shrinkage, deformation, and dislogement from flask surface were considered as nonviable.

Virus susceptibility for shrimp cells *in vitro* was tested by inoculating an extract of MBV infected hepatopancreas (HP) into cultured cells of lymphoid tissue from three species. Into each 2-day old confluent cell sheet, 0.1 m*l* of the extract (prepared from arrroximately 1 of HP in 4 m*l* of 2X L-15) was added. Inoculated cell cultures were then incubated at 28±1℃ for *P. monodon* or *P. penicillatus* and at 20 ±1℃ for *P. japonicus*, respectively. Monolayer cell sheets in culture medium alone were used as negative control. To ensure that the conditions were optimum for the experimental and control cells, a culture media was used that consisted of 2X L-15 plus 1 muscle extract of *P. monodon*, 10% foetal calf serum (GIBCO) and 10% ovary extract of *P. monodon*, 100 IU/m*l* penicillin and 100 μ g/m*l* streptomycin at an osmolarity of 760±10 mmol/kg. The confirmation of MBV infection in *P. monodon* used as source of the virus was determined by histopathological and electron microscopical techniques as described by Lightner *et al.* (1983a). All the cell cultures were observed for the presence of cytopathic effect (CPE) using light microscope. To confirm the infection in the cultured cells, 0.1 m*l* of medium from flasks with CPE was then diluted 1:100 in fresh 2X L-15 and then inoculated into a new confluent cell monolayer. The cells with CPE after the 4th subculture level were prepared for electron microscopic observations as described previously (Ueno *et al*., 1983). Under HITACHI 6000 transmission electron microscope, approximately 50 cells were observed for the presence of infection by MBV. To observe occuluded viruses, the cells with CPE were also fixed with Davidson's fixative and stained with Harris hematoxylin and eosin as described by Couch (1974). The infectivity of MBV to lymphoid tissue cells *in vitro* was also calculated.

RESULTS

Primary cell culture from lymphoid tissue

Fragments of lymphoid tissue from penaeid shrimps including *P. monodon*, *P.*

penicillatus and *P. japonicus* were cultured in a variety of culture media. Efficacy of each medium was evaluated by the ability of the cells to form a confluent cell sheet and by cell survival (Table 1 & 2). When lymphoid tissue fragments of penaeid shrimp were incubated at the appropriate temperature (28±1℃ for *P. monodon* and *P. penicillatus* and 20±1℃ for *P. japonicus*) and with an osmolarity range of 520-820 mmol/kg, a monolayer cell sheet was formed in the culture systems tested. In contrast, no cell sheet was formed in a culture medium with an osmolarity of 320 mmol/kg. Confluent cell sheets from penaeid shrimp have been subcultured for up to three times. After three passages, the cells became degenerate and dislodged from the flask surface. Primary cell cultures of lymphoid tissue consisted of epithelioid and spindle-shaped cells (Figs. 1-3). Approximately 7 days after incubation, the cell sheets became dense and obscured individual cell morphology (Fig. 4). After subcultivation, epithelioid cells became predominant and very few spindle-shaped cells were observed (Figs. 5&6).

Among tissue culture media tested, CMRL and L-15 were found to be superior to Tissue Culture Medium 199, Minimal Essential Medium (MEM) and Schneider's Dorsophila Medium in the *in vitro* cultivation of lymphoid cells. Table 2 also shows that lymphoid tissue cells remained alive for 7-11 days in 2X L-15 plus 20% FCS. However, when ovary and muscle extracts and lobster hemolymph were supplemented, the survival of lymphoid tissue cells was prolonged to more than 20 days.

Table 1. Composition of media used for cell cultures from penaeid shrimp in present study

Media	Abbreviations
Leibovitz's L-15 medium (GIBCO)	L-15
Tissue culture medium 199 (GIBCO)	199
CMRL tissue culture medium (GIBCO)	CMRL
Minimal essential medium (GIBCO)	MEM
Foetal calf serum (GIBCO)	FCS
Essential amino acids (GIBCO)	EAA
Non-essential amino acids (GIBCO)	NEAA
Hemolymph from spiny lobster, *Panulirus homarus*	LHL
Ovary extract of *P. monodon* *	OE
Extract from *Artemia salina* *	AE
Muscle extract of *P. monodon* *	ME

* protein concentration: 0.5 mg/m*l*

Table 2. Media for the *in vitro* cultivation of lymphoid tissue cells of penaeid shrimps, *Penaeus japonicus, P. monodon* and *P. penicillatus* and formation of confluent cell sheet or maximal survival of tissue cells in each medium

Culture No.	Medium	Serum (%FCS)	Osmolarity (mmol/kg)	Supplements	Formation of confluent cell sheet*	Maximal days of survival**
1	2X199	20	720		++	5 – 8
2	CNRL	20	720		++	7 – 9
3	2XMEN	20	720		+	4 – 5
4	Scheider's Drosophila	20	720		++	5 – 9
5	2XL15	20	720		++	7 – 11
6	2XL15	10	720		++	6 – 10
7	2XL15	5	720		++	5 – 7
8	2XL15	20	720		++	7 – 10
9	2XL15	20	720		++	3 – 5
10	2XL15	10	720		++	2 – 4
11	2XL15	10	720		±	0.5 – 1
12	2XL15	10	720	EAA	++	6 – 10
13	2XL15	10	720	NEAA	++	6 – 11
14	2XL15	10	720	NEAA+EAA	++	7 – 10
15	2XL15	10	720	20%LHL	++	12 – 14
16	2XL15	10	720	10%LHL	++	11 – 14
17	2XL15	10	720	5 %LHL	++	10 – 14
18	2XL15	10	720	10%AE	+	2 – 4
19	2XL15	10	720	10%AE	++	5 – 8
20	2XL15	10	720	10%OE	++	10 – 12
21	2XL15	10	720	5 %OE	+++	>20
22	2XL15	10	720	5 %OE +10%ME + 5 %LHL	+++	>20
23	2XL15	10	720	10%ME	+++	10 – 13

Each observation was summarized from cultivation *in vitro* of *P. japonicus, P. monodon* and *P. penicillatus*.

Tests for each species were performed at least 3 times.

Tissues from *P. monodon* and *P. penicillatus* were incubated at 28 ± 1℃ and *P. japonicus* at 20 ± 1℃.

2X : Double strengh of tissue culture medium.

± : few tissue fragments adhere on the flask surface

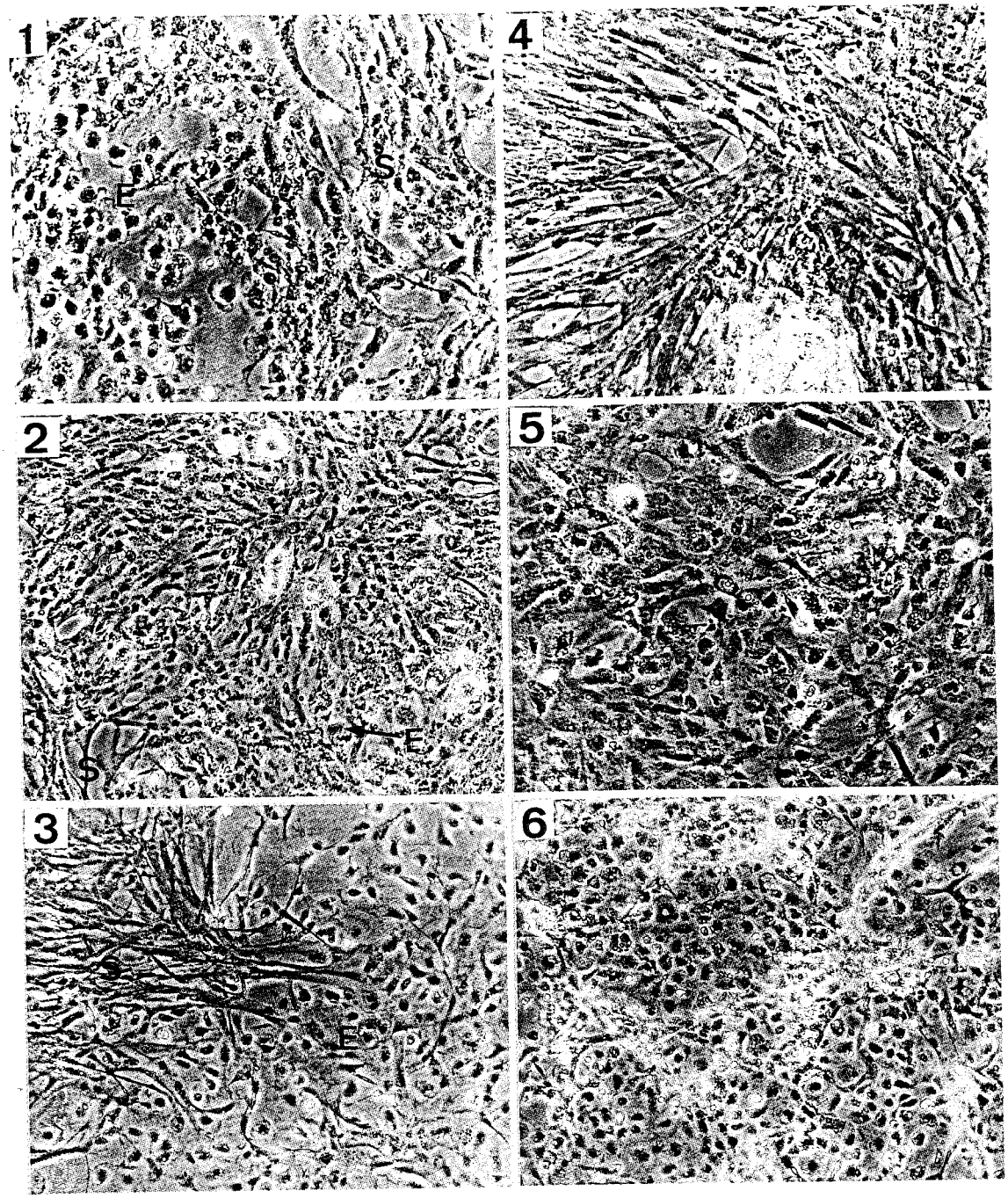
\+ : Approximately 30-50% of tissue fragments adhere on the flask surface

++ : Approximately 50-60% of tissue fragments adhere on the flask surface

+++ : Majority of tissue fragments adhere on the flask surface

* : Into each flask approximately 0.1-0.07 mg of cell fragments were inoculated.

** : Shrinkage or dislogement of cultured cells were recognized as dead cells.



Figs. 1-6. Confluent cell culture of lymphoid tissue (LT) of penaeid shrimps.

Figs. 1-3. Primary cell culture from LT of *Penaeus monodon* (Fig. 1, 50X) *P. penicillatus* (Fig.2, 400X) and *P. japonicus* (Fig.3, 400X) showing epitholioid (E) and spindle-shaped (S) morphology.

Fig. 4. 96 h after incubation of LT fragment in culture system. Note the cell sheet is too dense to recognize the cell morphology. (250X)

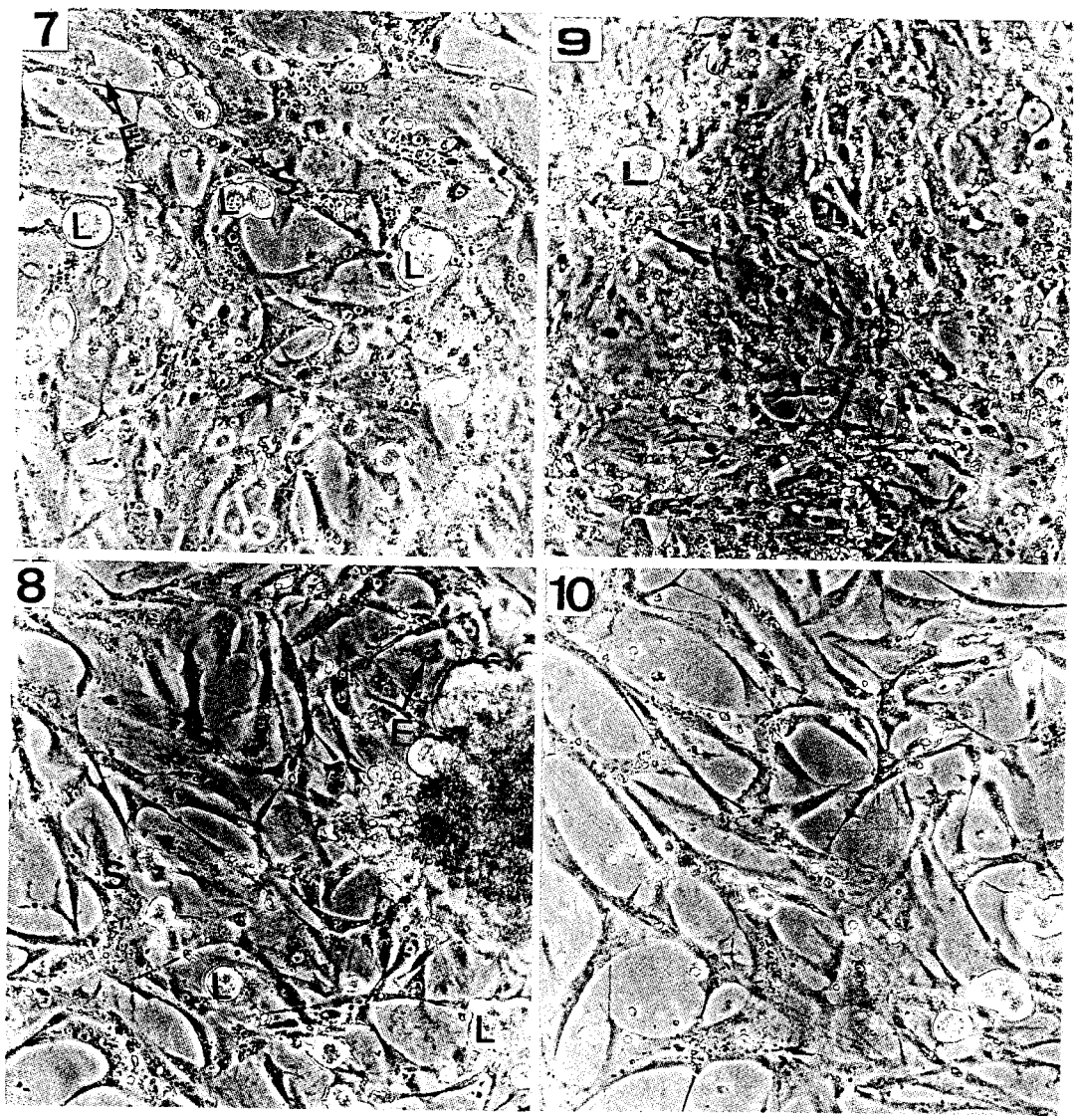
Figs. 5-6. Monolayer cultures of LT cell from *Penaeus monodon* (Fig. 5, 750X) and *P. japonicus* (Fig. 6, 400X) at passage level 2. Note the epithelioid cells become predominant.

[illegible] muscle extract and ovary extract also prolonged the survival of [illegible] ovary cells to more than 15 days (Table 3). A confluent cell sheet was [illegible] approximately three days after incubation. The majority of ovary cells [illegible] a spindle-type morphology (Figs. 7-9). A few ovoid-shaped lipid containing [illegible] epithelioid cells were also observed in the cell sheet from ovary (Figs. 7-9). [illegible] confluent ovary cell cultures from three species of penaeid shrimp hav been [illegible] for three times (Fig. 10). After three subcultures, cell degeneration and [illegible] from flask surface were observed.

Table 3. Media for the cultivation *in vitro* of heart, ovary fragments and hemocytes of penaeid shrimps, *Penaeus japonious, P. monodon, P. penicillatus* and formation of confluent cell sheet or maximal survival of cells in each medium

[illegible] No.	Medium	Osmolarity (mmol/kg)	Ovary		Heart		Hemocyte	
			A	B	A	B	A	B
1	[illegible] −[illegible]FCS	720	++	6 − 10	++	2 − 3	++	2 − 3
2	[illegible] −[illegible]FCS	720	++	6 − 10	++	2 − 3	++	2 − 4
3	[illegible] −[illegible]FCS	520	++	3 − 5	++	1 − 2	++	1 − 3
4	[illegible] −[illegible]FCS −[illegible]	720	+++	>15	++	3 − 4	++	2 − 3
5	[illegible] −[illegible]FCS −[illegible] −[illegible]	720	++	>16	++	2 − 3	++	2 − 4

A: Formation of confluent cell sheet
B: Maximal days of survival
Tests for each media were performed at least 3 times.
Ovary cells from *P. monodon* and *P. penicillatus* were incubated at 28±1℃ and those from *P. japonicus* at 20±1℃.
FCS: Double strength of tissue culture medium.
++: Approximately 30-50% of tissue fragments or cells adhered on the flask surface
+++: Majority of tissue fragments adhered on the flask surface
Stage of degeneration of cultured cells were recongnized as dead cells.



Figs. 7-9. Confluet cell cultures from ovary of *P. monodon* (Fig. 7, 750X), *P. japonicus* (Fig. 8, 750X), and *P. penicillatus* (Fig. 9, 400X). Note that most of cells reveal spindle-shaped (S) morphology and few epithelioid (E) and ovaid-shaped lipid cells (L) were observed.

Fig. 10. Monolayer cell culture of ovary cell at passage level 2 (750X). Note almost all of the cells reveal spindle-shaped morphology.

When heart fragments were cultured in five media as described in Table 3, confluent cell sheets were formed in 1-2 days. Similar results was obtained in cultivation *in vitro* of peripheral hemocytes of three species of shrimp. Maximal survival of approximately 4 days was obtained in the maintenance *in vitro* of hemocyte and heart fragments. Neither ovary extract nor muscle extract of shrimp enhanced significantly the survival of cell cultures derived from heart and peripheral hemocytes. Culture cells derived from cardiac tissue fragments of penaeid shrimps showed epithelioid morphology (Figs. 11&12). However, primary cell cultures from peripheral hemocytes revealed an irregular morphology (Fig. 13). Colonies of hyalin (Fig. 13a) and granulated cells (Fig. 13b) were observed in the cell sheets.

No confluent cell sheet was obtained when fragments of hepatopancreas were cultured in culture systems described in Table 3.

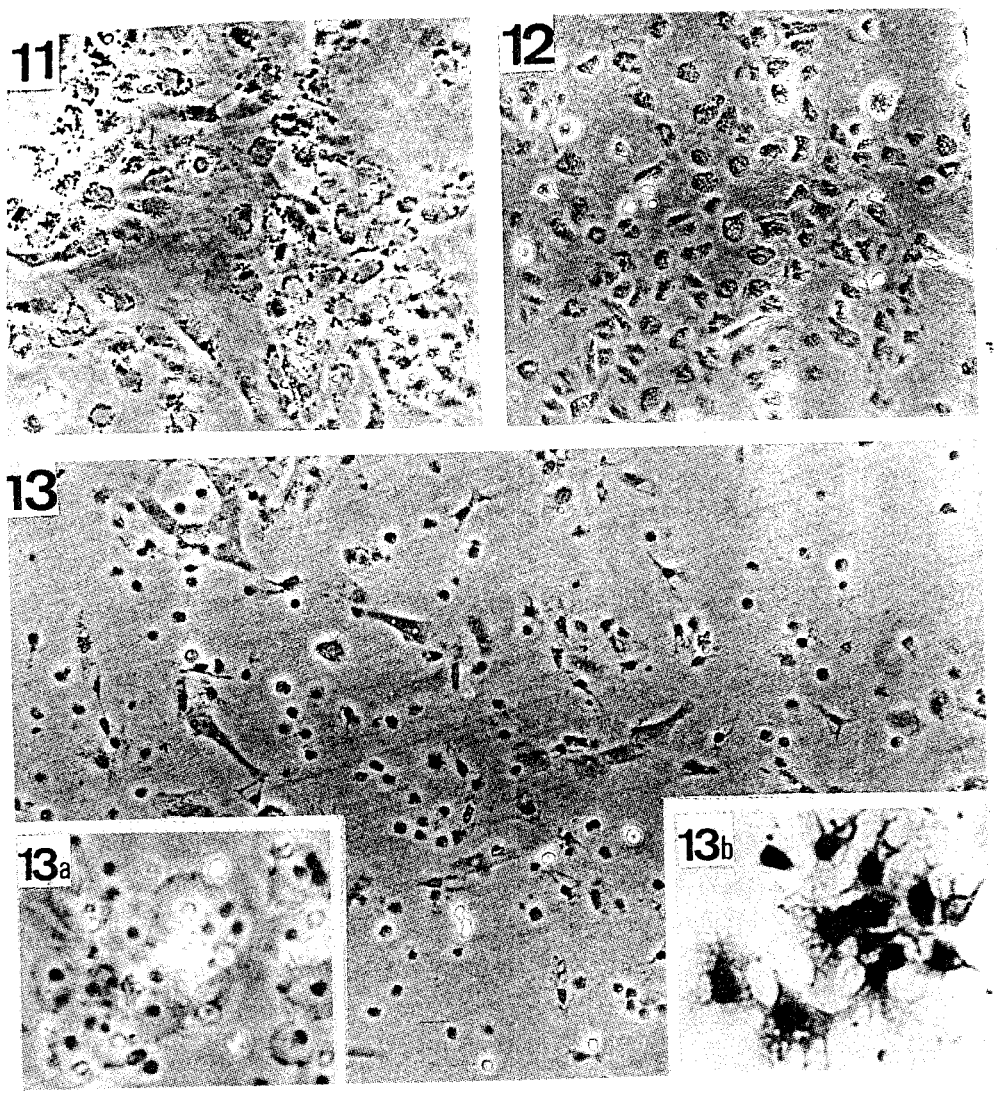
Susceptibility of lymphoid cell culture to MBV

When lymphoid tissue cells from *P. monodon* and *P. penicillatus* were inculated with extract of hepatopancreas containing MBV, CPE was observed 2-3 days after incubation at 28±1℃. CPE was limited to few areas of the confluent cell sheet (Fig. 14).

Lymphoid cell cultures from *P. japonicus* grew poorly at an incubation temperature of 28±1℃. Therefore, these cells were incubated 20±1℃. No CPE was observed within 7 days after inoculation of lymphoid organ cell culture from *P. Japonicus* with MBV infected hepatopancreas extract.

Using Harris hematoxylin and eosin staining technique, no occulsion bodies were observed in lymphoid organ cell cultures with or without CPE. However, electron micrographs of lymphoid cell cultures from *P. monodon* and *P. pecillatus* grown at 28 ± 1 ℃ , and exposed 3 days earlier to MBV, contained numerous rod-shaped nucleocapsids of MBV (measuring about 220×35 nm) present between the inner and outer lamella of the nuclear membrane (Fig. 15). In MBV-exposed cultures 72-96 h after inoculation, several free virions were observed in the degenerative cytoplasm of the infected cells as well as within the nuclear membrane (Fig. 16).

The susceptibility of lymphoid cells of *P. monodon* and *P. penicillatus* to MBV as evaluated by electron microscopy are shown in Table 4. When lymphoid tissue cells of *P. monodon* were observed 4 (8%) out of 50 cells were demonstrated to be infected by MBV. However, among 50 cells derived from *P. penicillatus* , only 2(4%) out of 50 cells were infected by MBV. In contrast, no cells from *P. japonicus* were found to be infected by MBV.



Figs. 11&12. Primary cell culture of cardiac fragments. From *Penaeus monodon* (Fig. 11, 750X) and *P. japonicus* (Fig. 12, 400X). Almost all the cells show epithelioid morphology.

Fig. 13. Primary cell culture of peripheral hemocytes (400X) showing irregular morphology of individual cells. Inserts showing population of hyaline (Fig. 13a, 750X) and granulated (Fig. 13b, Giemsa Stain, 750X) cell.

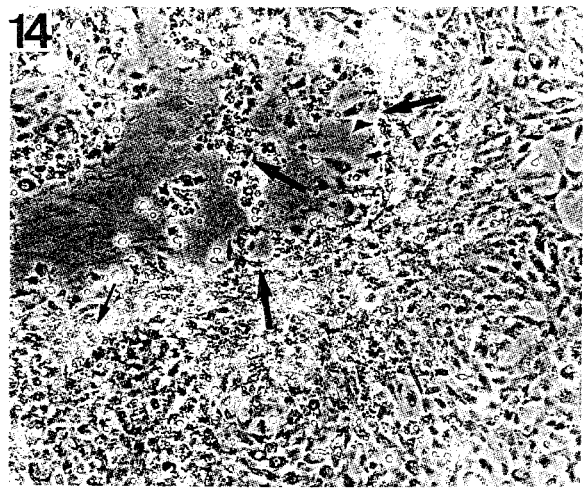


Fig. 14.
The occurrence of cytopathic effect (CPE, arrows) in confluent cell sheet derived from lymphoid tissue "Oka organ" of *P. monodon* which had been exposed to *Penaeus monodon* baculovirus (MBV) for 72 h at 28±1℃. Note only part of cells was affected. (400X)

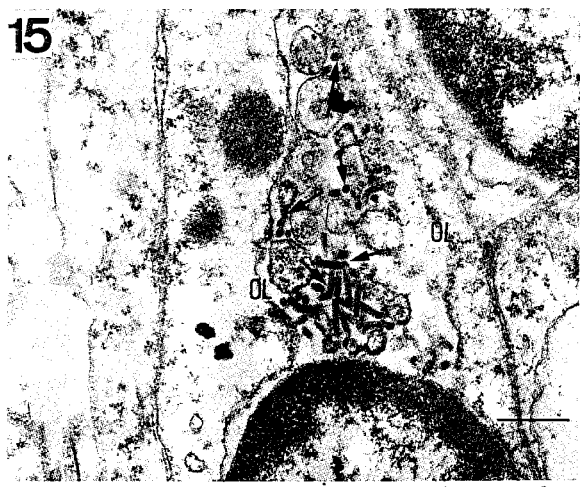


Fig. 15.
Electron micrograph showing the MBV infected (numerous) lymphoid cells from "Oka organ" of *Penaeus monodon* . Note numerous rod-shaped MBV virous (arrows) enclosed by outer lammella (OL) of nuclear membrane (Bar=400 nm)

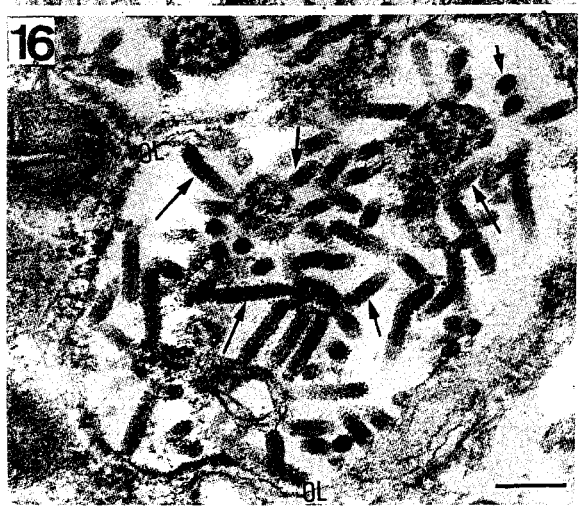


Fig. 16.
Electron micrograph showing that MBV virus enclosed by outer lamella of nuclear membrane was observed in degenerative cytoplam of the infected lymphoid tissue cells from "Oka organ" of *P. monodon* . (Bar = 166 nm)

Table 4. Suceptibility of cell cultures from lymphoid tissue, "Oka organ", of *Penaeus monodon, P. penicillatus* and *P. japonicus*

Cell cultures derived from	No. of cells observed	No. of cells infected	Infection rate (%)
P. monodon	50	4	8
P. penicillatus	50	2	4
P. japonicus	50	0	0
P. monodon (Control)	50	0	0

Each reading was obtained from at least 2 observations under electron microscope.

No extract from MBV infected hepatopancreas was inoculated into control culture.

MBV inoculated cell cultures from *P. monodon* and *P. penicillaturs* were incubated at 28±1℃ and those from *P. japonicus* were incubated at 20±1℃

DISCUSSION

Some of the culture systems investigated with penaeid shrimp in this study were adequate for the maintenance *in vitro* of lymphoid tissue and ovary cells for periods up to more than 20 days and for subcultivation *in vitro* of cells for up to 3 times. The optimal osmolarity for the maintenance of shrimp tissue cell was found to be between 620-760 mmol/kg, and the survival of lymphoid cells was markedly reduced at the osmolarity of the medium below 520 mmol/kg. A similar result was obtained when ovary of *P. monodon* was cultured (Chen *et al*., 1986).

Survival of the lymphoid tissue and ovary tissue of penaeid shrimp was found to be significantly better in culture systems which include muscle and ovary extracts. The culture media L-15 and CMRL plus foetal calf serum provided similar *in vitro* growth of lymphoid cells. However, Schneider's drosophila medium, MEM and 199 medium were demonstrated to be unsuitable for the cultivation of lymphoid tissue *in vitro*.

In the present study, conditions for the successful primary culture of penaeid shrimp cells have been established. The inadequacy of the methods for cell line establishment tested in the present study may suggst that more growth factors are needed for the establishment of penaeid shrimp cell lines.

Compared with the in vivo system, an *in vitro* culture system is equally important for the detailed study of ainmal viruses. Bacause no cell lines have been established from shrimp, several insect cell lines have been investigated for the

replication and production of shrimp baculoviruses. Summers (1977) exposed cell lines derived form *Culex tritaeniorhynohurs, Spodoptera frugiperda, Armigeres subalbatus* and *C. salinarius* to *Baculovirus penaei* (BP), a rebated shrimp baculovirus. Unfortunately, no successful viral infection was obtained in these cell lines. Similar attempts have been conducted with MBV using insect cell lines derived form *Spodoptera frugiperda, Nephotettrix* sp., *Trichopusia ni, Aedes aegypti* and *Heliothis zoe* and no replication or infection by MBV in these cell lines was obtained (Chen *et al*. unpublished data). The presen study showed that cultured lymphoid cells of *P. monodon* and *P. penicillatus* were susceptible to MBV. However, the cytopathology of MBV in infected lymphoid cells *in vitro* was different from that observed *in situ* in MBV-infected hepatopencreas epithelial cells. In addition, the susceptibility of lymphoid cells to MBV is too low to provide practical use. To improve viral susceptibility of lymphoid cells, selection of suceptible cells is needed. Since hepatopancreas is the main target tissue for MBV, establishment of cell culture or cell line from this tissue is urgently needed. Work is in progress in the establishment of cell culture system for hepatopancreas of penaeid shrimp and the infectivity of these cells against MBV is also being tested.

ACKNOWLEDGEMENT

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對蝦類組織的初級細胞培養與其對草蝦桿狀病毒的感受性

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由三種對蝦－*Penaeus monodon, P. japonicus, P. penicillatus* 的卵巢、心臟、淋巴組織及周圍血球細胞中，已可成功地建立出單層細胞培養。最適合的培養條件爲採用CMRL及L15培養基，滲透壓範圍爲620-760 mmol/Kg，而培養溫度如下：*P. japonicus* 組織爲20℃，另二種類的則爲 28℃。上述四種培養材料中，以淋巴組織或“Oka organ”最容易培養出單層細胞。來自淋巴組織與卵巢的細胞已可繼代 3 次並至少維持20天，而周圍血球細胞與心臟組織卻最多只能存活 4 天。此外培養肝胰臟時細胞無法附著也不能形成單層細胞。目前的研究發現：卵巢萃取液、肌肉萃取液和龍蝦的血淋巴等可延長對蝦類細胞在體外培養的存活時間。培養自 *P. monodon* 和 *P. penicillatus* 淋巴組織的細胞層對草蝦桿狀病毒有感受性，接種並培養於28±1℃之下，經 2 － 3 天後，單層細胞中的一些區域出現顯著的細胞病變效應。經由電子顯微鏡的觀察顯示病毒顆粒出現於感染細胞的內、外層核膜間。感染率爲：每50個 *P. monodon* 和 *P. pencillatus* 的淋巴組織細胞中，大約有 2 至 4 個細胞被感染。