

A Full Report

entitled

**An Electron Microscopic Study of
Haemocyte Changes in Giant Black Tiger
Shrimp *Panaeus monodon* Infected with
Yellow-Head Virus**

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การศึกษาการเปลี่ยนแปลงของเซลล์เม็ดเลือดกึ่งกลาดำ ที่ได้รับเชื้อไวรัสหัดเหลืองในระดับกล้องจุลทรรศน์อิเล็กตรอน

บทคัดย่อ

ได้ทำการศึกษาการเปลี่ยนแปลงทางโครงสร้างของเซลล์เม็ดเลือดกึ่งกลาดำหลังจากได้รับเชื้อไวรัสหัดเหลืองโดยใช้ (1) กล้องจุลทรรศน์ธรรมดา (2) กล้องจุลทรรศน์อิเล็กตรอน ที่ช่วงเวลาต่างๆ หลังการฉีดเชื้อไวรัสเข้ากล้ามเนื้อ พบว่าเมื่อเวลาผ่านไป 6 ชั่วโมงหลังการฉีด จำนวนเซลล์เม็ดเลือดลดลงเหลือประมาณครึ่งหนึ่งของกลุ่มควบคุม (0 ชั่วโมง) การลดลงมีมากขึ้นเรื่อยๆ ในทุกช่วงเวลา และเกือบไม่พบเม็ดเลือดเหลืออยู่เลยเมื่อเวลาผ่านไป 36 ชั่วโมงหลังการฉีด ลักษณะผิดปกติของนิวเคลียส ซึ่งได้แก่ pyknosis และ karyorhexis เริ่มสังเกตได้ในเซลล์เม็ดเลือดที่เวลา 12 ชั่วโมงหลังการฉีด นอกจากนี้ยังพบ chromatin margination และ cellular vacuolation ในเซลล์เม็ดเลือดที่เวลา 24 และ 36 ชั่วโมงหลังการฉีด เมื่อศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน (TEM) บ่งชี้ให้เห็นถึง nuclear margination, condensation และ fragmentation ของ chromatin โดยปราศจากการเปลี่ยนแปลงในไซโตพลาสซึมของเซลล์ ซึ่งลักษณะดังกล่าวเป็นคุณสมบัติของ เซลล์ที่กำลังตายแบบ apoptosis

**An Electron Microscopic Study of Haemocyte Changes in Giant
Black Tiger Shrimp *Panaeus monodon* Infected with Yellow-Head Virus**

Abstract

This study was carried out to investigate the morphological changes of haemocyte in giant black tiger prawn (*P. monodon*) following yellow head virus (YHV) infection at various time intervals using light and transmission electron microscopy. It was found that the number of haemocyte count decreased to about half of the control level (at 0 h) at 6 h post-infection. It declined progressively to almost zero at 36 h post-infection. Abnormal nuclei, including pyknosis and karyorrhexis, were observed in haemocytes starting from 12 h post-infection. At 24 h and 36 h post-infection, nuclear margination and cellular vacuolation were noticed. With TEM, there were evidence of marginated, condensed and fragmented chromatin without concurrent cytoplasmic damages observed in the YHV-infected haemocytes suggesting the apoptotic cells.

Introduction

Yellow-head disease (YHD) in giant black tiger prawn was first noticed in central part of Thailand by Limsuwan since in 1991. The causative agent of the disease was later found to be an RNA-type virus commonly known as yellow-head virus (YHV) (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Cowley et al., 1999). Now it still causes a serious problem to Thai shrimp farmers.

YHD was named because of the gross signs that can be observed in affected shrimp. The shrimp body is pale and the cephalothorax appears yellow due to the pale color of the hepatopancreas. The mass mortality tends to occur within 3-5 days of the first gross signs.

The haemocytes are one of the main targets involved in the disease process (Nash et al., 1995; Flegel et al., 1992). Lightner (1996) reported that chromatin margination and lateral displacement of the nucleolus in the cell might be observed in the early stage of YHD. In addition, some of the grossly normal shrimp in an affected pond may show abnormal haemocytes with pyknotic and karyorrhectic nuclei (Flegel et al., 1997). It is, therefore, of interest in this study to reveal the morphological changes in haemocytes following yellow-head virus (YHV) infection at various time sequences using light and transmission electron microscopy (LM & TEM).

Materials and Methods

Experimental animals

Experimental animals were healthy *P. monodon* of 10-15 g. BW. They were stocked in four concrete circular tanks of approximately 2 m. diameter and containing water up to 0.80 m. in depth. Each tank contained 50 shrimp, fed with commercial feed pellets at 3% BW daily. The water salinity was 10 ppt and adequately aerated through air stones, to ensure a dissolved oxygen level above 5 ppm. The shrimp were acclimated for 3 day before experiments.

Shrimps were divided into two groups, each composed of two tanks of shrimp as follows:

Group I : intact control

Group II : YHV-infected group treated by intramuscular injection with 0.1 ml YHV diluted 1:100 in lobster haemolymph buffer (LHB)

Crude extract of YHV was prepared from haemolymph of moribund shrimp from YHD outbreak culture ponds. Haemolymph was withdrawn from the ventral blood sinus of infected shrimp and diluted 1:100 with LHB. This diluted haemolymph was filtered through a sterile 0.45 µm membrane filter. All processes were carried out using sterile techniques and the diluted haemolymph was kept at -20°C until used.

Ten animals from each group were sampled at 0, 6, 12, 24, 36 hours after the infection of viral or carrier solution. Samples were processed further for routine light and electron microscopic techniques to investigate the morphological changes of the haemocyte.

Haemocyte preparation

For studying with light (LM) and transmission electron microscopes (TEM), 0.2 ml of haemolymph was withdrawn slowly from the ventral sinus by inserting a 26 gauge needle attached to a 1.0 ml syringe, containing 0.2 ml of 10% formalin in 0.85% NaCl solution (for LM) or 0.2 ml of 2% glutaraldehyde in phosphate buffer pH 7.4 (for TEM). The haemocytes in the haemolymph were therefore fixed immediately in fixative solution in the syringe.

For TEM, the haemolymph containing haemocyte were fixed by immersion with 2% glutaraldehyde in phosphate buffer, pH 7.4 and left 2 h in the same fixative at 4°C before taking them to centrifuge at 10,000 rpm. The precipitate of the haemocytes was then obtained. The sample were post-fixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol, infiltrated and embedded in Araldite 502 resin. Semithin sections (1 µm) were stained with toluidine blue and examined by light microscope (LM). Thin sections of 70-100 nm thick were stained with 1% uranyl acetate, then lead citrate and examine under a TEM at an accelerating voltage of 75 kV.

Results

The present study showed that the density or the number of the haemocyte in the shrimp reduced progressively from 6, 12, 24 and 36 h post-injection, respectively. The haemocytes almost disappeared from the haemolymph at 36 h post-injection.

At 0 h, the types of haemocytes in normal shrimp contained numerous hyalinocytes, semigranulocytes and granulocytes (Fig. 1). Hyalinocytes were fusiform cells with oval-shaped nuclei and scanty cytoplasm at both sides of the cell. Semigranulocytes also had oval nuclei but with more area of the cytoplasm than the hyalinocytes. Some granules were observed in its cytoplasm. Granulocytes had somewhat round nuclei with distinctive clear cytoplasm; relatively large granules could be observed.

Histology of haemocytes in YHV-infected shrimp began to reveal abnormality at 6 h post-injection by presence of a few pyknotic and karyorhetic nuclei in the cells (Fig. 2). At 12 h, extensive nuclear pyknosis and nuclear karyorhexis could be observed in the haemocytes. The abnormal nuclei seemed to be maximal at 24 and 36 h post-injection, the time at which shrimp were moribund stage. In addition to these abnormalities, at 24 and 36 h post-injection, nuclear chromatin margination and vacuolation could be seen in haemocytes (Figs. 3-10). There was no evidence of inflammation in the YHV-infected haemocytes. It was observed that, in YHV-infected haemocytes, the nuclear envelope and organelles such as mitochondria, ribosomes, rough endoplasmic reticulum were still well preserved.

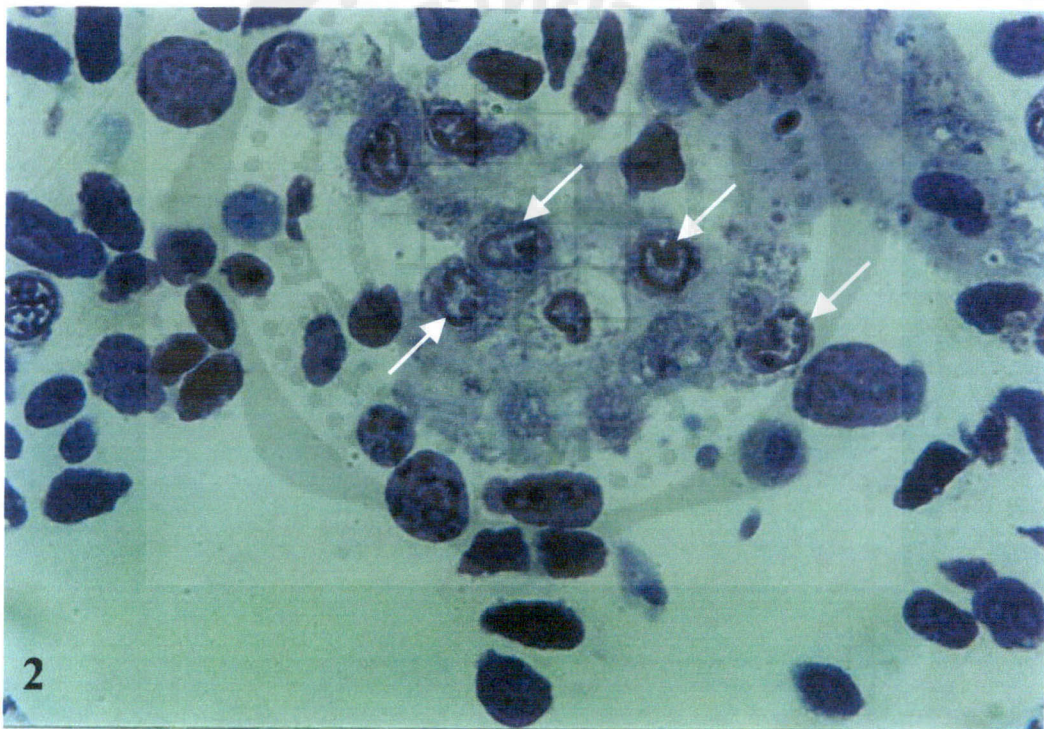
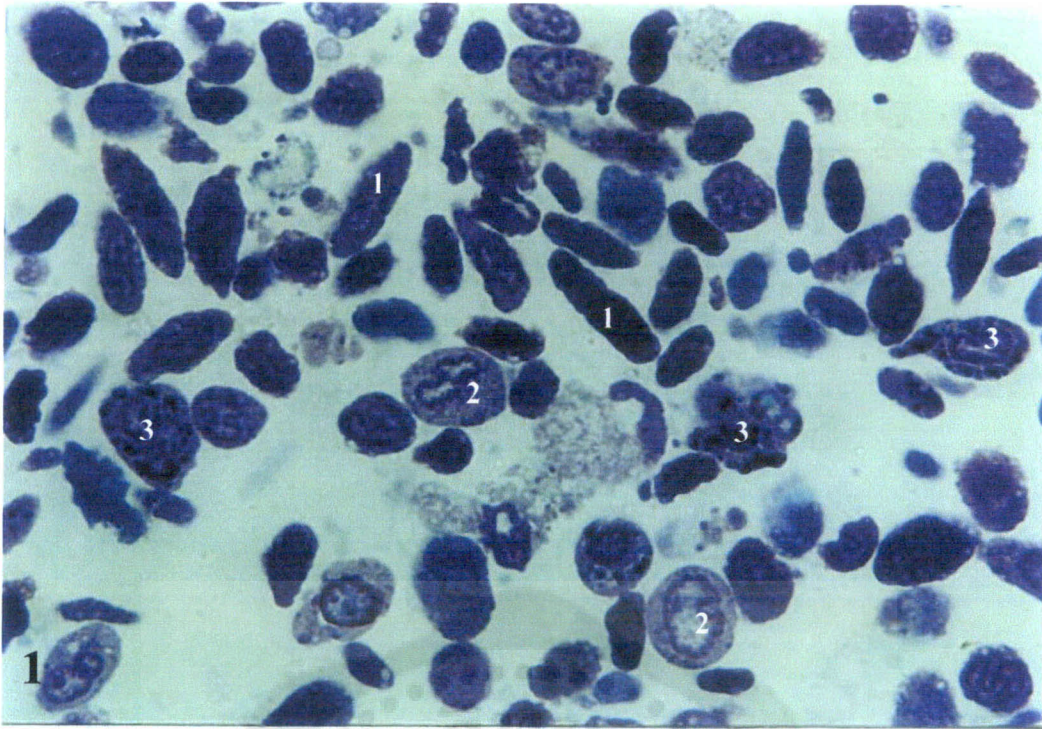
The normal nuclei of intact haemocytes contained heterochromatin in peripheral patches and a central mass (Figs. 11-12). At 12 h post-injection, the evidence of apoptosis began to appear. Early apoptotic nucleus was observed as the nuclear chromatin became condensed and marginated into sharply delineated masses

abutting the nuclear envelope (Fig. 13). The cytoplasm was also markedly condensed. At 24-36 h post-injection, many apoptotic cells were detected. Their nuclear chromatins were condensed, and either fragmented or non-fragmented (Figs 14-15). Despite severe pathological changes of the nucleus, the nuclear envelope and cytoplasmic organelles such as mitochondria, ribosomes, rough endoplasmic reticulum were well preserved. Apoptotic bodies frequently occurred in clusters in the intracellular space (Fig. 15). In the moribund stage, degeneration of the cytoplasmic organelles occurred (Fig. 16).

Therefore, the morphological changes of the haemocytes in YHV-treated shrimp seemed to gradually change from normal healthy stage to severe non-functional stage at 36 h post-injection. It was found that each type of the haemocyte was equally effected by yellow head virus. Moreover, no inflammatory responses were observed.

Fig. 1. Three types of *P. monodon* haemocytes in normal shrimp. LM. 1, hyalinocyte; 2, semigranulocyte; 3, granulocyte. Methylene blue. 3900x.

Fig. 2. *P. monodon* haemocytes in YHV-infected shrimp at 6 h post-injection. LM. Note cells with marginated condense-heterochromatin, early stage of apoptotic cell (arrows). Methylene blue. 3900x.



Figs. 3-10. *P. monodon* haemocytes in YHV-injected shrimp at 24 h, and 36 h post-injection. LM. All of the depicted cells were abnormal. Note the cells with nuclear pyknosis (Figs. 3-6), nuclear chromatin condensation (Figs. 3-6), and margination (arrow in Fig. 3) as well as with nuclear vacuolation (Figs. 4, 8). Also apoptotic cells were detected (Figs 9-10). Their nuclear chromatin were condensed, and either non-fragmented (Fig. 9.) or fragmented (Fig. 10). Methylene blue. 3700x.

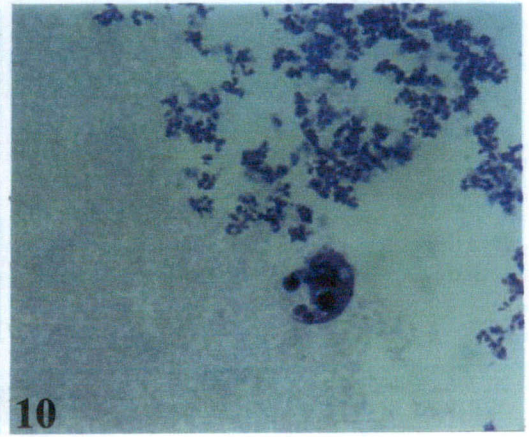
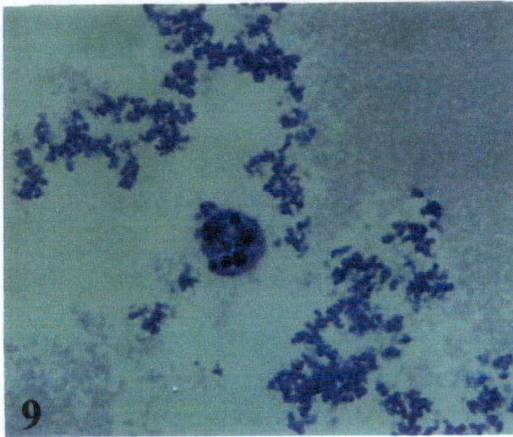
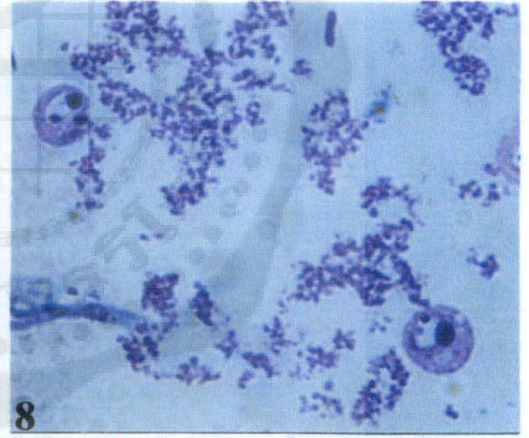
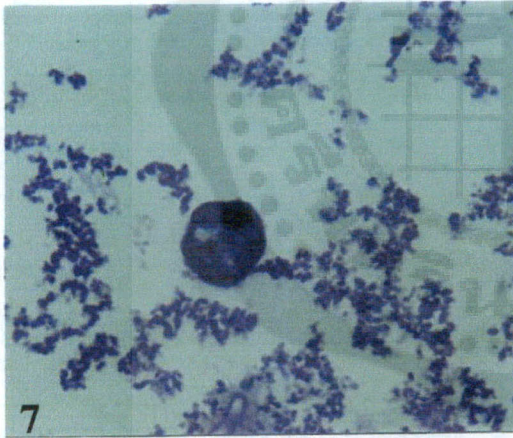
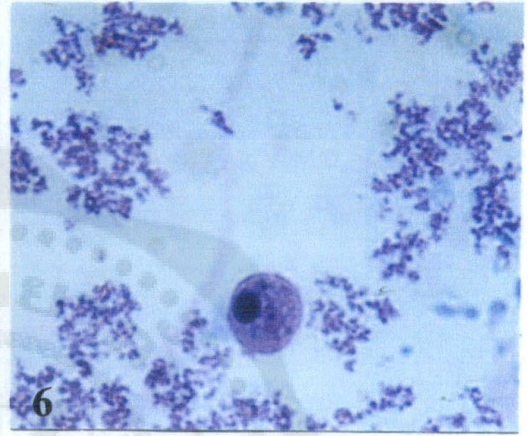
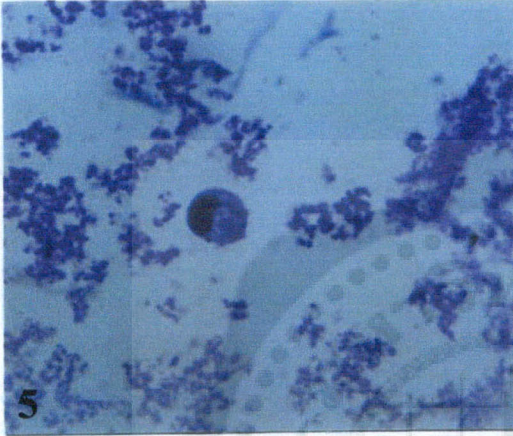
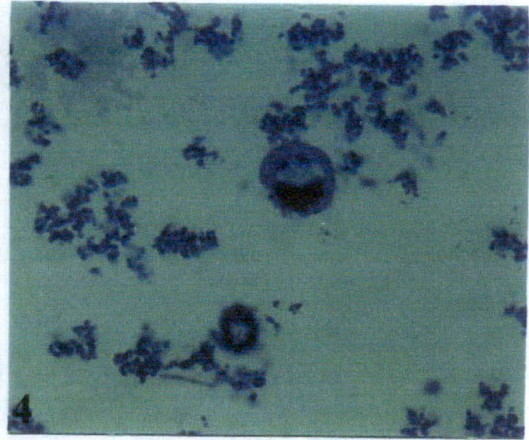
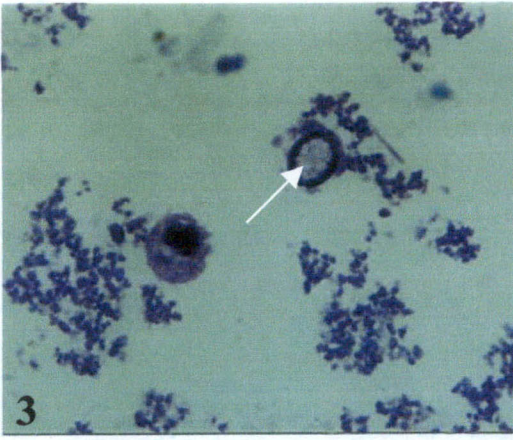
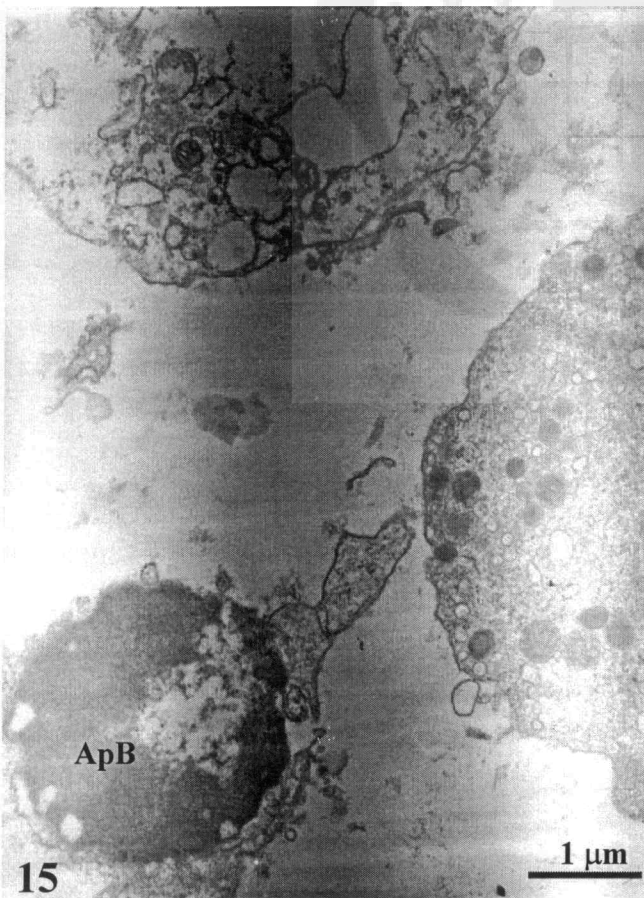
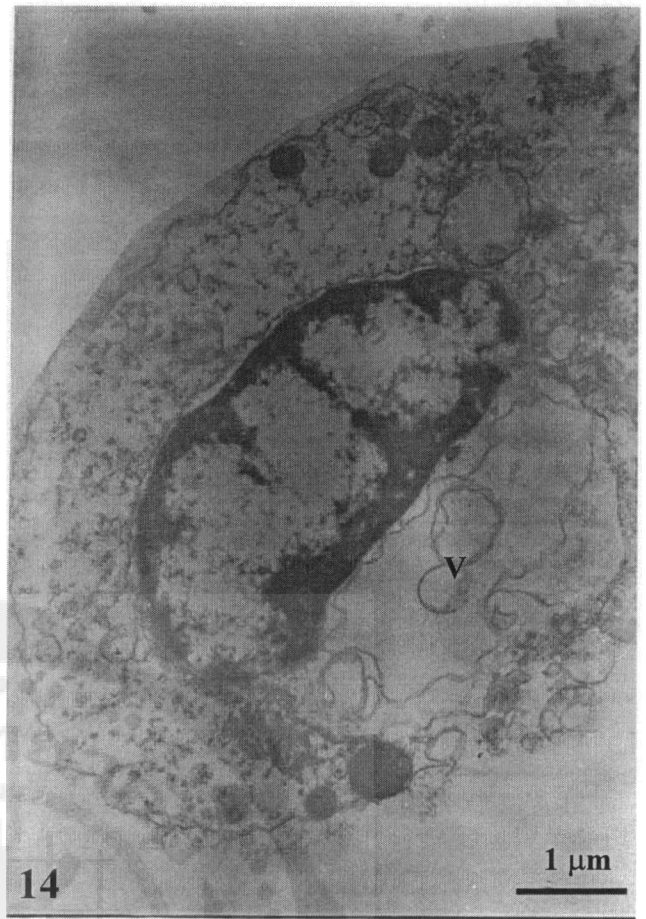
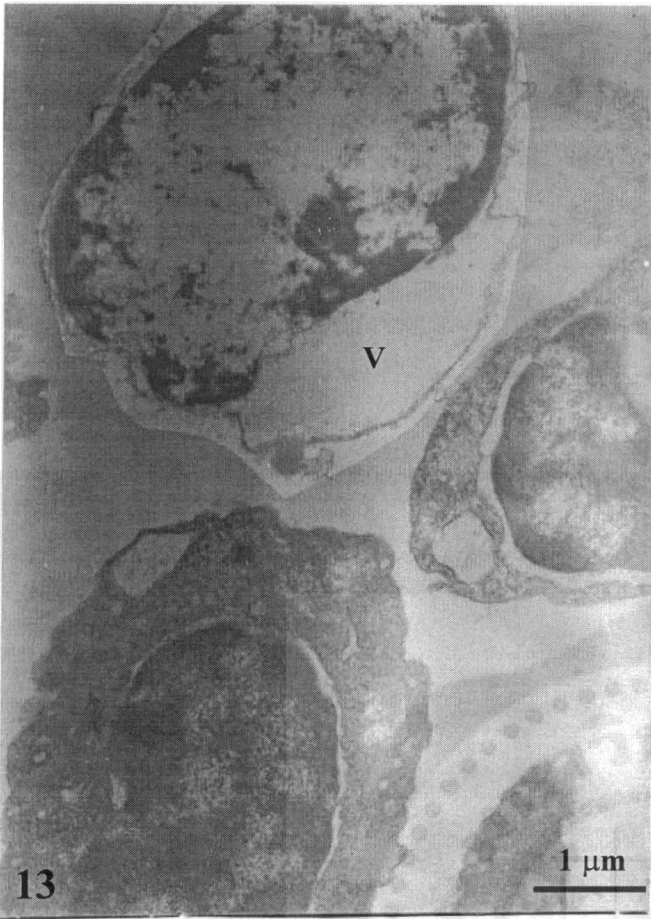


Fig. 11. *P. monodon* haemocytes in normal shrimp. TEM. 1, hyalinocyte; 2, semigranulocyte. Note some small granule in semigranulocyte. Bar = 1 μm .

Fig. 12. *P. monodon* granulocyte (3) in normal shrimp. TEM. Note intact nuclear membrane. Bar = 1 μm .



Figs. 13-16. *P. monodon* haemocytes in YHV-injected shrimp at 12 h (Figs.13-14), 24 h (Fig. 15), and 36 h (Fig. 16) post-injection. TEM. Pathological features were gradually severe as post-injection time increased. The nuclei of the infected cells were either in early stages of apoptosis (in Figs.13-14) or in the late stages which were markedly condensed and either nonfragmented as apoptotic body (ApB) (in Fig. 15) or fragmented (in Fig. 16). Despite these drastic changes in the nucleus, the cell, especially in 12 h and 24 h post-injection, still had well-preserved nuclear envelopes and normal cytoplasmic organelles. *V*, vacuolation. Bars = 1 μ m.



Discussion

The present study showed that the number of haemocyte of the YHV-infected shrimp decreased progressively from 6, 12, 24 and 36 post-injection, respectively. This finding agreed with the previous study (Khanobdee, 2000) in that the total density of haemocyte began to decline at 6 h post-injection and progressively reduced at every sampling time thereafter. A decrease in total haemocyte count has also been reported in shrimp infected with penaeid rod-shaped DNA (PRDV) virus, which is the same as or closely related to white spot syndrome virus (WSSV) (Maeda et al., 1997; Hennig et al., 1998). The authors suggested that haemocytes were a target tissue of the virus and that a large fraction of them were destroyed during infection. The same explanation is offered here for YHV infection in which the haemocyte underwent nuclear pyknosis and karyorhexis. Beckage (1996) proposed that the decrease in total haemocyte count against WSSV infection was provoked by the shrimp immune system as it attempted to eliminate infected haemocytes. Strand and Pech (1995) also found that *Microplitis demolitor* polydnavirus (MdPDV) induces apoptosis of granular cells, the primary haemocytes that involved in regulation of the cellular immune response toward *M. demolitor* and other parasites. This virus promoted its own survival by inducing apoptosis of host immune cells, which would otherwise kill the developing *M. demolitor* egg. The mechanisms by which a virus maintained its survival in a host could either be by induction of apoptosis (e.g. in MdPDV, papilloma viruses and parvovirus), or by suppression of apoptosis (e.g. in African swine fever virus, baculoviruses and vaccinia virus), or both (e.g. in Adenovirus) (Shen and Shenk, 1995, O'Brien, 1998).

In the present study, evidence of apoptosis was found in the YHV-infected haemocyte of the shrimp by light and electron microscopy. Ultrastructurally, early

apoptosis was shown by nuclear chromatin margination, followed by partial and complete chromatin condensation. The final stages involved nuclear budding or fragmentation (Kurr, 1971; Kurr et al., 1972). The fragment of nucleus, termed apoptotic bodies, were surrounded by an envelope. The cytoplasm was also markedly condensed. Despite severe pathological changes of the nucleus, the nuclear envelope and cytoplasmic organelles such as mitochondria, ribosomes and rough endoplasmic reticulum were well preserved. In this study, all of these ultrastructural changes were demonstrated.

Apoptosis is often implicated in the elimination of cells whose survival might be harmful to the organism as a whole. For example, cells infected by certain viruses may become apoptotic to prevent extensive viral replication in the cell. On the other hand, it has been proposed that many viruses can subvert the cellular apoptotic program to promote their own dissemination within apoptotic bodies and to ensure the release of progeny virions in a form that provides isolation from extracellular effector molecules. A good example is the role of Sindbis virus in apoptosis to assist its own replication and dissemination (Giffin and Hardwick, 1997; Hardwick, 1998).

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