Characterization of white spot syndrome virus replication in \textit{in vitro}-cultured haematopoietic stem cells of freshwater crayfish, \textit{Pacifastacus leniusculus}

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Replication of \textit{White spot syndrome virus} (WSSV) was investigated in haematopoietic cells (hpt cells) derived from haematopoietic tissue (hpt) of freshwater crayfish, \textit{Pacifastacus leniusculus}. Temperature and type of inoculum for virus replication were studied. The cell culture remained viable at a wide range of temperatures ranging from 4 to 25 °C. WSSV replicated in cells, as evidenced by \textit{in situ} hybridization, RT-PCR and by the presence of virions visualized with an electron microscope. Moreover, the results showed that the infectivity of WSSV to hpt cells is dependent on temperature and a supplemented growth factor (cytokine) astakine. WSSV replicated more rapidly at higher temperatures than at lower temperatures. No virus replication was observed at 4 °C. Detectable WSSV-infected cells were present as early as 36 h post-inoculation, demonstrated by \textit{in situ} hybridization or RT-PCR of VP28 expression at 25 °C. Hpt cells can survive a few weeks at 25 or 16 °C and longer than several months at 4 °C.

INTRODUCTION

Among cultured crustaceans, penaeid shrimp are of considerable economic importance. Rapid development and expansion of shrimp farming have occurred throughout the world especially in South East Asia. However, diseases, mainly viral, have resulted in the collapse of the shrimp aquaculture worldwide and hence caused considerable economic losses. Over the past decade, several viral diseases have been discovered and they have had large economic losses. Over the past decade, primary cultures obtained from various sources have been used and are reported with increasing frequency in the literature. Explants or dissociation techniques are often used to obtain cells from various tissues and organs of penaeid shrimp, including the lymphoid (Oka) organ (Chen & Kou, 1989; Chen & Wang, 1999; Hsu et al., 1995; Kasornchandra et al., 1999; Itami et al., 1999; Owens & Smith, 1999; West et al., 1999; Wang et al., 2000), the heart (Chen et al., 1986; Chen & Wang, 1999; Owens & Smith, 1999), nerve cord (Nadala et al., 1993; Owens & Smith, 1999), gut (Nadala et al., 1993), hepatopancreas (Owens & Smith, 1999) and gonads (Chen et al., 1986; Luedeman & Lightner, 1992; Nadala et al., 1993; Chen & Wang, 1999; Fraser & Hall, 1999; Owens & Smith, 1999). In addition, different media have been described to establish shrimp cell lines. Most media have been modified by adding supplements to commercially available media (L15 and M199), often at double strength (Toullec, 1999; Wang et al., 2000). Cells derived from the ovary and lymphoid tissues have been used and some are susceptible to virus infection, for example, primary cell cultures of the lymphoid organs that are susceptible to WSSV are from \textit{Penaeus monodon} (Kasornchandra et al., 1999; Wang et al., 2000), from the blue shrimp, \textit{Litopenaeus stylirostris} (Tapay et al., 1995, 1997) and from the kuruma shrimp, \textit{Marsupenaeus japonicus} (Itami et al., 1999) as well as ovarian primary cultures from kuruma shrimp (Maeda et al., 2003, 2004). Primary cell cultures of lymphoid organ were also reported to be susceptible to YHV (Lu et al., 1995; Assavalapsakul et al., 2003). Only one report (West et al., 1999) used a mixture of haematopoietic tissues (hpt) and lymphoid cells from...
P. monodon to develop primary cell cultures, but these were not used for viral studies.

To date the mechanism of how WSSV gains entry into the host cell is unknown. Since various tissues are susceptible to WSSV, the viral receptor on the surface of the cells might be a common receptor such as for example integrins, which are present in the membranes of haemocytes (Holmblad et al., 1997) and haematopoietic cells (hpt cells) in crayfish (unpublished data). Several studies have reported that many RGD (Arg–Gly–Asp)-containing viral proteins, such as the penton protein of adenovirus (Huang et al., 1995), the foot-and-mouth virus coat protein (Lea et al., 1995) and the Coxsackie type virus (Roivainen et al., 1994) serve as ligands through which these viruses bind to integrins on the cell surface and then gain entry into the cell. Some proteins of WSSV such as VP28 or VP36B, VP31, VP36A, VP110, VP136A and VP664 also contain RGD motifs (Huang et al., 1997, 1998). Taken together, it is reasonable to suggest that a WSSV infection might be mediated by the adhesion of their RGD structural proteins to the target cells. However, further studies are needed to confirm this.

The present study is aimed at examining the susceptibility of freshwater crayfish, Pacifastacus leniusculus to WSSV. Hpt cells of crayfish can be established in an in vitro system for several months (Söderhäll et al., 2005). Thus, besides pathological studies the use of hpt cell culture may provide a tool to gain a better understanding of the molecular mechanism of WSSV or other virus infections.

**METHODS**

**Cell culture and maintenance.** Cell culture from hpt of P. leniusculus was established as described previously (Söderhäll et al., 2005). Briefly, hpt was isolated from the dorsal part of the cardiac stomach of crayfish as described previously (Chaga et al., 1995). Hpt was washed with CPBS (crayfish phosphate buffer saline; 10 mM Na2HPO4, 10 mM KH2PO4, 150 mM NaCl, 10 mM CaCl2, 10 mM MnCl2 and 27 mM KCL, pH 6-8) and then immediately submerged in 500 μl 0-1% collagenase (type I and IV) in CPBS for 40 min at room temperature to dissociate hpt cells. The isolated cells were washed twice with CPBS by centrifugation at 2500 g for 5 min at room temperature. The pellet was resuspended with modified L15 medium (Sigma-Aldrich) supplemented with 5 μM 2-ME, 1 μM phenylthiourea, 60 μg penicillin ml⁻¹, 50 μg streptomycin ml⁻¹, 50 μg gentamicin (Sigma-Aldrich) ml⁻¹ and 2 mM 1-glutamine then subsequently seeded at a density of 1-2 x 10⁵ cells per 150 μl in 96-well plates or 1-2 x 10⁶ cells per 2-5 ml in six-well plates. Hpt cells were allowed to attach for 30 min at room temperature. The cell monolayer was then supplemented with a crude astakine fraction (AF) (Söderhäll et al., 2005). Complete medium change was performed after incubation with WSSV particles for 6 h to overnight depending on the assigned experiment and thereafter 50% of the medium was changed at 48 h intervals. All plates were further incubated at various temperatures.

**Viral inoculation preparations.** The principal inocula used for virus infection experiments in this study were (i) plasma from WSSV-infected crayfish; (ii) sucrose-gradient purified WSSV or (iii) a gill homogenate from WSSV-infected crayfish. To prepare plasma-containing WSSV, haemolymph was withdrawn from moribund WSSV-infected crayfish. Haemolymph was centrifuged at 2500 g for 10 min at 4°C and then the supernatants were pooled together and centrifuged at 16000 g for 1 h at 4°C. Supernatants were then filtered through 0.45 μM Millipore membrane. To prepare purified WSSV, a gill homogenate from WSSV-infected crayfish was purified by sucrose-gradient ultracentrifugation (van Hulten et al., 2001), except that crayfish saline (CFS; 0-2 M NaCl, 5-4 mM KCl, 10 mM CaCl2, 2-6 mM MgCl2, 2 mM NaHCO₃, pH 6-8) or L15 medium was used to resuspend the pellet. A gill homogenate from WSSV-infected crayfish was prepared as described previously (Jiravanichpaisal et al., 2001). As a control, mock-infected cell culture was treated with plasma, sucrose-gradient purified gill solution or a gill homogenate from normal crayfish, and was then prepared in the same manner as above or UV-inactivated plasma-containing WSSV. The same amount of protein was used as a mock infection in control experiments.

**Treatment with UV-irradiated virus.** WSSV was UV irradiated at 254 nm at a distance of 10 cm for 5 min on ice to eliminate its infectivity. The loss of infectivity was confirmed by injection into crayfish. All WSSV stock solutions or UV-inactivated WSSV solution were kept at -80°C until used.

**WSSV replication in cell culture analysed by RT-PCR.** Total RNA from cells was extracted using Trizol (Gibco-BRL) according to the manufacturer’s instructions. The precipitated RNA was then resuspended in 100 μl diethyl pyrocarbonate (DEPC)-treated sterile water and stored at -80°C until used. These RNA samples were then subjected to DNase treatment with RNase-free DNase I (Ambion) at a concentration of 2 U per sample for 30 min at 37°C. Next, the DNase I enzyme activity was terminated and RNA was extracted once with phenol plus chloroform and once with chloroform only. Finally, RNA samples were precipitated with 2-propanol and the pellets were washed with 70% ethanol and resuspended in 15 μl DEPC-treated sterile water. These RNA samples were used for conventional PCR and RT-PCR to detect the presence of DNA contamination and VP28 gene of WSSV, respectively. For RT-PCR, aliquots (250 ng) were reverse transcribed using 50 μg Oligo(dT)20 primer and ThermoScript reverse transcriptase (Invitrogen). PCRs were subsequently performed according to the manufacturer’s specifications with the following primers: VP28 (GenBank accession no. AF502435) forward, 5'-TCACCTCTTCGGTGCTGGTCG-3' and reverse, 5'-CCACACAAAAAGGTCACACAC-3', and a control RT-PCR analysis of expression of the crayfish housekeeping gene, 40s ribosomal protein, was also undertaken; 40s primers forward, 5'-CCAGGACC-CCAAATCTTCTTAG-3' and reverse, 5'-GAAACTGACACGCGGTTG-3', respectively. Amplification reaction of VP28 was performed for 32 cycles in a 50 μl reaction solution, containing 1-5 μl of the first strand cDNA reaction solution and 10 μM each of the forward and reverse primers. Similarly, amplification reaction of 40S was performed for 28 cycles in the same manner from the same RNA for comparison. The products were then analysed by agarose gel stained with ethidium bromide and visualized by UV light. The expected size of VP28 and 40S were 506 and 359 nt, respectively. In all RNA RT-PCR reactions, RT-PCR without reverse transcriptase was also performed in order to prove that no DNA was present in RNA samples.

**In situ hybridization.** To determine that WSSV replicated in the nuclei of hpt cell culture, hpt cells were fixed in 10% formaldehyde on coverslips and kept in 70% ethanol at -20°C. A digoxigenin (DIG)-labelled WSSV-specific probe was prepared. Hybridization and staining procedures were carried out as described previously (Jiravanichpaisal et al., 2001) except that the concentration of Proteinase K (Roche) was decreased to 0·5 μg ml⁻¹.

**Temperature-dependent viral binding and replication of WSSV in hpt cell culture.** Hpt cells were aliquoted onto plates and then each plate was incubated at different temperatures: 4, 16,
25 and 32 °C for different time intervals 36 h, 3, 5 and 7 days. To test whether temperature affects virus binding to hpt cells, the cells were allowed to attach to the well surface for 30 min at 4, 16 or 25 °C, respectively. WSSV particles were added and then further incubated for 6 h. Free virions were removed by washing the cells and all plates were incubated at 25 °C. After 4 and 5 days, the infected cells from all plates were checked for virus replication by using RT-PCR.

**Effect of astakine in plasma on the susceptibility of hpt cells to WSSV.** Hpt cells from crayfish were prepared and cultured in 96-well plates. Then, the same amount of WSSV was added to all wells. After adsorption overnight at 25 °C, the inoculum suspension was removed and the cells were washed twice with medium. Then, fresh medium with 5% AF was added to six wells containing hpt cells and only medium was added to another six wells. For the duration of the experiment, 50% of incubation medium was changed every second day. On day 4 and 6, cells from all wells were washed twice with CPBS and then total RNA was extracted from both treatments to monitor WSSV infection by using RT-PCR.

To investigate whether integrin receptors might be involved in virus entry, hpt cells were incubated with a peptide including an RGD motif [Gly–Arg–Gly–Asp–Ser (Sigma)] or a peptide with reverse amino acids, Ser–Asp–Gly–Arg–Gly (Sigma) as a control, for 2 h at 25 °C. The final concentrations of both peptides in the medium were 50 and 100 μM, respectively. Then, WSSV was added to all wells. After incubation with WSSV overnight, the cultures were washed with medium three times to remove viral particles and were further incubated for 5 days at the same temperature. WSSV-infected cells were monitored by using *in situ* hybridization and RT-PCR methods.

**Transmission electron microscope.** At 5 days post-infection, hpt cells were washed three times with CPBS and then fixed in 2.5% glutaraldehyde in CPBS at room temperature for 1 h. These cells were mechanically harvested using a scraper and then centrifuged at 9500 g for 10 min. The pellet was post-fixed in 1% osmium tetroxide for 1 h then washed again with CPBS. The fixed cells were dehydrated in a series of ethanol followed by acetone and then embedded in Epon resin. Sections were collected with a LKB microtome and a diamond knife. Sections were stained with uranylacetate and lead citrate.

**Virulence of WSSV replicated in hpt cell culture.** Cell-free WSSV was prepared from WSSV-producing hpt cell culture. Hpt cells were cultured in a six-well plate at a density of 1·0 × 10⁵ cells per well and then incubated at 25 °C. Following 5 days of viral inoculation, cells were washed with CPBS buffer three times, harvested, homogenized in CFS buffer and then filtered through a 0.45 μm filter (Millipore) aliquoted and stored at −80 °C until use. Before the infectivity test, all cell-free virus solutions were adjusted to the same amount of protein (0·85 mg ml⁻¹) and 200 μl of each solution was injected into each crayfish. Each injected crayfish was monitored by PCR amplification of viral sequences in haemocytes.

**RESULTS**

Hpt cells formed monolayers of round cells of different sizes (Fig. 1a). When a crude AF was added to the medium, three types of cells could be observed after 2–3 days: round, epithelial-like and fibroblast-like cells (Fig. 1b) and these cells proliferated and adhered well to the substratum (Söderhäll *et al.*, 2005). The cells could be maintained in culture for more than 1 month at 16 °C with supplemented AF. AF stimulated the formation of round cells to fibroblast-like cells (Fig. 1c) and more than 80% of cells differentiated into an elongated shape in a dose-dependent manner of AF protein (1·5, 3 or 4·5 μg per well). However, at 25 °C and a low concentration of AF (1·5 μg per well) hpt cells survive for a longer period of time than if a high concentration of AF was added to the cells. At 4 °C, hpt cell culture supplemented with AF in concentrations ranging from 1·5 to 4·5 μg per well could survive for several months. More elongated cells were observed when higher concentration of AF was added to the culture.

Various WSSV preparations were tested for their efficiency as inoculum in hpt cell culture. The first WSSV inoculum was extracted from the gills, the second was crayfish plasma-containing WSSV and the third was sucrose-gradient purified WSSV from gill tissue. All tissues used were obtained from heavily infected crayfish. The different inocula were confirmed for the presence of WSSV and then injected back into healthy crayfish. For controls, the inoculum was prepared in the same manner, but all tissues were obtained from healthy crayfish and then injected back...
into healthy crayfish. The results showed that the non-puri-
fied gill extract inoculum was harmful to hpt cells, whereas
the purified gill extract or plasma-containing WSSV were
both non-toxic. In subsequent experiments, only the latter
WSSV preparations were used.

If hpt cells were induced to proliferate by AF for 3 days at
16°C, the cell monolayers showed approximately 80% of
fibroblast-like cells (Fig. 1c). Then, plasma-containing
WSSV was added and the fibroblast-like cells rounded up
(Fig. 2a). In contrast, if the fibroblast-like cells were
treated with the same plasma-containing WSSV, in which
WSSV had been inactivated with UV light for 5 min,
then the appearance of fibroblast-like cells still remained
(Fig. 2b).

Infected cells shrunk, were rounded and were also to a large
extent detached from the surface of the culture vessels when
compared with the control. All detached cells in the medium
were collected by centrifugation and the resuspended pellet
was used to detect WSSV by using PCR, an intense positive
band for the presence of WSSV was found.

To verify that WSSV was replicating inside the nucleus of
hpt cells, we detected WSSV particles by using in situ
hybridization (Fig. 3a and b). Infected cells attached to the
surface of the coverslip had a dark-brown signal as shown
in Fig. 3(b) when compared with the control in Fig. 3(a).

Electron microscope examination of ultrathin sections of
uninfected and infected hpt cells (Fig. 4a–c), showed several
WSSV particles in the nucleus of infected cells (Fig. 4c,
arrows). Hpt cells from another species of freshwater
crayfish, *Astacus astacus*, were isolated and cultured with the
same medium supplemented with *P. leniusculus* AF. Then,
these cells were used to replicate WSSV and this virus was
also able to replicate in these cells.

Based on our previous study, susceptibility of WSSV in
crayfish was shown to be highly dependent on tempera-
ture (Jiravanichpaisal et al., 2004). To confirm this, a time-
course study of WSSV replication in hpt cells in vitro was
performed at various temperatures: 4, 16, 25 and 32°C. As
shown in Fig. 5, replication of WSSV was determined by
RT-PCR of VP28. The amount of WSSV gradually increased
from 36 h to day 7 at both 16 and 25°C. This suggests that
WSSV replicated efficiently at temperatures from 16°C and
higher. At 4°C, hpt cells were still healthy with a high
survival rate, but the cells did not proliferate, and hardly
any spreading and no virus replication occurred at 4°C. In
contrast, at 32°C, we found that the cultured hpt cells
were unhealthy with a low degree of survival. However,
replication of WSSV could be observed at this temperature
after incubation for 36 h.
We also performed experiments to reveal how efficient WSSV particles were at binding hpt cells at different temperatures for a short period of time (6 h). After incubation with virus for 6 h at different temperatures, hpt cells were washed to remove non-bound virus and the cultures were incubated for 5 days at 25 °C. As shown in Fig. 6, only a very small amount of WSSV was found to infect the cells at 4 °C, whereas at 25 °C WSSV efficiently infected cells and replication at the same temperature was very high.

To demonstrate that WSSV particles that replicated in hpt cell culture maintained their virulence, WSSV-infected hpt cells were homogenized in CFS and then injected into three crayfish. These crayfish died within 5–6 days, while no crayfish died after injection with only homogenized non-infected hpt cells.

Astakine is necessary to promote cell proliferation and differentiation of hpt cells and hence the effect of crude AF on WSSV susceptibility was studied. As a result, WSSV replication in hpt cells with AF was higher than in hpt cells without AF as shown in Fig. 7, and the intensity of WSSV bands at days 4 and 6 were higher than in hpt cells without any AF addition.

Experiments were also carried out to reveal whether WSSV infection could be affected by a peptide containing an RGD motif (GRGDS). We expected that this peptide would block integrin receptors on hpt cells and therefore infection would be inhibited if WSSV utilizes integrin as a receptor for entry into host cells. A synthetic peptide in which the RGD motif was changed to DGR (SDGRG) was used as a control, since this peptide will not bind to integrin. However, although the RGD peptide was incubated with cells 2 h before WSSV was added to the medium and the concentrations of the synthetic peptides were maintained throughout the experiment, no effect of this peptide on WSSV infection was observed.

**DISCUSSION**

In a previous study, conditions for a successful proliferating culture system for hpt cells from *P. leniusculus* have been described (Söderhäll et al., 2005). Compared with the in vivo system, an in vitro culture system is important to allow a
any CPE foci. Therefore, we suggest that WSSV affects WSSV particles in the nucleus of target cells during virus infection. Wang et al. (2002) also observed massive WSSV particles in the nucleus of target cells in vivo without any CPE foci. Therefore, we suggest that WSSV affects individual cells of hpt cell culture in vitro in the same manner as in vivo.

Interestingly, when plasma-containing WSSV was added to differentiate hpt cells these fibroblast-like cells rounded up, whereas UV-inactivated plasma-containing WSSV had no effect. This clearly shows that live WSSV is the causative agent of this rounding up of hpt cells.

Although the amount of WSSV particles inside hpt cultured cells was much lower compared with in vivo infected cells, WSSV that replicated in the cell culture was highly infective because crayfish died after injection of homogenized infected cultured cells. This strongly suggests that replication of WSSV was complete and mature virions were produced in the cell culture. Furthermore, the cells can survive at a wide range of temperatures that make them very useful for studies on virus isolation, replication and pathology. To date more than six viruses have been found to infect crayfish, but only pathological studies and electron microscopical examinations have been reported so far (Edgerton et al., 2002). Our hpt cell culture is a useful tool for studying in detail the interaction between crayfish and shrimp viruses and their target cells.

Since astakine promotes hpt cell proliferation (Söderhäll et al., 2005) as well as virus replication, it is likely that dividing cells favour the replication of WSSV. This is also supported since at low temperature there is no virus replication or cell proliferation, whereas at higher temperatures, above 16 °C, both virus replication and cell proliferation are high (Jiravanichpaisal et al., 2004). This clearly suggests that temperature affects WSSV replication regardless of the host-immune response and/or host variation.

Virus entry is initiated when a virion first binds to the surface of a potential host cell. The events that occur during the first encounter are unclear and are likely to vary for different viruses. Viral attachment is mediated by viral attachment proteins on the surface of the virus particle and virus receptors expressed on the target cell (Klasse et al., 1998). Integrins are one member of virus receptors for enveloped viruses (Wang et al., 1992). However, in the present study, GRGDS peptides containing the RGD motif did not display any effects on the infection efficiency of WSSV. This may suggest that this virus is not using integrins of the host cell as a receptor to gain entry into host cells and that they use alternative receptors when integrins are not available. In many systems, viruses appear to bind directly to the cell surface molecules that mediate internalization. These can be considered primary receptors. In the absence of these primary receptors, viruses may use other cell surface components for entry into cells. For instance, HIV-1 can bind to both CD4 and galactosylceramide and Semliki Forest virus can bind to MHC class I and to an unknown receptor (Klasse et al., 1998). Moreover, threonine (T) at the fourth position (RGDT) has been reported to be important for any protein to bind to integrins via the RGD motif (Plow et al., 2000). Tsai et al. (2004) has suggested that two structural

![Fig. 7. RT-PCR analysis of VP28 (a) and 40S (b) in hpt cells cultured with or without AF. Lanes 1, 2 and 5, 6: hpt cells cultured with AF for 4 or 6 days post-viral inoculation, respectively. Lanes 3, 4 and 7, 8: hpt cells cultured without AF for 4 or 6 days post-inoculation, respectively.](image-url)
proteins, VP36A and VP31, contain a threonine at the fourth position after RGD. Hence, these two structural proteins might interact with integrin of the host cell and generate the infection.

Most insect cell lines are cultured at 25–29 °C. These temperatures are probably suitable for most insect cells, since they are consistent with the temperatures at which the insects live and thrive. However, Winstanley & Crock (1993) showed that initiating and maintaining culturing moth cell lines at lower temperatures (18 or 21 °C) was instrumental in keeping them permissive to an insect baculovirus. In freshwater crayfish, although 16 °C is suitable for hpt culture (Söderhäll et al., 2005), a higher temperature is required to make these cells more susceptible to WSSV in both viral binding and replication. This result supports our previous study in vivo (Jiravanichpaisal et al., 2004) that replication is more efficient at a higher temperature. Since, 32 °C is not a suitable temperature for hpt culture, the efficiency of WSSV replication is also low as shown in Fig. 5, where at day 3 WSSV replication is reduced when compared with 36 h. However, this result does not clearly explain that 32 °C or higher temperatures will affect WSSV replication directly. This problem can be elucidated by using warm-adapted crayfish or shrimp to investigate this problem. However, it has been suggested that hyperthermia, at 32 °C, might facilitate apoptosis in WSSV-infected shrimp and might be one of the mechanisms responsible for increased survival of infected shrimp maintained at 32 °C (Granja et al., 2003).

In conclusion, hpt cell culture can be used to replicate WSSV and most likely other crustacean viruses, especially those which are specific for freshwater crayfish and infect hpt cells. But perhaps of greater importance is the possibility to use this proliferating cell culture to study the ways in which different viruses bind to and gain entry into host cells and further the interaction between virus and host cells. This is particularly important for crustaceans since molecular genetic tools are still not available.

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REFERENCES


