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White Spot Syndrome Virus Envelope Protein VP28 Is Involved in the Systemic Infection of Shrimp

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White spot syndrome virus (WSSV) is a large DNA virus infecting shrimp and other crustaceans. The virus particles contain at least five major virion proteins, of which three (VP26, VP24, and VP15) are present in the rod-shaped nucleocapsid and two (VP28 and VP19) reside in the envelope. The mode of entry and systemic infection of WSSV in the black tiger shrimp, *Penaeus monodon,* and the role of these proteins in these processes are not known. A specific polyclonal antibody was generated against the major envelope protein VP28 using a baculovirus expression vector system. The VP28 antiserum was able to neutralize WSSV infection of *P. monodon* in a concentration-dependent manner upon intramuscular injection. This result suggests that VP28 is located on the surface of the virus particle and is likely to play a key role in the initial steps of the systemic WSSV infection in shrimp.

Key Words: Penaeus monodon; white spot syndrome virus; envelope; VP28; shrimp infection; *in vivo* neutralization.

INTRODUCTION

White spot syndrome virus (WSSV) is a major disease agent of penaeid shrimp in Southeast Asia, the Indian continent, and in South and Central America (Rosenberg, 2000). The disease is caused by an ovoid-to-bacilliform virus with a rod-shaped nucleocapsid and a tail-like appendix at one end of the virion (Durand *et al.,* 1997; Nadala *et al.,* 1998). The virus contains a double-stranded DNA with an estimated size of 290 kbp (Yang *et al.,* 1997). Genetic analysis indicates that WSSV is a representative of a new virus group provisionally named whispovirus (Van Hulten *et al.,* 2000b; Tsai *et al.,* 2000).

WSSV has a broad host range, infecting several crustacean species, like shrimp, crab, and crayfish (Wang *et al.,* 1998). Little is known about WSSV infection and morphogenesis *in vivo.* Upon infection per os, infected cells are observed first in the stomach, gill, and cuticular epidermis of the shrimp. The infection subsequently spreads systemically in the shrimp to other tissues of mesodermal and ectodermal origin (Chang *et al.,* 1996). Research on virus replication and virion morphogenesis shows that DNA replication and *de novo* envelope formation take place in the nucleus (Durand *et al.,* 1997; Wang *et al.,* 2000). The mechanism of virus entry into the shrimp and of the spread of the virus in the crustacean body is not known.

The virus particle consists of at least five major proteins with estimated sizes of 28 kDa (VP28), 26 kDa (VP26), 24 kDa (VP24), 19 kDa (VP19), and 15 kDa (VP15). VP28 and VP19 are associated with the virion envelope and VP26, VP24, and VP15 with the nucleocapsid (Van Hulten *et al.,* 2000c). Amino acid analysis of VP28, VP26 and VP24 indicated that these proteins have about 40% amino acid identity and that their genes may have evolved from a common ancestral gene (Van Hulten *et al.,* 2000a). The role of the envelope and its proteins in the establishment of the systemic infection process has not been determined.

Neutralization experiments have often been performed to study the role of virion proteins or their domains in the infection process. Neutralizing antibodies bind to envelope spikes on the virion and prevent attachment of the virus to the cell surface, cell entry, or virus uncoating (Burton *et al.,* 2000). For many vertebrate viruses like poxviruses (Galmiche *et al.,* 1999) and hepadnaviruses (Sunyach *et al.,* 1999) *in vitro* neutralization experiments involving cell cultures (plaque reduction assays) have been used for this purpose. For invertebrate baculoviruses, *in vitro* neutralization experiments have been exploited to show that *Autographa californica* nucleopolyhedrovirus (AcMNPV) can be neutralized by complexing the budded virions with specific antibodies against the viral envelope protein (GP64) (Volkman and Goldsmith, 1985). These authors showed that the mechanism of neutralization is by inhibition of virus entry and adsorptive endocytosis. However, standardized (primary) shrimp cell cultures are not available and therefore an *in vivo* approach is followed.

In vivo neutralization experiments have been widely used for many vertebrate viruses and have even led to

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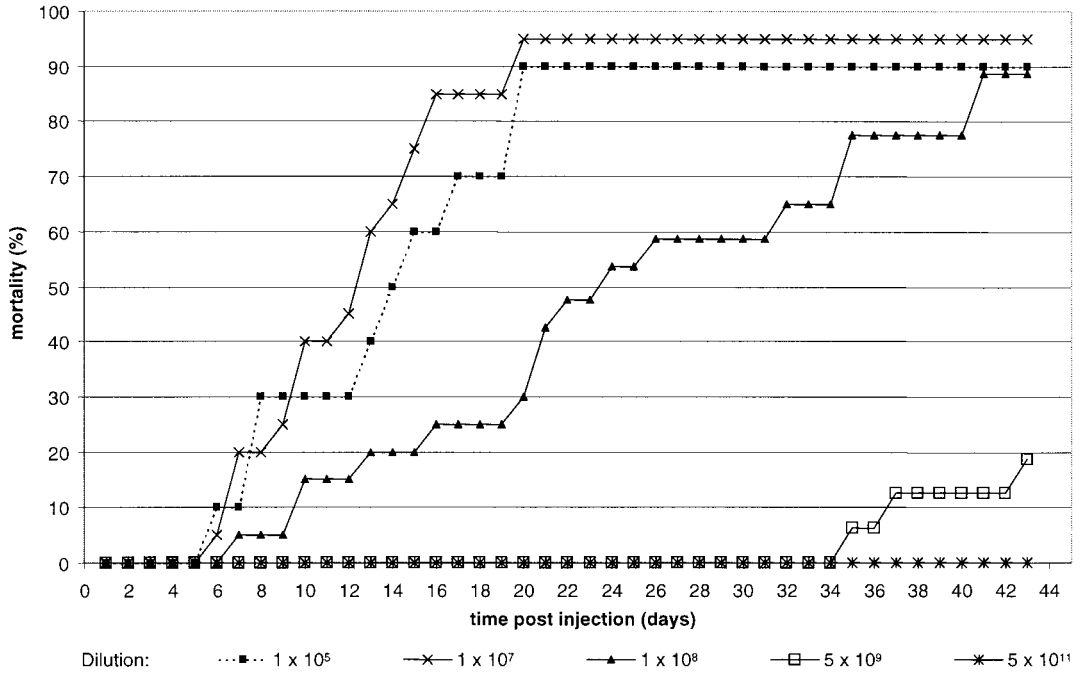

FIG. 1. Titration of WSSV in *P. monodon* shrimp. Days postinjection of the virus are shown on the abscissa and the accumulated mortality (in %) on the ordinate. Ten microliters of a 1×10^5, 1×10^7, 1×10^8, 5×10^9, or 5×10^{11} diluted virus stock was injected.

passive immunization strategies. When combined with the use of monoclonal antibodies, this strategy has been used to identify the virion protein epitope(s) involved in the neutralization (e.g., Schofield *et al.,* 2000). *In vivo* neutralization assays have also been successfully used in insects, e.g., in inhibiting infection of larvae of the Douglas fir tussock moth *Orgyia pseudotsugata* with its nucleopolyhedrovirus (OpMNPV) using OpMNPV antiserum (Martignoni *et al.,* 1980). This strategy has now been applied to shrimp with the added advantage that it is as close as possible to the *in vivo* situation. In this paper we provide evidence that VP28 is directly involved in the systemic infection of the shrimp *P. monodon* by WSSV.

RESULTS

Virus titration

A WSSV virus stock was produced in the crayfish *P. clarkii* by intramuscular injection of purified WSSV. To determine the dilution resulting in 90–100% mortality in the black tiger shrimp *P. monodon,* an *in vivo* virus titration was performed using animals of approximately 1 g in weight. The virus stock was diluted in steps from 1×10^5 to 5×10^{11} times in 330 mM NaCl as indicated (Fig. 1) and for each dilution 10 μl was injected intramuscularly into 10 shrimp. Shrimp that were injected with 330 mM NaCl served as negative control for the infection. All shrimp serving as negative control (not shown) and those having received the 5×10^{11} virus dilution survived, whereas mortality due to virus infection occurred in all groups with a lower virus dilution (Fig. 1). Administration of virus dilutions of 1×10^5 and 1×10^7 resulted in almost 100% mortality in a period of 20 days. A delay in mortality was observed when virus dilutions of 1×10^8 and 5×10^9 were used. The 1×10^8 dilution resulted in 90% final mortality, but the time of mortality was delayed and spanned a period of 40 days. The experiment was repeated with the 1×10^7, the 1×10^8, and the 5×10^9 dilution yielding essentially the same results. The dilution of 1×10^8 was chosen as the virus dose for further experiments as this condition was expected to give the optimal response to the neutralization in terms of mortality reduction.

Antibody against recombinant VP28

The major WSSV envelope protein VP28 was expressed under control of the polyhedrin promoter in insect cells using recombinant baculovirus AcMNPV-WSSVvp28 (Van Hulten *et al.,* 2000c) and was used after purification to raise specific polyclonal antibodies in rabbits. The VP28 polyclonal antiserum reacted strongly with baculovirus expressed as well as with bacterial expressed VP28 (data not shown).

As VP28 shows a considerable degree of amino acid homology with nucleocapsid proteins VP26 and VP24 (Van Hulten *et al.,* 2000a), the specificity of the polyclonal was tested against purified WSSV virions and WSSV nucleocapsids (Fig. 2). All major proteins are present in the virion fraction (Fig. 2A, lane 2) and only VP26, VP24 and VP15 are present in the nucleocapsid fraction (Fig.

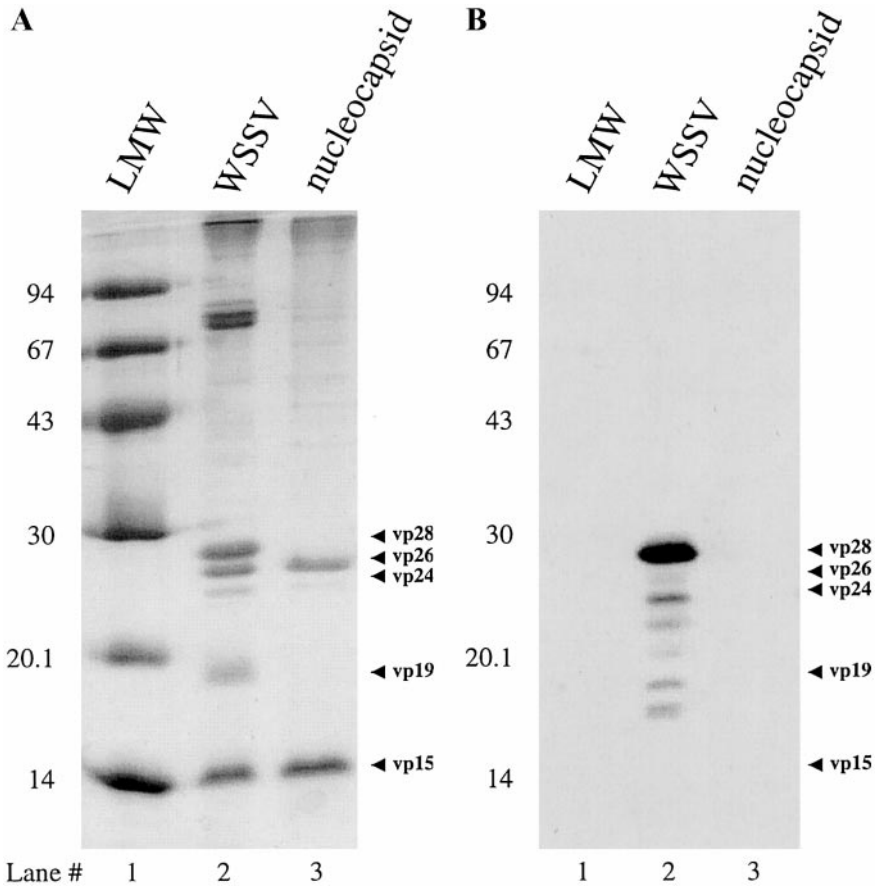

FIG. 2. (A) Fifteen percent Coomassie brilliant blue-stained SDS–PAGE gel of purified WSSV. Lane 1, low-molecular-weight protein marker. Lane 2, purified WSSV virions. Lane 3, purified WSSV nucleocapsids. (B) Western blot of the virions (lane 2) and nucleocapsid (lane 3) of A. VP28 polyclonal antiserum is used and detection is performed with the ECL kit.

2A, lane 3). In the Western analysis (Fig. 2B) the VP28 antiserum (1:5000 dilution) shows a clear reaction with the VP28 present in the WSSV virion (Fig. 2B, lane 2). A minor reaction was observed with smaller products, most likely VP28 breakdown products. There was no reaction with proteins of the WSSV nucleocapsids (Fig. 2B, lane 3). This shows that there is no cross-reactivity of the VP28 polyclonal antiserum with VP26 or VP24, despite the notable degree of amino acid homology.

WSSV neutralization *in vivo*

The VP28 polyclonal antiserum was used in an *in vivo* neutralization assay in *P. monodon*. A constant amount of WSSV was incubated with various antiserum concentrations (Table 1) and injected into shrimp. No shrimp died in the negative control injected only with 330 mM NaCl (group 6). The shrimp in the positive control, which were injected with WSSV only (group 1), showed a 100% mortality at day 23 (Fig. 3). Addition of the preimmune serum (group 2) resulted in a small initial delay in shrimp mortality, which reached 100% at day 25 (Fig. 3). When the virus was preincubated with a 10-fold dilution of the VP28 antiserum (group 3), shrimp mortality was 100% at day 22. Apparently the VP28 antiserum at this dilution is not able to neutralize the virus. When the VP28 serum is diluted only two times (group 4) or used undiluted (group 5), none of the shrimp died, indicating that WSSV can be

TABLE 1

Constitution of Injection Solutions

Group No.	Type	Injection (10 μl total)	No. shrimp
1	Positive control	WSSV	10
2	Preimmune serum control	WSSV + 9 μl preimmune serum	15
3	10× dilution of VP28 antiserum	WSSV + 1 μl VP28 antiserum	15
4	2× dilution of VP28 antiserum	WSSV + 5 μl VP28 antiserum	15
5	VP28 antiserum	WSSV + 9 μl VP28 antiserum	15
6	Negative control	330 mM NaCl	10

Note. The first column shows the group numbers, the second the treatment, the third the content of the 10-μl injected volume, and the fourth the number of shrimp used in each group. The total amount of WSSV injected is the same for groups 1 to 5.

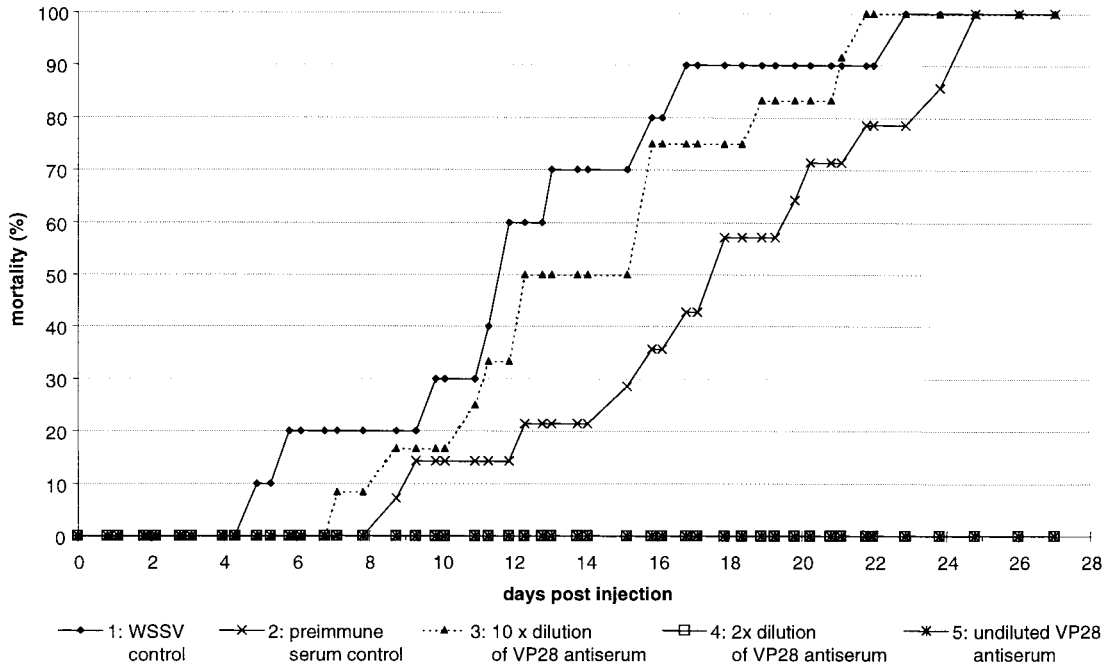

FIG. 3. Neutralization of WSSV infection in *P. monodon* using VP28 polyclonal antiserum. Days postinjection are shown on the abscissa and percentage mortality on the ordinate. Treatments of the five groups used are described in Table 1.

neutralized by the VP28 antibodies in a dose-dependent manner (Fig. 3).

DISCUSSION

To study the role of the major WSSV envelope protein VP28 in WSSV infection, a specific antiserum was produced against this protein. As there is a significant degree of homology between the envelope protein VP28 and the nucleocapsid proteins VP26 (41% amino acid similarity) and VP24 (46% amino acid similarity) (Van Hulten *et al.,* 2000a), a Western analysis (Fig. 2) was performed, confirming the specificity of the VP28 polyclonal antiserum for VP28.

A WSSV virus stock was produced from infected *P. clarkii.* Since an absolute measure of virus activity in infectious units cannot be given, we have determined the highest virus dilution of the virus stock that results in almost 100% mortality upon injection of a fixed volume (10 μl) into shrimp. Shrimp were used for the titration of the virus stock, as no reliable cell culture system is available to measure WSSV infection and the effect of neutralization. Since the 1×10^8 dilution of the virus stock was the lowest dose still resulting in almost 100% final mortality (Fig. 1), this dilution was used for neutralization experiments. In the latter experiments (Fig. 3) the dilution of 1×10^8 resulted in a somewhat quicker mortality (Fig. 3, group 1: WSSV) than in the titration experiment (Fig. 1). This difference in response might be the consequence of the use of a different batch of shrimp. However, this does not influence the results of the neutralization experiment, as a control (no antiserum) was included.

To study the role of VP28 in WSSV infection in shrimp, an *in vivo* neutralization test was performed. This test showed that WSSV infection was neutralized by the VP28 polyclonal antiserum (Fig. 3) and that VP28 is involved in this process. The preimmune serum control resulted in a small delay of shrimp mortality. This could be due to compounds present in the serum stimulating the shrimp defense system. WSSV neutralization using the VP28 polyclonal antiserum was concentration dependent; only the two highest antibody concentrations used in this study resulted in neutralization (Fig. 3).

VP28 is the major protein in the WSSV envelope, but its location in this structure is not known. The neutralizing activity of the VP28 antiserum shown here might depend on the relative abundance of this protein on the virion envelope. However, mere binding of the antibody to the surface of the virus does not automatically result in virus neutralization. The existence of nonneutralizing antibodies, which bind to virus without diminishing infectivity, has long been recognized (Dimmock, 1984). In other virus systems only anti-envelope antibodies binding to the envelope spike on the virion will be neutralizing or show antiviral activity (Burton *et al.,* 2000). Therefore, we postulate that VP28 or its neutralization domain is located in the envelope spike of WSSV virions.

Further research is required to reveal the exact role of VP28 in WSSV infection. Neutralization of viral infectivity by antibodies is a complex and, as yet, poorly under-

stood phenomenon. Studies on the functional domains of proteins suggest that neutralization sites and virus attachment sites are often distinct (Ramsey *et al.,* 1998). Neutralizing antibodies often inhibit a subsequent stage of infection, which is then responsible for the loss of infectivity. There are only a few examples of residues within neutralization epitopes that are also involved in the attachment of the virus to its cellular receptor (Sunyach *et al.,* 1999). For VP28 a similar situation could exist as for the major envelope protein of the budded viruses of AcMNPV, where the virus can be neutralized using antibodies to GP64 (Volkman *et al.,* 1984). Further studies with AcMNPV showed that the mechanism of neutralization is not by inhibition of adsorption, but by inhibition of the fusion of the viral envelope with the cell membrane (Volkman and Goldsmith, 1985). However, alternative mechanisms of neutralization are possible. Inhibition can also take place during uncoating of virus or transport of DNA in the nucleus. Furthermore, binding of antibodies can induce conformational changes in virus proteins and these may be relevant for the neutralization process.

The *in vivo* neutralization experiments on WSSV in *P. monodon* with VP28 antibodies suggest that VP28 is located in the "spikes" of the WSSV envelope and this protein may thus be involved in the systemic infection of WSSV in shrimp. It cannot be excluded that other WSSV envelope proteins, such as VP19, are also involved in this process, either alone or in concert with VP28. Antibodies against VP19 will assist in the elucidation of this point. Future experiments using *in vivo* neutralization will demonstrate which part of VP28 is involved in the neutralization process and what the role of VP28 in WSSV attachment and entry in the systemic infection is.

MATERIALS AND METHODS

Shrimp culture

Cultures of healthy shrimp were performed in a recirculation system at the Laboratory of Fish Culture and Fisheries at Wageningen University. For the experiments shrimp were transferred to an experimental system located at the Laboratory of Virology, Wageningen University, and kept in groups of 10–15 individuals in 60-liter aquariums with an individual filter (Eheim, Germany) and heating (Schego, Germany) at 28°C. *P. monodon* shrimp of approximately 1 g were used in the titration and neutralization experiments.

White spot syndrome virus stock production

The virus isolate used in this study originates from infected *P. monodon* shrimp imported from Thailand in 1996 and was obtained as described before (Van Hulten *et al.,* 2000b). Crayfish *Procambarus clarkii* were injected intramuscularly with a lethal dose of WSSV using a 26-gauge needle. After one week the hemolymph was withdrawn from moribund crayfish and mixed with modified Alsever solution (Rodriguez *et al.,* 1995) as an anticoagulant. The virus was purified by centrifugation at 80,000*g* for 1.5 h at 4°C on a 20–45% continuous sucrose gradient in TN (20 mM Tris, 400 mM NaCl, pH 7.4). The visible virus bands were removed and the virus particles were subsequently sedimented by centrifugation at 45,000*g* at 4°C for 1 h. The virus pellet was resuspended in TE (pH 7.5) and the virus integrity was checked by electron microscopy. The virus stock was stored at −80°C until use in the experiments.

VP28 polyclonal antibody

The major WSSV structural envelope protein VP28 was expressed in insect cells using baculovirus *Ac*MNPV-WSSVvp28 (Van Hulten *et al.,* 2000c). The protein band containing the VP28 was purified using a Model 491 PrepCell (Bio-Rad) according to the instruction manual. Fractions were collected using a Model 2110 fraction collector (Bio-Rad) and analyzed in a silver-stained SDS–PAGE gel. Western blotting using a polyclonal WSSV antibody was employed to determine the VP28-containing fractions. These fractions were pooled and SDS was removed by dialysis against several volumes of 0.1× TE. The protein was subsequently concentrated by freeze-drying and resuspended in 0.1× TE (pH 7.5). The purified VP28 protein (100 μg) was injected into a rabbit to produce a polyclonal antibody. The rabbit was boosted with 300 μg of VP28 after 6 weeks and the antiserum was prepared 2 weeks thereafter.

In vivo injection

P. monodon shrimp of approximately 1 g were injected intramuscularly with 10 μl of virus solution in 330 mM NaCl in the fourth or fifth tail segment of the shrimp with a 29-gauge needle (Microfine B & D). The shrimp were subsequently cultured for a period of 40 days and the mortality was monitored twice daily. For each group 10–15 shrimp were used. Deceased shrimp were monitored for WSSV infection by viewing hemolymph extracts under the electron microscope.

Neutralization assay

Shrimp of 1 g were injected with WSSV in the presence or absence of VP28 antibody. A negative (330 mM NaCl) and a positive control (virus only) were included (Table 1). The total amount of virus administered per shrimp is constant in all groups and is equivalent to 10 μl of the 1×10^8 dilution of the virus stock. The preimmune serum was included as a control for the effect of the serum on shrimp mortality. Several dilutions of the antiserum were incubated with the virus for 1 h at room temperature, prior to injection in shrimp. After injection, the shrimp were monitored for 28 days and dead shrimp were ex-

amined for the presence of WSSV by electron microscopy.

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