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Fish & Shellfish Immunology 23 (2007) 164–170

Fish & Shellfish Immunology

www.elsevier.com/locate/fsi

Haemocyte reactions in WSSV immersion infected *Penaeus monodon*

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Received 24 August 2006; revised 5 October 2006; accepted 16 October 2006
Available online 24 October 2006

Abstract

White spot syndrome virus (WSSV) has been a major cause of shrimp mortality in aquaculture worldwide in the past decades. In this study, WSSV infection (by immersion) and behaviour recruitment of haemocytes is investigated in gills and midgut, using an antiserum against the viral protein VP28 and a monoclonal antibody recognising haemocytes (WSH8) in a double immunohistochemical staining and in addition transmission electron microscopy was applied. More WSH 8^{+} haemocytes were detected at 48 and 72 h post-infection in the gills of infected shrimp compared to uninfected animals. Haemocytes in the gills and midgut were not associated with VP28-immunoreactivity. In the gills many other cells showed virus replication in their nuclei, while infected nuclei in the gut cells were rare. Nevertheless, the epithelial cells in the midgut showed a clear uptake of VP28 and accumulation in supranuclear vacuoles (SNV) at 8 h post-infection. However, epithelial nuclei were never VP28-immunoreactive and electron microscopy study suggests degradation of viral-like particles in the SNV. In contrast to the gills, the midgut connective tissue shows a clear increase in degranulation of haemocytes, resulting in the appearance of WSH8-immunoreactive thread-like material at 48 and 72 h post-infection. These results indicate recruitment of haemocytes upon immersion infection in the gills and degranulation of haemocytes in less infected organs, like the midgut.

Keywords: *Penaeus monodon*; White spot syndrome virus; Haemocyte; Infection; Gills; Gut

1. Introduction

White spot syndrome virus (WSSV) is an economically important viral pathogen occurring widely in shrimp culture worldwide [1]. In cultured shrimp, WSSV infection can reach a cumulative mortality of up to 100% within 3–10 days [2]. It affects penaeid shrimp such as Chinese shrimp (*Fenneropenaeus chinensis*), Kuruma shrimp (*Marsupenaeus japonicus*), black tiger shrimp (*Penaeus monodon*) and other crustaceans, such as salt, brackish and fresh water crayfishes, crabs and lobsters [3–5].

Tissues of ectodermal and mesodermal origin are the main targets for viral replication [6]. Early in infection, stomach, gills, cuticular epidermis and the connective tissue of the hepatopancreas, but not midgut are reported to be

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doi:10.1016/j.fsi.2006.10.003

WSSV-infected [7–9]. A significant reduction at 48 h after infection in the total haemocyte count is observed after shrimp are infected with WSSV [10,11]. WSSV can be transmitted by predation of diseased individuals, or by free virus particles released in the water. In this study the reaction of haemocytes upon WSSV infection is studied in gills and midgut by immunohistochemistry and transmission electron microscopy. In addition, WSSV infection was followed up to 3 days after immersion in both organs using an antibody against an essential viral peptide (VP28).

2. Materials and methods

2.1. Animals and infection

Healthy *P. monodon* were imported as post-larvae from Malaysia and maintained in a recirculation system at ''de Haar vissen'' at Wageningen University. Each shipment was tested for the presence of WSSV, Monodon baculovirus, yellow head virus, Taura syndrome virus and infectious hypodermal and hematopoietic necrosis virus by a virus specific PCR. Shrimp of approximately 15 g were immersed in 6 μl virus solution (as described by van Hulten et al. [12])/500 ml seawater/shrimp for 4 h. This relative high dose was used to synchronise infection in the gills. After immersion, shrimp ($n = 18$) were transferred to 180 l aquaria and cultured for up to 72 h at 28 °C and a salinity of 20 parts per thousand. WSSV infection in the shrimp was determined in the gills by PCR with VP26 (WSSV) and shrimp actin-specific primers [13] on genomic shrimp DNA and WSSV DNA.

2.2. Immunohistochemistry

Gills and midgut were isolated from shrimp ($n = 3$ at each time point) at 0, 4, 8, 24, 48 and 72 h after infection and immediately fixed for 24–48 h at room temperature (RT) in freshly prepared Davidson's fixative [14] (29 ml Milli Q water, 29 ml 100% ethanol, 19 ml 37% neutral formalin and 10 ml 100% acetic acid). After fixation the organs were stored in 50% ethanol, subsequently dehydrated in ethanol series and embedded in paraffin. Sections of 5 μm were made and after deparaffination in xylol, endogenous peroxidase was inactivated in methanol and H_2O_2 for 30 min (at RT). After hydration, sections were treated with 10% normal goat serum (DAKO, Glostrup, Denmark) followed by washing in PBS. Subsequently, the sections were incubated for 1 h at room temperature with a rabbit VP28 polyclonal antibody (1:200; [12]) and a mouse haemocyte specific mAb (WSH 8; 1:100; [15]). After the first incubation, slides were washed with PBS–T (1 M PBS pH 7.2, 0.1% Triton) followed by a second incubation for 1 h at room temperature with goat-anti-rabbit Ig conjugated to horse radish peroxidase (GAR–HRP: 1:200; DAKO) and goat-anti-mouse Ig conjugated to alkaline phosphatase (GAM–AP: 1:200; DAKO). After washing again, HRP was stained using 3-amino-9-ethyl-carbazole (260 mg/l Na-acetate buffer pH 5) (Sigma; red) and AP was stained with 5-bromo-4-chloro-3-indolyl-phosphate (100 mg/ml; Sigma) and 4-nitro blue tetrazolium chloride (50 mg/ml) (Sigma; blue). All necessary controls were performed and if not specially mentioned found to be negative.

2.3. Transmission electron microscopy

Gills and midgut were fixed for 1 h (at 4 °C) in 1% (w/v) $K_2Cr_2O_7$, 2% (v/v) glutaraldehyde and, after storage, in 1% (w/v) OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.2) and subsequently washed in double distilled water, dehydrated in ethanol followed by propylene oxide and embedded in Epon 812 (Electron Microscopy Science, Fort Washington, USA). Ultra-thin sections were cut on a Reichert Ultracut S (Leica, Rijswijk, The Netherlands) and stained with uranyl acetate and lead citrate. Sections were studied with a Philips EM208 electron microscope (FEI, Eindhoven, The Netherlands) equipped with a SIS digital camera (Soft Imaging System, Münster, Germany).

3. Results

3.1. Gills

Staining with the anti-VP28 antibody against an envelope protein (VP28) of WSSV showed that shrimp were infected with WSSV. Detection of WSSV by PCR with primers designed on another envelope protein (VP26) also confirmed infection (data not shown). With both methods first signs of infection could be detected between 24 and