

Neutralization of White Spot Syndrome Virus by Monoclonal Antibodies against Viral Envelope Proteins

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ABSTRACT: Two monoclonal antibodies (MAbs) recognizing envelope proteins of the white spot syndrome virus (WSSV), 6E1 against VP28 and 3E8 against VP19, were applied to demonstrate their neutralizing ability to this virus by using both *in vitro* and *in vivo* assays. Mixtures of MAb 6E1 with virus filtrate were inoculated into the primary explant monolayer culture derived from the lymphoid Oka organs of *Penaeus monodon*. MAb was likely to neutralize the infectivity of virus to monolayer since cytopathic effects were apparently blocked in experiment group. WSSV was titrated using Blue-Cell ELISA and the neutralizing index was calculated to be 6.90 for 6E1 and 5.83 for 3E8. Neutralized virus fluids injected intramuscularly into post larvae of *P. monodon*. The shrimp in the positive control, which were injected with WSSV only showed an increasing mortality and a 100% mortality was reached at day 34, whereas no shrimp died in the negative control. The mortality for 6E1 was 6.7% and for 3E8 was 13.3%. These results suggest that MAbs recognizing the WSSV envelope proteins could neutralize viral infectivity to both cultured cells and shrimp.

KEY WORDS: White spot syndrome virus, WSSV, MAbs, Neutralization, Primary culture.

INTRODUCTION

White spot syndrome is a major shrimp disease worldwide. The disease has also been found in other invertebrate aquatic organisms including crab and crayfish (Flegel, 1997). The causative agent is an enveloped, non-occluded and rod-shaped virus, shrimp white spot syndrome virus (WSSV) (Chou *et al.*, 1995). The virus contains a 305 kb double-stranded circular DNA (Yang *et al.*, 2001) and has been approved recently to be a representative of a new genus, *Whispovirus*, by the International Committee on Taxonomy of Viruses (ICTV) (Mayo, 2002). The virus particle consists of at least five major proteins: VP28, VP26, VP24, VP19 and VP15 (28, 26, 24, 19 and 15 kDa, respectively). VP28 and VP19 are associated with the virion envelope and VP26, VP24, and VP15 with the nucleocapsid (van Hulten *et al.*, 2001). Although the protein profiles of WSSV have been revealed already, epitopes located on these proteins responsible for virus neutralization or playing roles in virus cell interaction and in the establishment of the systemic infection process were still obscure. Except that VP28 had been suggested to be likely to play a key role in the initial steps of the systemic WSSV infection of shrimp recently (van Hulten *et al.*, 2001).

Since there is no established cell line, which can be infected with WSSV, the role of the envelope proteins VP19 or VP28 in virus neutralization is less well studied. Primary lymphoid cultures can be reproducibly established (Chen *et al.*, 1986) and are infectable with WSSV (Shih, 2002), permitting an investigation into the neutralizing activity of antibodies independent of the effects of other defense mechanisms *in vivo*. In this study, panel monoclonal antibodies (MAbs) specific to two envelope proteins, VP28 and VP19 (Shih

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et al., 2001), are applied and their neutralizing ability for WSSV infectivity either *in vitro* or *in vivo* are evaluated.

MATERIALS AND METHODS

Virus and primary Oka lymphoid cell cultures

White spot syndrome virus used in this study was obtained from infected, farmed *Penaeus japonicus* in Taiwan. A homogenate of the head soft tissues was prepared in TNE buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.4) after centrifugation and filtration (Shih, 2002). Primary cell cultures were prepared from Oka organs. Lymphoid Oka organs from *Penaeus monodon* were harvested, pooled and processed for primary cell culture according to procedures described previously (Chen *et al.*, 1986). The explant cultures were grown in 2x Leibovitz's L15 medium (2xL15) supplemented with 20% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B and 10% shrimp muscle extract as reported before (Shih, 2002). Briefly following these protocols, confluent primary cultures of 24 h old were ready for use to titrate WSSV-containing fluids before and after neutralization by monoclonal antibodies.

Neutralization test of MAbs to WSSV in primary cultures

Two monoclonal antibodies recognizing viral envelope proteins produced in my lab (Shih *et al.*, 2001) were used in this study. MAb 6E1 against VP28 was IgG₁ with a κ light chain, and MAb 3E8 against VP19 was IgM with a κ light chain too. Ascites, containing 6E1 or 3E8, or the mock medium (FBS-free L15) was mixed with an equal volume of ten-fold diluted WSSV filtrate and incubated at room temperature for 1 h with gentle shaking. To determine the titer of WSSV filtrate remained after neutralization, the mixtures were ten-fold diluted and inoculated into 24 h old primary monolayer cultured in 48-well plates. Virus adsorption was carried out for 1 h with gentle agitation at room temperature. The supernatant was replaced with 0.5 mL of fresh L15 medium containing 2% FBS in each well. The plates were incubated at 20 °C for 3 days.

The TCID₅₀ titer of each mixture was determined by observation of blue cell after the procession of the Blue-Cell ELISA described previously (Shih, 2002). The neutralization index (NI) was calculated by the difference between the titer of control and MAb treated samples, and expressed as log₁₀ NI. All neutralization assays were performed in duplicate and reproduced twice by using two series of WSSV filtrates prepared independently.

In vivo neutralization assay

Post larvae of *P. monodon* at PL20 stage were injected intramuscularly in the fourth tail segment of the shrimp by a 29-gauge needle with 10 µL of each mixture described above. A negative (330 mM NaCl) and a positive control (WSSV only) were included. For each group 15 shrimp were used. The shrimp were subsequently cultured for a period of 35 days and the mortality was monitored daily.

The presence of WSSV in shrimp pre- and post-injection was tested by a PCR protocol (Wang *et al.*, 1997) with DNA templates extracted from gill tissues using a GenomicPrep Cell and Tissue DNA Isolation Kit (Pharmacia Biotech, Sweden). The results of the analysis showed that the experimental batch of shrimp was WSSV-free before injection but that all became infected after injection.

RESULTS

WSSV infectivity to primary lymphoid cells could be neutralized by MABs against its envelope proteins VP28 (Fig. 1). The morphology of monolayer cultured from the Oka lymphoid organ of *P. monodon* 3 days after seeding showed that the confluence was still remained. Besides any signs of deterioration of this culture, such as granularity around the nucleus, vacuolation within cytoplasm or monolayer detaching from the substrate were not observed (Fig. 1A). Mock medium could not neutralize WSSV infectivity and cytopathic effects appeared (Fig. 1B). On the contrary, neutralization was successively produced by MAB 6E1 against VP28 and the cytopathic effects was although not totally achieved but evidently delayed (Fig. 1C).

Results of the neutralization assays titrated by Blue-Cell ELISA methods showed those two MABs possessed neutralizing activities. Neutralization index (\log_{10} NI) of 6E1 was 6.90 and of 3E8 was 5.83, respectively (Table 1).

During *in vivo* neutralization assays, no shrimp died in the negative control injected only with 330 mM NaCl (Fig. 2). The shrimp in the positive control, which were injected with WSSV only showed an increasing mortality as the test proceeded and a 100% mortality was reached at day 34. WSSV could be neutralized by those two MABs, for 6E1 a 6.7% mortality appeared at day 15 and lasted until the end of this assay. For 3E8, a 6.7% mortality appeared at day 16 and increased to 13.3% at day 20, then remained until the end of this experiment.

DISCUSSION

To demonstrate the role of the major WSSV envelope protein in WSSV infection, a specific polyclonal antiserum against VP28 was generated. And the VP28 antiserum was

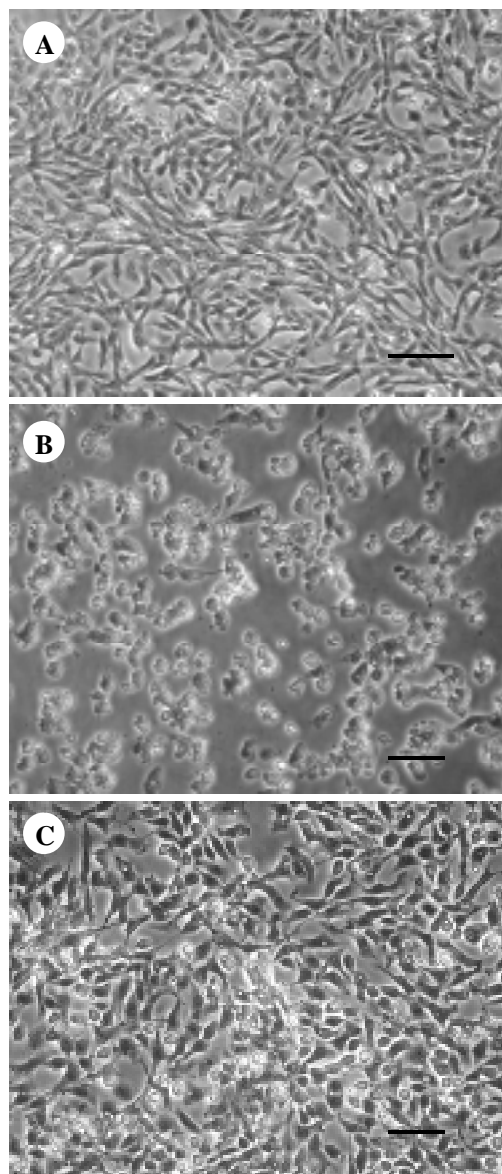


Fig. 1. WSSV infectivity to primary lymphoid cells could be neutralized by MAB against its envelope proteins VP28. Neutralized mixtures of MABs and WSSV-containing filtrate were inoculated into 24 h old primary monolayer and incubated for 3 days at 20 °C. A: The morphology of monolayer cultured from the Oka lymphoid organ of *P. monodon* 3 days after seeding. B: Mock medium could not neutralize WSSV infectivity and cytopathic effects are showed. C: Neutralization produced by MAB 6E1 against VP28. Since the total neutralization was not achieved, the cells became ovoid but cytopathic effects was evidently delayed in (C). Scales = 50 μ m in (A) and 30 μ m in (B) & (C).

Table 1. Neutralization index (\log_{10} NI) of 2 MAbs against WSSV envelope proteins, VP28 and VP19.

MAB	Epitope ^a	NI ^b
6E1	VP28	6.90
3E8	VP19	5.83

^a As determined by Western blotting against WSSV (Shih *et al.*, 2001).

^b The means of duplicate determinations and reproduced twice by using two series of inocula prepared independently are presented.

suggested to be able to neutralize WSSV infection of *P. monodon* in a concentration-dependent manner upon intramuscular injection (van Hulten *et al.*, 2001). VP28 had been pronounced to locate in the spikes of the WSSV envelope and it was likely to play a key role in the initial steps of the systemic WSSV infection in shrimp (van Hulten *et al.*, 2001). However, this observation did not prove the neutralizing activity of WSSV VP28 specific antibodies alone, because protective defense mechanisms might have coincidentally been induced and involved during virus elimination in shrimp. In contrast, the *in vitro* neutralization studies preformed with WSSV-infected primary shrimp lymphoid cells herein provide unequivocal evidence for the neutralization activity of the corresponding antibodies alone. In this study, the *in vitro* neutralization test was processed within 72 h to decrease the time for possible horizontal spread of virus, which may prevent the measurement of more effective neutralization.

Although the neutralizing activities of 3E8 and 6E1 were high (NI are 5.83 and 6.90, respectively), and mortalities could be extremely reduced (13.3% and 6.7%, respectively) but complete neutralization was never achieved. A constant residual infectivity was observed under several assay conditions. The observation of only partial neutralization is consistent with related studies which showed that for virtually all viruses a small fraction escapes neutralization even when a large excess of neutralizing antibodies is used (Burton *et al.*, 2000).

Binding to epitopes located on VP19, the other envelope protein of WSSV, with MAb 3E8 could reduce the infectivity of this virus *in vitro* and *in vivo* as revealed for the first time in this study. But researches concerning the WSSV envelope proteins have been merely focused on VP28 so far. Polyclonal or monoclonal antibodies specific to VP28 had been generated against different geographical virus isolates (Liu *et al.*, 2002; Shih *et al.*, 2001; van Hulten *et al.*, 2001) to detection, identification and localization of the WSSV. In addition, a gene (*vp28*) encoding VP28 had been cloned and expressed either in *Escherichia coli* (Zhang *et al.*, 2002) or in insect cell (van Hulten *et al.*, 2000b). A computer-assisted analysis which revealed a striking similarity between WSSV VP24, VP26 and VP28 at the amino acid and nucleotide sequence level, but still neglected VP19 (van Hulten *et al.*, 2000a). Further research to reveal the distribution of VP19 on the viral envelope and its possible role in WSSV infection will be required. Such information might be useful to realize the mechanisms of neutralization and to control the spreading of this virus.

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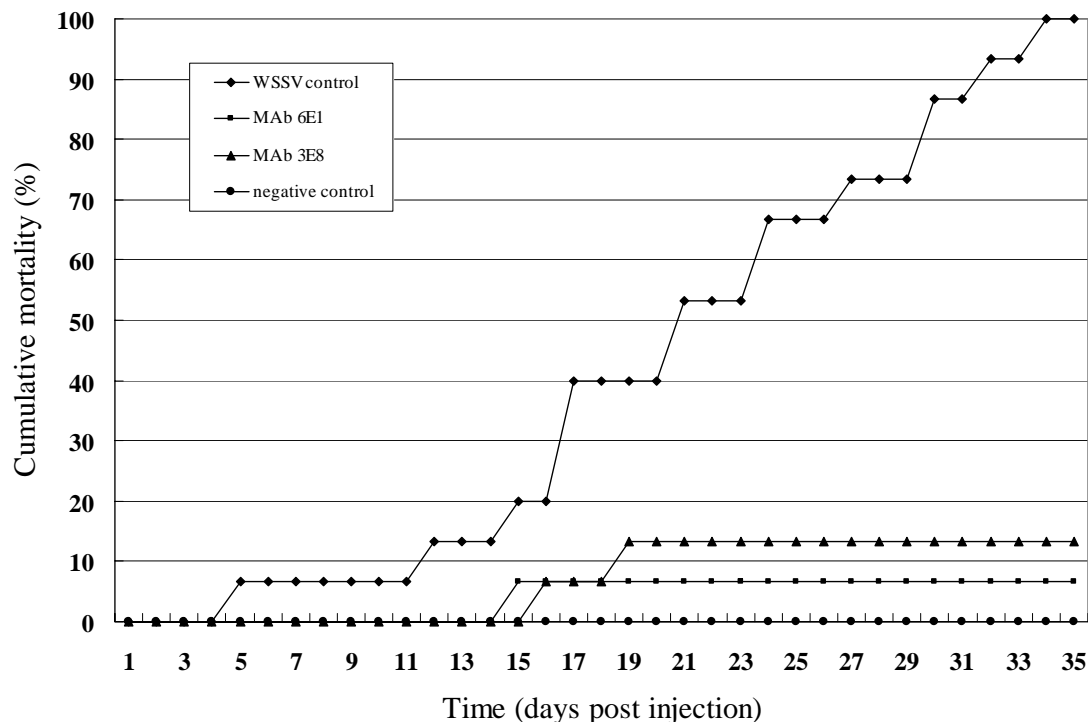


Fig. 2. Neutralization assay of WSSV infection in *P. monodon* using monoclonal antibodies 6E1 against VP28 or 3E8 against VP19.

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辨識白斑病病毒套膜蛋白質之單株抗體對病毒之中和作用

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摘 要

本研究利用辨識白斑病病毒套膜蛋白質 VP28 和 VP19 之兩株專一性單株抗體 - 6E1 和 3E8，經由體外培養和活體實驗兩系統測試其對病毒之中和作用。體外系統實驗係先將草蝦淋巴器官組織塊培養出初級培養之單層細胞，再接種與 6E1 反應過之病毒液，結果發現實驗組中細胞病變效應被顯著遏止，顯示抗體可中和病毒對細胞之感染力。以 Blue-Cell ELISA 滴定中和反應前後之病毒液力價並計算抗體之中和指數，6E1 的指數為 6.90 而 3E8 的為 5.83。活體系統實驗是將抗體中和後之病毒液注射入草蝦後期幼生之腹部肌肉，飼養觀察 35 日；發現注射病毒液之陽性對照組的死亡率持續上升而在第 34 日達到 100%，陰性對照組的死亡率維持於 0，感染經抗體 6E1 或 3E8 中和之病毒液，其累積死亡率分別為 6.7% 及 13.3%。本研究顯示辨識白斑病病毒套膜蛋白質之單株抗體可中和此病毒對體外初級培養淋巴細胞及對蝦體之感染力。

關鍵詞：白斑病病毒、單株抗體、中和作用、初級培養。

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