**N-Linked glycosylation is essential for the yellow head virus replication cycle**

Chumporn Soowannayan,1,2 Nhuengtida Chanarpakorn,2,3 Mongkholl Phanthura,2,3 Nattawoot Deekhlai,2 Chanon Kunasol2 and Siriporn Sriurairatana2

1National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand Science Park, Klong Luang, Patumthani 12120, Thailand
2CENTEX SHRIMP, Faculty of Science, Mahidol University, Rama VI Road, Bangkok, 10400, Thailand
3Department of Biotechnology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok, 10400, Thailand

Yellow head virus (YHV) particles contain a nucleocapsid protein (p20) and two envelope glycoproteins (gp116 and gp64). The glycans attached to the two glycoproteins are N-linked and are complex and high mannose types, respectively. Here, we show that treatment with the N-linked glycosylation inhibitor tunicamycin in YHV-infected black tiger shrimp (*Penaeus monodon*) resulted in less severe yellow head disease and reduced mortality when compared with untreated control shrimp. Quantitative real-time reverse transcription PCR analysis also revealed lower YHV copy numbers in the haemolymph of treated than control shrimp. This was concurrent with less intense immuno-reactions in tissues of treated versus untreated shrimp using mAbs against all three YHV structural proteins. In addition, transmission electron microscopy of lymphoid organ tissue of the treated and untreated shrimp [eight collected at 36 h and eight at 48 h post-infection (p.i.)] revealed only unenveloped nucleocapsids in all but one of the treated shrimp (collected at 48 h p.i.). By contrast, all the untreated shrimp showed a mixture of many unenveloped and enveloped virions. These results were supported by purification of YHV from the cell-free haemolymph of treated and untreated shrimp followed by YHV structural protein analysis by SDS-PAGE. It revealed three expected structural protein bands (116, 64 and 20 kDa) from the untreated shrimp but no structural protein bands from the tunicamycin-treated shrimp (confirmed by Western blot analysis). Overall, the results indicated that blocking glycosylation with tunicamycin inhibited the formation of mature YHV virions and their subsequent release into shrimp haemolymph, reducing the severity of disease.

**INTRODUCTION**

Yellow head virus (YHV) is one of the most important penaeid shrimp viruses. It was first reported as the cause of mass mortality of cultivated native *Penaeus monodon* in Thailand in 1991 (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Flegel et al., 1995; Limsuwan, 1991) and later also in cultivated exotic *Penaeus* (*Litopenaeus*) *vannambei* (Senapin et al., 2010). Other variants that cause less severe disease or no disease were later reported from Australia and from other countries in South-East Asia (Spann et al., 1995, 1997; Wijegoonawardane et al., 2008). In Australia, in the north-eastern states of Queensland and New South Wales, the endemic YHV genotype is called gill-associated virus and is associated with gradual death of *P. monodon* from 3–3.5 months after stocking (12–15 g in weight) until harvest (Cowley et al., 1999; Spann et al., 1995, 1997), and it is considered to be the main cause of mid-crop mortality syndrome (Oanh et al., 2011). Currently, six genotypic variants of YHV have been identified (Wijegoonawardane et al., 2008), but only type 1 from Thailand and type 2 (gill-associated virus) have been reported to cause disease.

YHV is a positive-sense ssRNA virus that has been classified in the order *Nidovirales* and in the genus *Okavirus* (Cowley & Walker, 2002; Cowley et al., 1999; Walker et al., 2005; Wontteerasupaya et al., 1995; Ziebuhr et al., 2003). YHV particles contain three structural proteins: a nucleocapsid protein or p20 protein and two envelope glycoproteins (gp116 and gp64) (Cowley et al., 2004; Jitrapakdee et al., 2003). The nucleocapsid protein (smallest of the three) is encoded by ORF2, which is located in the YHV genome at the N terminus of the two viral envelope protein genes. The nucleocapsid protein of
gill-associated virus has been shown to bind RNA in a non-sequence-specific manner, and an RNA-binding domain on the protein has been identified as an arginine-rich region near its N terminus (Soowannayan et al., 2011). The two envelope glycoproteins are generated by post-translational cleavage of the viral ORF3 gene product, potentially by a signal peptidase type I enzyme (Jitrapakdee et al., 2003). These cleavages occur at two sites in the newly synthesized protein, one at aa 228–229 and another at aa 927–928. This generates three protein fragments of 228 aa, 899 aa (gp116) and 359 aa (gp64). The smallest fragment (228 aa) has not been reported either from YHV virions or from YHV-infected cells.

Carbohydrates attached to both glycoproteins have recently been characterized and all were found to be N-linked glycans (Soowannayan et al., 2010). Using a combination of mass spectrometry and enzyme digestion, it was found that most of the putative N-linked glycosylation sites of gp116 (6/7) and gp64 (3/4) were utilized. The unutilized, putative N-linked site of gp64 was identified whilst that of gp116 was not. The carbohydrates or glycans attached to the gp64 and gp116 proteins were found to consist mostly of mannose but also a variety of other glycans (Soowannayan et al., 2010).

Glycosylation of envelope proteins of many viruses has been shown to affect various steps in viral replication cycles, including receptor binding, virus infection, transcription, replication and virulence (Beasley et al., 2005; Davis et al., 2006; Deshpande et al., 1987; Panda et al., 2004; Shirato et al., 2004; reviewed by Vigerust & Shepherd, 2007). One such example is the glycosylation of gp120 of human immunodeficiency virus type 1 (HIV-1), in which removal of glycans attached to the protein was found to reduce its CD4 receptor binding ability (Matthews et al., 1987) and thus reduce HIV-1 infectivity (Fenouillet et al., 1990). The role of glycans in YHV structural proteins is not known. However, it has been shown that gp116 may play a major role in binding with its host receptor during infection, since mouse antiserum against it neutralized the virus in primary lymphoid organ (LO) cell cultures whereas antiserum against gp64 did not (Assavalapsakul et al., 2005). In addition, a possible viral receptor with gp116-binding specificity has been identified and it has been shown that its downregulation by RNA interference resulted in complete inhibition of viral entry (Assavalapsakul et al., 2006).

In this study, tunicamycin (an inhibitor of N-linked glycosylation via blocking of N-acetylglucosamine-1-phosphotransferase activity required in dolichol metabolism) was used to examine the importance of YHV envelope protein glycosylation in the replication cycle, virion assembly and infectivity of YHV.

RESULTS

Tunicamycin delays mortality of YHV-infected shrimp

Shrimp mortality started in both YHV-injected groups at 24 h post-infection (p.i.), whether treated with tunicamycin or not. However, no obvious signs of yellow head disease (e.g. erratic swimming, lying on the side, yellow coloration of the cephalothorax and pale bodies) were observed in the moribund shrimp at this stage. Clearer signs of disease were observed in YHV-infected shrimp that did not receive tunicamycin at 36 h p.i. when four shrimp from this group died compared with two in the YHV-injected group treated with tunicamycin. As time progressed, many more grossly diseased shrimp were observed in the untreated YHV-infected group. However, none of the grossly diseased shrimp displayed yellow coloration of the cephalothorax, in agreement with several early reports of experimental YHV infections (Anantasomboon et al., 2008; Chantananchook et al., 1993; Fleget al., 1995; Lu et al., 1994). All shrimp in the untreated YHV-infected group showed gross signs of disease and died by 55 h p.i., whereas those in the tunicamycin-treated group showed similar signs only beyond 48 h p.i., and by 72 h p.i. only seven out of 13 shrimp in the group had died. Shrimp in the tunicamycin-infected control group and the uninfected control group showed no signs of disease (as expected), and only one and three shrimp died in these two groups, respectively. The dead shrimp in these groups were found to be newly moulted and had been partially cannibalized by their peers.

Fig. 1(a) shows the cumulative death results from this experiment.

Less severe pathology in tunicamycin-treated YHV-infected shrimp

At 36 h p.i., histological observation of haematoxylin and eosin (H&E)-stained tissue sections from shrimp in both YHV-injected groups revealed typical yellow head disease pathology, including pyknotic and karyorrhectic nuclei or apoptotic cells in many vital organs and tissues including the LO, gills, heart, interstitial cells of the hepatopancreas and haemocytes. Among these tissues, the LO (one of the earliest targets for YHV) was chosen to determine infection severity.

The normal LO is composed of tubules but in defence states may also contain spheroidal collections of cells (spheroids) between the tubules. The latter develop in response to both biotic and non-biotic foreign materials (Anggraeni & Owens, 2000; Hasson et al., 1999b; Kondo et al., 1994; Rusaini & Owens, 2010) and may contain apoptotic cells similar to those caused by YHV infection. For example, spheroids formed in response to Tuara syndrome virus infection (Hasson et al., 1999a, b) are indistinguishable from those resulting from other causes. Thus, spheroid development in H&E-stained tissues cannot be assigned to any particular cause without additional supporting evidence. In our study, spheroid bodies of various types were observed in all our shrimp groups and contained apoptotic cells in varying numbers, regardless of YHV infection status. Thus, spheroids were not considered alone in scoring the severity of YHV infection. The main
criterion was the presence of cells with pyknotic and karyorrhectic nuclei (or apoptotic cells) in the tubules.

Light microscope observation of H&E-stained cephalothorax sections of shrimp in the no-tunicamycin, YHV-infected group revealed only a few apoptotic cells in functional tubules of the LO as early as 24 h p.i., but many in samples collected at 36 h p.i. and later. All shrimp in this group died within 48 h p.i. By contrast, LO of the YHV-infected shrimp that received tunicamycin showed generally less severe pathology or fewer apoptotic cells at the same times (see Fig. 1b for mean severity scores, details of which are in Methods). No YHV pathology was observed in LO tubules of shrimp in the uninfected control group or the group injected with tunicamycin alone, although some specimens showed spheroids.

**Less intense YHV immunopositive reactions in tunicamycin-treated shrimp**

Immunohistochemical analysis using specific mAbs against the three YHV structural proteins (i.e. envelope glycoproteins gp116 and gp64 and nucleocapsid protein p20) in YHV-challenged shrimp revealed less intense immunoreactive signals in tissues of tunicamycin-treated shrimp than in tissues of untreated shrimp. The reactions were generally stronger for the nucleocapsid protein than for the envelope glycoproteins. In the untreated shrimp, positive immune reactions for all three antibodies were first observed in the LO at 24 h p.i. and they became more intense as time progressed in various tissues including the gills, the heart, connective tissues, haematopoietic tissues and interstitial cells of the hepatopancreas. The positive
signals in the LO were generally observed first in cells of the tubule stromal matrix, in haemocytes, in the haemocoel and in the cells of newly formed spheroids. Reactions in the outer margins of pre-existing spheroids occurred later, as previously reported (Soowannayan et al., 2002). These results correspond well with those of H&E-stained tissues. Examples of immunoreactions in shrimp samples collected at 36 h p.i. are shown in Fig. 1(c), where few focal positive immunoreactions for p20 can be seen in LO tubules of tunicamycin-treated shrimp whilst more generalized positive immunoreactions are seen in tubules of the untreated shrimp. No immune reactions were observed in tissues of the control shrimp not injected with the virus and treated or not with tunicamycin.

**Tunicamycin reduces the replication of YHV**

To determine the effects of tunicamycin on viral replication, quantitative real-time reverse transcription PCR (qRT-PCR) assays targeting the YHV polymerase gene were performed to estimate relative virus copy numbers in whole haemolymph of YHV-challenged shrimp treated or not with tunicamycin. At each time (12, 24, 36 and 48 h p.i.), RNA was extracted from eight shrimp in each group and used as the template for cDNA synthesis followed by real-time PCR. For each cDNA, three qRT-PCRs were performed. An equal quantity of extracted RNA (12.5 ng) from each shrimp was used as the template to synthesize the cDNA template for real-time PCR amplification. It was found that YHV copy numbers in the haemolymph of tunicamycin-treated shrimp were significantly lower ($P<0.05$) than those of untreated shrimp at all times p.i. (Fig. 2).

**Tunicamycin inhibits the formation of enveloped YHV particles**

Observations by transmission electron microscopy (TEM) of thin sections of LO tissue from 16 tunicamycin-treated shrimp (eight shrimp each from 36 and 48 h p.i.) revealed only thread-like nucleocapsids of YHV in the cytoplasm of infected cells (Fig. 3b), except for the presence of a few enveloped particles in one shrimp specimen collected at 48 h p.i. In contrast, many enveloped virions together with thread-like unenveloped nucleocapsids were observed in the tissues of all 16 untreated YHV-challenged shrimp (eight each from 36 and 48 h p.i.) (Fig. 3a).

**Tunicamycin reduces the quantity of YHV particles released into the haemolymph**

YHV was purified from the cell-free haemolymph of tunicamycin-treated and untreated shrimp at 36 and 48 h p.i. Analysis by SDS-PAGE and Coomassie Brilliant Blue (CBB) staining showed that only preparations from untreated shrimp showed three bands corresponding with the YHV structural proteins (gp116, gp64 and p20) at both times. None was found for the tunicamycin-treated shrimp (Fig. 4a). However, both preparations showed bands at approximately 75 and 73 kDa indicating similar levels of haemocyanin contamination and indicating that tunicamycin treatment did not affect the level of haemocyanin. Western blot analysis using mAbs against YHV gp116 (V3-2B) and gp64 (Y-18) confirmed the CBB staining result (Fig. 4b).

![Fig. 2.](http://vir.sgmjournals.org)
Staining of the SDS-PAGE gel with glycoprotein-specific Pro-Q Emerald 300 dye gave positive signals for two glycoproteins corresponding to the positions of gp116 and gp64 only for the preparation from untreated shrimp and not from tunicamycin-treated shrimp (Fig. 4c).

To confirm these results, YHV purified from cell-free haemolymph of tunicamycin-treated or untreated shrimp at 36 and 48 h p.i. was subjected to TEM with negative staining. Results from 36 and 48 h were the same, so only those from 36 h are shown in Fig. 5. The preparation from untreated shrimp showed many enveloped virions (Fig. 5a) and the presence of gp116 envelope protein in the virions was confirmed by immuno-negative staining (Fig. 5b). By contrast, no enveloped virions (or even non-enveloped nucleocapsids) were observed in the negatively stained sample prepared from tunicamycin-treated shrimp (Fig. 5c).

**Production of gp64 and gp116 in haemocytes reduced by tunicamycin**

When proteins from haemocytes isolated from YHV-infected shrimp treated and not treated with tunicamycin were analysed by SDS-PAGE and Western blotting using mAbs against YHV structural proteins gp64 and gp116, positive
bands for both were strongest for the untreated shrimp and weakest for the tunicamycin-treated shrimp (Fig. 6).

**DISCUSSION**

Our investigation of the roles of N-linked glycosylation of the two envelope proteins of YHV in shrimp (*P. monodon*) revealed that blocking of N-linked glycosylation with the inhibitor tunicamycin had dramatic effects on viral replication, assembly and dispersal in the host. This was confirmed by microscopic examination of H&E-stained tissues, immunohistochemistry, TEM, SDS-PAGE and Western blotting, qRT-PCR and staining of proteins from purified viral preparations for glycosylation. The overall result in YHV-challenged shrimp was reduced mortality in the tunicamycin-treated group when compared with the untreated group.

Tunicamycin has been shown to have similar effects on many viruses including Sindbis virus and vesicular stomatitis virus (Leavitt *et al.*, 1977b) for which 0.5 mg tunicamycin ml$^{-1}$ was found to inhibit virus particle formation and virus multiplication by 99.9 % in baby...
hamster kidney (BHK)-21 cells, without any effect on cell survival. For these two viruses, non-glycosylated forms of their envelope proteins were formed but were unable to migrate to the cell surface where virus envelopment occurred (Gibson et al., 1979; Leavitt et al., 1977a). In a study of mouse hepatitis virus strain A59 grown in Sac(−) cells, it was found that tunicamycin (0.5 μg ml−1 or higher) inhibited infectious particle formation by >99%, whilst production of non-infectious particles that lacked gp90/E2, gp180/E2 and p110 was not inhibited (Rottier et al., 1981). In our study, the amount of tunicamycin used [2 mg (kg−1 body weight)] was found to be effective for inhibition of YHV replication, envelopment and spreading, with no detectable negative effect on shrimp health or survival. Although a few enveloped particles were observed in one tunicamycin-treated, YHV-infected shrimp specimen, this may have been due to inhibition leakage or inability of the tunicamycin to reach all YHV-infected cells in that particular shrimp. Since the two envelope proteins produced at low levels in tunicamycin-treated, YHV-infected shrimp had similar masses to their respective counterparts in untreated shrimp, evidence suggests that they too were glycosylated. This result supports our proposal that these proteins may be produced by some cells not affected by tunicamycin. It is also possible that differences in shrimp genetic make-up may render resistance to tunicamycin treatment in particular shrimp individuals. Indeed, differences in sensitivity to tunicamycin treatment among cells and tissues are well known, and a gene responsible for this resistance has recently been identified [major facilitator domain containing 2A (MFSD2A) transporter] (Reiling et al., 2011). Human cells over-expressing this protein were found to be super-sensitive to tunicamycin whereas cells without it were found to be resistant (Reiling et al., 2011). Although no protein similar to MFSD2A has been identified in shrimp, our demonstration that shrimp cells sensitive to tunicamycin treatment suggests that a similar protein or a protein with similar function may be present.

Reduction in the quantity of all three YHV structural proteins occurred in tunicamycin-treated shrimp (as shown in the immunohistochemical studies of the tissue sections and Western blot analysis of haemocyte proteins from treated and untreated animals). This reduction was unlikely to be the result of a general protein synthesis inhibition effect of tunicamycin, since haemocyanin protein bands found in the tunicamycin-treated and untreated samples were comparable. Further, a study done on BHK cells clearly showed that tunicamycin treatment did not have a general inhibitory effect on protein synthesis (Leavitt et al., 1977b). In addition, lack of general protein synthesis inhibition by tunicamycin has been clearly proven in another study done on chick embryo fibroblasts in which tunicamycin treatment was found to decrease the total amount of the major cell surface glycoprotein (by ~50–65%) whilst three other membrane associated proteins actually increased in amount. It was concluded that the decrease of cell surface glycoprotein was not due to protein synthesis inhibition but to accelerated degradation (two- to threefold) of the unglycosylated cell surface glycoprotein (Olden et al., 1978). Similar conclusions regarding possible increased rates for degradation of un-glycosylated proteins were proposed in other studies on tunicamycin homologues (Duksin & Mahoney, 1982; Mahoney & Duksin, 1979). Many proteins have been shown to be unstable if not glycosylated. In a pulse-chase study of MHV-A59-infected Sac(−) cells in the presence of tunicamycin, several host and viral glycoproteins that lacked glycosylation were shown to be misfolded and retained in the endoplasmic reticulum. Protein folding in the endoplasmic reticulum is facilitated by many molecular chaperones and is closely monitored by a stringent endoplasmic reticulum quality control system that allows only properly fold proteins to be transported to the Golgi apparatus. The misfolded proteins are retained and are targeted by endoplasmic reticulum-associated protein degradation or by autophagy (Määtänen et al., 2010). Whether these are the fates of the unglycosylated gp116 and gp64 proteins after tunicamycin treatment might be further investigated in vitro with shrimp cells.

Inhibition of viral assembly is likely to be a key event that limits succeeding rounds of infection in new host cells. It is known that each cell has the ability to produce a limited number of virus particles (Dulbecco & Vogt, 1954). Inability to infect more cells limits the virus’s ability to spread in the host. This effect was evident in reduced viral copy numbers seen by qRT-PCR and in less intense and fewer immunoreactive signals for all three YHV structural proteins in the tunicamycin-treated shrimp. The end result was less severe disease in the treated group.

To determine which glycosylation site(s) is or are important for viral enveloped particle formation will require the use of good reverse genetic and cell culture systems. Unfortunately, neither is yet available for shrimp virus studies. Empirical studies done on shrimp infected with atypical YHV isolates may shed some light on the process. For example, a non-virulent strain of YHV called atypical YHV produced only non-enveloped nucleocapsids in infected shrimp tissues, and it was speculated that this and its lack of virulence may have resulted from a 162 bp mutation in the ORF3 gene region that codes for gp116 protein (Gangnombre et al., 2009). The 162-base deletion included two potential N-linked glycosylation sites, at least one of which is utilized in the virulent prototype YHV (YHV genotype 1a) (Soowannayan et al., 2010). However, in a later report also from Thailand, another virulent YHV isolate (called YHV type 1b) found in cultured Penaeus vannamei (Sittidilokratna et al., 2009) contained the same 162-base deletion. Although no TEM study was done, evidence from SDS-PAGE and Western blot analysis of the purified virus preparation suggested that enveloped particle formation did occur, and this was confirmed later
by TEM revealing YHV type-1b particle morphology indistinguishable from that of the prototype YHV type 1a (Senapin et al., 2010). Unlike YHV type 1a, the amount or molar ratio of the truncated gp116 protein that was incorporated into virions of type 1b was found to be less than that of gp64 (Sittidilokratna et al., 2009). From these results, the absence of these two glycosylation sites on the gp116 protein did not have any detrimental effect on virion formation but may have been responsible for its slightly reduced virulence when compared with the prototype (Sittidilokratna et al., 2009).

In summary, our data indicate that N-linked glycosylation is necessary for production of mature, enveloped viral particles of YHV. Inhibition of the process with tunicamycin results in blockage of viral spread from initially infected cells to uninfected cells, reducing the severity of disease. Thus, targeting glycosylation alone or together with other more obvious processes such as the viral RNA replication might be a good strategy for development of antiviral therapy in shrimp.

METHODS

Shrimp. Juvenile giant tiger shrimp P. monodon (180 shrimp, ~20 g each) were obtained from the Shrimp Genetic Improvement Center (SGIC, Suratthani, Thailand). The animals were acclimatized for 7 days in aerated aquaria containing artificial seawater at 5 parts per thousand salinity and 24–30 °C. The shrimp were fed twice daily with commercial shrimp pellets throughout the experiment.

Tunicamycin. Tunicamycin from Streptomyces chartreusis was purchased from Fermentek. Stock tunicamycin was made by dissolving 50 mg tunicamycin in 1 ml DMSO and was further diluted with sterile water to 400 µg ml⁻¹ before use.

YHV stock preparation. The YHV strain used in this study originated from moribund shrimp obtained in 1999 from a commercial shrimp farm in Chonburi, Thailand, where outbreaks of yellow head disease had occurred. Crude virus or virus inoculum was prepared as described earlier (Soowannayan et al., 2003, 2010). Briefly, haemolymph from an infected shrimp was drawn into a syringe containing an equal volume of shrimp salt solution (450 mM NaCl, 10 mM KCl, 10 mM EDTA, 10 mM HEPES), mixed thoroughly before haemocytes were pelleted by centrifugation at 2000 g and kept at ~80 °C. The supernatant containing virus was filtered through a 0.45 µm membrane filter. The filtrate was then aliquoted and stored at ~80 °C for use as inoculum for experimental infections. This virus has been used in many studies in our laboratory since it was first isolated from infected shrimp (Soowannayan et al., 2002, 2003, 2010, 2011).

Tunicamycin injection experiment. Two experiments were performed to test the effect of tunicamycin, the N-linked glycosylation inhibitor, on YHV infection in P. monodon. The first experiment was set up to test if tunicamycin had any effect on the mortality of YHV-challenged shrimp. For this, 52 shrimp were divided into four groups of 13. Shrimp in the first group were injected intramuscularly at the third abdominal segment. Shrimp in the third group were injected with 100 µl containing 400 µg tunicamycin ml⁻¹ only. Shrimp in the last group were kept as an un.injected control. Shrimp mortalities were monitored at 12, 24, 36, 48, 55 and 72 h p.i.

The second experiment was designed to examine the effects of N-linked glycosylation on YHV replication, envelopment and infectivity. For this, 136 shrimp were divided into four groups (44 shrimp per group) and injected as in the first experiment. Eight shrimp from each group were randomly collected at 12, 24, 36 and 48 h p.i. From each shrimp sample, 0.5 ml haemolymph was drawn into a syringe containing 0.5 ml salt solution, and one lobe of LO was collected and fixed with 0.5 ml electron microscopy fixative (4 % paraformaldehyde, 0.1 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) for 1 h. These LO tissues were then processed for embedding with the water-miscible resin LR White (London Resin) following the manufacturer’s method. The remainder of the shrimp body was fixed with Davidson’s fixative and processed for paraffin embedding as described by Bell & Lightner (1988).

Histology. Paraffin-embedded shrimp tissues were cut at 6 µm thickness. The sections were dewaxed rehydrated and stained with H&E as described by Bell & Lightner (1988). The stained tissues were studied under a light microscope. Severity scores (1–5) were assigned for each shrimp sample. The scoring system was as follows.

The highest score of 5 was given to samples where generalized pyknotic and karyorrhectic (apoptotic cells) were observed in both spheroids and in functional tubules of the LO and to samples where the spheroid and tubules had degenerated.

A score of 4 was given to samples where generalized apoptotic cells were observed in both functional tubules and in spheroid bodies of the LO but with these structures intact.

A score of 3 was given to samples where apoptotic cells were observed in both functional tubules and in pre-existing spheroids (generally types B and C) and not in newly formed spheroids (type A).

A score of 1 was given to samples where few apoptotic cells were observed in functional tubules and in pre-existing spheroids.

A score of 0 was given to samples where no apoptotic cells were observed in functional tubules but where a few might be observed in spheroid bodies. Spheroids are formed in shrimp as a non-specific response to both infectious and non-infectious agents.

Immunohistochemistry for light microscopy. Immunohistochemistry for light microscopy was carried out using mAbs against all three YHV structural proteins [i.e. the nucleocapsid protein (clone Y-19), gp116 (clone 3-2B) and gp64 (clone Y-18)] (Sithigorngul et al., 2000, 2002). All antibodies were kindly provided by Professor Paisarn Sithigorngul of Srinakarinriwroat University, Thailand. The immunodetection was done as described previously (Soowannayan et al., 2003). Briefly, three adjacent paraffin sections of each shrimp sample were dewaxed, rehydrated and incubated with one of the three mAbs against YHV for 1 h at 37 °C before they were washed three times with PBS at room temperature. The tissues were then incubated with goat anti-mouse antibody labelled with HRP (GAM–HRP; Vector Laboratory) for 30 min at 37 °C before the unbound antibodies were removed by washing three times with PBS at room temperature. The enzyme reactions were then detected by incubation with a solution containing the chromogenic substrate (30 mg diaminobenzidine, 30 µl 30 % H₂O₂ in 100 ml PBS) for 3–10 min or until a brown precipitate was observed. The reactions were stopped by washing in
water. The tissues were then counter stained with H&E as described earlier and examined under a light microscope.

TEM. To study the effect of tunicamycin on viral particle envelopment or viral maturation, a piece of LO from each shrimp was fixed and processed for embedding with LR White resin. Thin sections were cut using an ultramicrotome (Leica), stained or contrasted with uranyl acetate and lead citrate for study by TEM (Hitachi H7100).

Virus purification, negative staining, SDS-PAGE analysis, Western blotting and glycoprotein staining. To study the effect of tunicamycin on the release of viral particles from infected cells into the shrimp haemolymph, purified virus was prepared/separated from the haemolymph sample of both treated and untreated shrimp groups. Virus purification was carried out as previously described (Soowannayan et al., 2003). The virus preparations obtained were placed onto carbon-coated, formvar-treated nickel grids, exposed to a mouse mAb against YHV envelope protein gp116 (clone V3-2B), exposed to a 10 nm gold particle-labelled goat anti-mouse antibody and contrasted with uranyl acetate and lead citrate (Soowannayan et al., 2003) before observation using TEM.

Aliquots (~6 µg) of purified viral preparation from treated and untreated shrimp together with molecular mass standard proteins (PageRuler Prestained Protein ladder; Thermo Scientific) were separated SDS-PAGE (12.5 % acrylamide) and electrotransferred onto a nylon membrane (Hybond-C; Amersham Pharmacia) in a Towbin transfer buffer (25 mM Tris/HCl, pH 8.3, 192 mM glycine, 20 % methanol and 1 % SDS) at 80 mA constant current for 1 h using a Semiphor semiblotting system (Hoefer). The membrane was blocked for 30 min in incubation in a blocking buffer (5 % skimmed milk in TBS-T (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, containing 0.1 %, v/v, Tween 20)) before incubation with a mixture of two mAbs against YHV gp116 (clone 3-2B) and gp64 (clone Y-18) proteins diluted 1:500 in blocking buffer for 1 h at room temperature. The membrane was washed by three 10 min incubations in TBS-T, before it was incubated for 30 min at room temperature with GAM–HRP diluted 1:1000 in TBS. The unbound GAM–HRP was removed by three 10 min incubations in TBS. Colour development was achieved by 1–5 min incubation in 100 ml PBS containing 30 mg diamino- benzidine (Sigma) and 30 µl 30 % H2O2. The reaction was stopped by washing the membrane in distilled water and the membrane was scanned using an image scanner (Epson Perfection 1650).

To determine if envelope glycoproteins of the virus were present in the virus preparations, aliquots (~8 µg) of purified viral preparation from treated and untreated shrimp were separated by SDS-PAGE (12.5 % acrylamide) and stained with glycoprotein-specific Pro-Q Emerald 300 dye (Molecular Probes, Invitrogen) following the manufacturer’s instruction and visualized with a 300 nm UV transilluminator equipped with an image documentation system (Syngene). This method relies on the oxidation of carbohydrate groups by periodic acid, which can then conjugate to the fluorescent hydrazide Pro-Q Emerald 300 dye and allow glycoproteins to be detected under UV light. After glycoprotein-specific staining, the same gel was stained with CBB and the stained gel was scanned using an image scanner (Epson Perfection 1650).

RNA extraction and YHV qRT-PCR. To determine the relative copy numbers of YHV in the stock virus inoculum and in shrimp haemolymph by qRT-PCR, a standard curve was first created. Eight serial dilutions of a solution containing a plasmid carrying the YHV polymerase gene (0, 10, 102, 104, 106, 108, 1010 and 1012 copies µl−1) were prepared and used as templates for PCRs. Triplicate PCRs were performed using iQ SYBR Green Supermix reagent (Bio-Rad) and a 7900 Real-Time PCR System (Applied Biosystem). The PCR primers used were YHV Pol F (5′-CAATCAAGTGCCCGAAGAAT-3′) and YHV Pol R (5′-GATTGATGATGGCGAGAAT-3′). This pair of primers was designed to target a 164 bp fraction of the virus polymerase gene. The PCR cocktail (20 µl) contained iQ SYBR Green Supermix (10 µl), DNA template (1 µl), primers (0.5 µl 10 µM primer for each) and sterile water (8 µl). The PCR conditions used were as follows: 50 °C for 5 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were collected at these steps. Dissociation conditions were set as the defaults of the machine. Each RT-PCR generated a Ct value, the cycle number at which the fluorescent intensity of PCR amplicons rose above that of background level. Mean Ct values for each dilution were then calculated. A standard curve was made by plotting plasmid copy numbers against their respective Ct values (mean).

Shrimp total RNA and stock viral RNA were extracted from 200 µl of 1:1 mixture of cell-free haemolymph or pelleted cells homogenate and shrimp salt solution. RNA extraction was done using TRIzol reagent (Life Technologies) following the manufacturer’s protocols. The total RNA obtained was resuspended in 25 µl DEPC-treated water, quantified by spectrophotometry measuring absorbance at 260 nm (A260) and stored at −20 °C. Total RNA (250 ng) from each sample was transcribed into cDNA (20 µl per reaction) using a iScript cDNA synthesis kit (Bio-Rad Laboratory) following the manufacturer’s protocols. The cDNAs obtained were collected and kept at −20 °C before use as templates for RT-PCR.

To estimate the YHV RNA copy number in the stock virus and in haemolymph or haemocytes of shrimp in both YHV-infected groups, cDNA prepared from these samples (1 µl per 20 µl reaction or equivalent to 12.5 ng cDNA per 20 µl reaction assuming that the reverse transcriptase reaction was 100 % efficient) were used as templates for RT-PCR using the same conditions as above, in triplicate. Ct values (mean) obtained were then compared with those in the standard curve to extrapolate copy numbers. Based on the assumption that the RNA extracts were dominated by host RNA and that viral RNA would proportionally constitute a much smaller amount, fixing the RNA template concentration for the RT step to be equivalent for each sample type and fixing the volume of the cDNA solution from the RT step made it possible to determine relative copy numbers of YHV for each test.

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