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Crayfish hematopoietic tissue cells but not hemocytes are permissive for white spot syndrome virus replication

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ABSTRACT

Hemocytes are the major immune cells of crustaceans which are believed to be essential for the pathogenesis of white spot syndrome virus (WSSV) infection. Crayfish hemocytes and hematopoietic tissue (HPT) cells have been found to be susceptible to WSSV infection, but the procedure of WSSV infection to both cell types has not yet been carefully investigated. In this study, we analyzed the infection and proliferation of WSSV in crayfish hemocytes as well as HPT cells in detail through transmission electronic microscopy (TEM). The results showed that WSSV could enter both hemocytes and HPT cells through endocytosis, but the production of progeny virus was only achieved in HPT cells. Further investigation demonstrated that although WSSV could transcribe its genes in both cell types, viral genome replication and structural protein expression were unsuccessful in hemocytes, which may be responsible for the failure of progeny production. Therefore, we propose that both hemocytes and HPT cells are susceptible to WSSV infection but only HPT cells are permissive to WSSV replication. These findings will extend our knowledge of the interaction between WSSV and the host immune system.

1. Introduction

White spot syndrome virus (WSSV) is a DNA virus that replicates in the nucleus of infected cells [1]. It has a broad host range among aquatic crustaceans including shrimp [2,3], crayfish [4,5], crabs [6,7] and lobsters [8,9], and is believed to infect cells of ectodermal and mesodermal origins [10,11].

Hemocytes are critical players in crustacean immune system which can function in non-self recognition, phagocytosis, encapsulation, melanization, and coagulation [12]. Because of their crucial role in crustacean immunity, there are a number of studies concerning the responses of hemocytes to WSSV infection. Notably, at the late stage of infection, a dramatic decrease in the number of circulating hemocytes is observed in both penaeid shrimp [13,14] and crayfish [15,16], which suggests a link between hemocyte depletion and the death of infected animals. The lifespan of mature hemocytes and the efficiency of hematogenesis are two key factors that determine the number of the circulating hemocytes. Previous researches have demonstrated that both hemocytes and the hematopoietic tissue (HPT) cells of crayfish are susceptible to WSSV infection. The uptake of WSSV virions and transcription of viral genes were observed in both cell types [17–19]. In addition, WSSV has been found to induce apoptosis of host hemocytes [5]. However, the fate of the virus within hemocytes and HPT cells has not yet been carefully investigated.

In this study, by comparing cell entry, gene expression, genomic replication and progeny production of WSSV in crayfish hemocytes and HPT cells, we propose that HPT cells but not hemocytes are permissive for WSSV replication.

2. Materials and methods

2.1. Animals

Crayfish *Procambarus clarkii* were purchased from a local market in Xiamen, China, and *Cherax quadricarinatus* were purchased from Xiamen Xinrongteng Aquatic Technology Development Company. *P. clarkii* were used in the *in vivo* experiments. *C. quadricarinatus* were

Abbreviations: WSSV, white spot syndrome virus; HPT, hematopoietic tissue; MOI, multiplicity of infection; hpi, hour post-infection; FBS, fetal bovine serum; TEM, transmission electronic microscopy.

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used in the *in vivo* and *in vitro* experiments. These animals were determined to be WSSV free by PCR [20].

2.2. Virus preparation

WSSV China isolate WSSV-CN02 [16] were propagated in *P. clarkii* and purified by differential centrifugation as previously described [21]. The purified virions were resuspended in TNM (20 mM Tris–HCl, 150 mM NaCl, 2 mM $MgCl_2$, pH 7.5) and quantified by spectrophotometry [22]. The virus stock was supplied with 10% DMSO and then stored at −80 °C.

2.3. Primary cell culture

Hemolymph was taken from the *pericardial sinus* of *C. quadricarinatus* using a sterile syringe preloaded with equal volume of anticoagulant solution (26 mM sodium citrate, 30 mM citric acid, 100 mM glucose, 140 mM NaCl, pH 5.8). Hemocytes were collected by centrifugation 500 × g for 5 min and resuspended in L-15 medium (Gibco) supplemented with 15% fetal bovine serum (FBS). The cells were seeded in 24-well culture plates at a density of 2×10^5 cells/well and maintained at 27 °C. Hpt cells of *C. quadricarinatus* were prepared as described before [17] with some modifications. In brief, HPT was removed from the dorsal part of the stomach, washed once with CPBS (10 mM Na_2HPO_4, 10 mM KH_2PO_4, 0.15 M NaCl, 10 μM $CaCl_2$, 10 μM $MnCl_2$, 2.7 μM KCl; pH 6.8), and then incubated in 500 μl of 0.1% collagenase (type I and IV) (Sigma) in CPBS at 30 °C for 40 min to dissociate HPT cells. HPT cells were pelleted by centrifuging at 500 g for 5 min at room temperature, and washed twice with CPBS. The cells were then resuspended in L-15 medium supplemented with 15% FBS, seeded in 24-well culture plates at a density of 1×10^6 cells/well and maintained at 27 °C. These cells were grown for 24 h before viral infection.

2.4. In vivo WSSV challenge and ultrastructural analysis

Crayfish were injected with purified WSSV virions (at a dose of 10^9 virions each), via the base of the fourth walking leg. At each time point post-infection, hemocytes and HPT samples were collected from three individuals then fixed for ultra thin sectioning. For each crayfish, hemocytes were pelleted from one milliliter of hemolymph and HPT was collected as described in section 2.3. The hemocyte pellets and HPT were fixed with 2.5% glutaraldehyde plus 2% paraformaldehyde in PBS for 2 h at 4 °C, and post-fixed in 1% osmium tetroxide for another 2 h. After stepwise dehydration in ethanol, the specimens were embedded in Epon resin and polymerizated at 60 °C for 24 h. The samples were ultrasectioned and the slices were stained with 2% uranyl acetate and 0.4% lead citrate sequentially, and visualized with a transmission electron microscope (JEM-1230, JEOL). The experiment was carried out in triplicate.

2.5. WSSV infection to primary cultured cells and ultrastructral analysis

Primary cultured hemocytes and HPT cells (grown in 24-well plates) were infected with WSSV at a multiplicity of infection (MOI) of 500. Cells were washed three times with CPBS at 2 h post-infection (hpi) to remove free virions, and grown in fresh medium at 27 °C for indicated periods of time.

At each sampling time point, cells from three wells were directly fixed in-well with 1% glutaraldehyde plus 2% paraformaldehyde for 10 min, collected by centrifugation at 500 × g for 5 min, and post-fixed in 1% osmium tetroxide for 2 h. After stepwise dehydration, embedding and polymerization, the samples were sectioned and observed by transmission electronic microscopy (TEM) as described in section 2.4.

2.6. RT-PCR analysis of WSSV gene transcription

Primary cultured hemocytes and HPT cells were inoculated with WSSV as described in section 2.5. At each sampling time point, cells from three wells were lysed with TRI Reagent (Molecular Research Center). Total RNA was extracted from the mixed lysate from three wells, and residual genomic DNA was digested by RNasefree DNase I (Takara). First-strand cDNA was synthesized with oligo dT(18) primer using Transcriptor First-Strand cDNA Synthesis Kit (Roche), and the genes of interest were detected by PCR with primers listed in Supplementary Table 1. Among the WSSV genes analyzed in this study, *wsv083* and *wsv051* are immediate early genes [19], while structural proteins genes *vp28*, *vp19* and *vp664* are late genes [23]. In RT-PCR experiments, crayfish β-actin gene was used as a loading control and an intergenic region in WSSV genome was used as a control for DNA contamination in the samples [24]. The experiment was repeated three times.

2.7. Real-time PCR analysis of WSSV genomic DNA

Primary cultured hemocytes and HPT cells were inoculated with WSSV as described in section 2.5. At each sampling time point, DNA was extracted from three wells of cells (the culture supernatant was discarded). The viral copies in each well of cells were determined by absolute qPCR using a fluorescently labeled TaqMan MGB probe together with a pair of primers specific to WSSV genomic DNA following the instructions of WSSV-qPCR detection kit (Xiamen Lulong Biotech Co., Ltd., Xiamen, Fujian, China). The amplification reactions were performed as the following: denaturing at 95 °C for 2 min, annealing at 94 °C for 10 s, and extending at 60 °C for 30 s, 40 cycles).

2.8. Western blotting

Primary cultured hemocytes and HPT cells were infected with WSSV as described in section 2.5 at an MOI of 1. At each sampling time point, cells were lysed in-well with SDS-PAGE loading buffer. Protein samples were separated on SDS-PAGE gels, transferred to PVDF membranes, and detected with appropriate primary and secondary antibodies sequentially. Purified WSSV virions were used as a positive control. Anti-VP28 and anti-VP26 monoclonal antibodies were produced by Shanghai Immune Biotech Company (China). Anti-VP51 monoclonal antibody was produced by Abmart (China). The HRP conjugated Goat anti-mouse IgG secondary antibody was purchased from Pierce.

2.9. Immunofluorescence microscopy

Hemocytes and HPT cells were grown in 24-well plate containing a cover slide in each well. Cells were infected with WSSV as described in section 2.5. At each sampling time point, cells from three wells were fixed in 4% paraformaldehyde for 10 min at room temperature, and treated with 0.5% Triton X-100 for 1 min. The samples were probed with anti-VP26, anti-VP28 or anti-VP51 monoclonal antibodies, and incubated with Alexa Fluor 488 Donkey Anti-Mouse IgG (H + L) secondary antibody (Life Technology) sequentially. The nucleus was stained with DAPI. Cells were observed by a Nikon Eclipse 80i fluorescence microscope or a Leica TCS SP5 confocal microscope.