

Production and Characterization of Monoclonal Antibodies of Shrimp White Spot Syndrome Virus Envelope Protein VP28

Wan-gang GU¹, Jun-fa YUAN¹, Ge-lin XU², Li-juan LI¹, Ni LIU³,
Cong ZHANG¹, Jian-hong ZHANG¹, Zheng-li SHI^{1**}

(1. State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China; 2. Wuhan Institute of Biological Products, Ministry of Health, Wuhan 430060, China; 3. College of Life Science, Central China Normal University, Wuhan 430079, China)

Abstract: BALB/c mice were immunized with purified White spot syndrome virus (WSSV). Six monoclonal antibody cell lines were selected by ELISA with VP28 protein expressed in *E. coli*. In vitro neutralization experiments showed that 4 of them could inhibit the virus infection in crayfish. Western-blot suggested that all these monoclonal antibodies were against the conformational structure of VP28. The monoclonal antibody 7B4 was labeled with colloidal gold particles and used to locate the VP28 on virus envelope by immunogold labeling. These monoclonal antibodies could be used to develop immunological diagnosis methods for WSSV infection.

Key words: White spot syndrome virus (WSSV); Mab; Envelope; Localization; Neutralization

Since it was identified in Taiwan in the early 1990's, White spot syndrome virus (WSSV) has spread throughout the world and has become the main pathogen in shrimp cultures. It has a wide host range and can infect most aquatic crustacean species. The infected shrimps reach an accumulative mortality of 100% within 3 to 10 days (1,3,4,8,21). WSSV has a genome size of 305kb, the biggest genome of animal viruses (12,19). According to the morphology and genome structures, WSSV has been assigned to a new virus family Nimaviridae by ICTV (9). The study of virucation and assembly are hampered because of

lacking permissive shrimp cell lines. At least 39 structural proteins have been identified with proteomics methods, of these proteins, 7 were reported to be involved in virus infection (2,6,5,10,13,17). VP28 is the main envelope protein of WSSV, and an target for developing diagnostic methods (11, 20). Previous reports have shown that anti-VP28 polyclonal antibodies could neutralize the virus and delay the death of infected shrimp (13). Expressed VP28 and its subunit in prokaryotic cells can protect shrimp from WSSV (16), suggesting that VP28 has an important role during the virus entry. In this paper, six

Received:2006-04-24, Accepted:2006-09-07

** Corresponding author. Tel: 86-27-87197240, Fax:86-27-87197240, E-mail: zlshi@wh.iov.cn

anti-VP28 monoclonal antibodies have been screened by ELISA with VP28 expressed in procaryotic cells and their characteristics have been identified.

1. Materials and methods

1.1 Materials

The crayfish *Procambarus clarkii* was used in viral proliferation and neutralization assays. Crayfish were purchased from a Wuhan market (Hubei Province, China). They were reared for at least 3 days before the experiment. BALB/c mouse and Kunming mouse were purchased from the Center for the Disease Control in Hubei Province. The SP2/0 cell line was from the Wuhan Institute of Biological Products.

1.2 Virus proliferation and purification

Virus proliferation and purification were performed according to the protocol described by reference (18).

1.3 Immunization

Six-week BALB/c mice were immunized with purified virus. 30 μ g virus was injected into the spleen of each mouse. After 3 weeks, each mouse was injected subcutaneously with 50 μ g virus mixed with Freund's adjuvant. This was repeated once more after another 3 weeks. Ten days after third immunization, one mouse was sacrificed for antibody titer assay. The serum was kept as positive control. One immunization was added 3 days before the cell fusion.

1.4 Cell fusion and positive clone screening

The cell feeder was prepared one day before fusion. Cell fusion was performed with spleen cell and SP2/0 in a ratio of 4:1 by using PEG 1500. Hybridomas were cultured in RPMI1640 medium with a complement of 20% FCS and HAT(Invitrogen). The positive clones were screened by ELISA with the VP28 expressed in *E.coli* (5 μ g/mL).

1.5 Ascites production

A volume of 0.5ml pristane was injected into the BALB/c mouse paunch. After 7 days, 105 hybridomas were injected into the BALB/c mouse paunch to produce ascites. Ten days later, the mouse was sacrificed and the ascites were collected.

1.6 Isotype determination and titer test

The immunoglobulin isotype of the monoclonal antibodies(mAb) was determined using the mouse monoclonal antibody isotyping kit(HyCult biotechnology b.v.) according to the manufacturer's instructions. The antibody titer was tested by ELISA with diluted purified virus.

1.7 Western blot

Purified virus and expressed VP28 protein were transferred to PVDF membrane after 12% SDS-PAGE. Western blot was performed with 6 mAbs supernatant as the first antibody and goat anti mouse HRP-IgG as the second antibody. Polyclonal antibody against WSSV was used as positive control and non-specific supernatant as negative control.

1.8 Dot blot

One microlitre of purified virus was added to a 1 \times 1 cm PVDF membrane. The ascites were diluted in 1:50 and added as the first antibody to perform a dot blot. The polyclonal antibody was used as positive control and non-specific ascites as negative control.

1.9 Neutralization assay

The in vivo neutralization assay using 6 mAbs was performed as described by Huang *et al* (2). A volume of 100 μ L virus (104 particles per microliter) was mixed with 100 μ L ascites diluted in TNE buffer (0.5 mol/L Tris-HCL, 0.1 mol/L NaCL, 0.01mol/L EDTA, pH 7.4) and incubated at 37 $^{\circ}$ C for 2 h. The mix of virus and non-specific ascites were injected as positive control. The mix of non-specific ascites and TNE were injected as negative control. Ten crayfish were used

for each group and three replicates were performed. Crayfish mortality was monitored every day.

1.10 Purification of mAb and labeling with colloidal gold

The mAb 7B4 was purified using the antibody purification kit (Oncogene) according to the manufacturer's instructions. The purified antibody was labeled with 15 nm colloidal gold according to the manufacturer's protocol (14).

1.11 Localization of VP28 on virus

Purified viral particles and nucleocapsids were absorbed to Formvar-supported and carbon coated nickel grids for 10 minutes and then blocked with 4% BSA (in PBS pH7.4) for 30 minutes. The grid was incubated with 4-fold diluted mAb 7B4 (in TBS, pH8.2,) conjugated with colloidal gold for 60 minutes, and then washed three times with TBS (Tris-Base 20 mmol/L, NaCl 137 mmol/L, pH8.2, with 0.1% BSA) for 5 minutes. After extensively washing several times, the grid was stained with 2% phosphotungstic acid (pH 7.2) for 3 min and then observed with electron microscopy. The colloidal gold labeled BALB/c mouse IgG was used as negative control.

2. Results

2.1 Hybridomas screening, isotype and titer determination of mAb

We obtained 370 hybridomas after 2 fusions and succeeded in establishing 6 stable clones designated 5B7, 3A10, 1E7, 7E12, 7B4 and 6F6. All the 6 clones were characterized and used for neutralization assays.

Table 1. Monoclonal antibody isotype and titer

Cellstrain	5B7	3A10	7E12	1E7	6E6	7B4
Isotype	IgG16	IgG1	IgG3	IgG2a	IgG2a	IgG3
Titer	10 ⁷	10 ⁷	10 ⁷	10 ⁷	10 ⁷	10 ⁷

The titers of 6 clones determined by ELISA were between 10⁴~10⁶. The results are shown in Table 1.

2.2 Western blot

Purified virus and expressed vp28 protein were transferred to PVDF membrane after 12% SDS-PAGE. Western blot was performed with 6 mAbs as first antibody, but no positive signal appeared. This suggested that all these monoclonal antibodies were against the conformational structure of VP28. Denatured VP28 protein cannot be recognized by the 6 mAbs.

2.3 Dot blot

All 6 mAbs reacted strongly with virus. But no reaction was detected to negative control. This suggested that all these monoclonal antibodies were specifically against virus protein (Fig. 1.).

2.4 Neutralization assay

The results of the neutralization assay are shown in Fig. 2. No crayfish died in the negative control, i.e. those injected with non-specific ascites and TNE. The group injected with virus and non-specific ascites showed 100% mortality at day 12 post-infection (p.i.). The neutralization assay with mAbs 3A10, 1E7, 7B4, and 6F6 showed a delayed onset of mortality compared with positive controls. However, the mAbs of 5B7 and 7E12 didn't show any inhibition effect on virus in the assay.

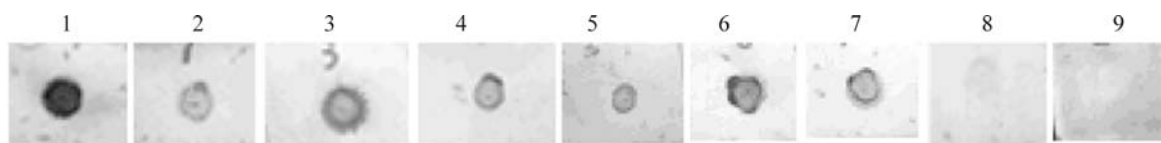


Fig.1 Dot-blot Assay of VP28 mAbs with purified viral particles. 1, Polyclonal antibody; 2-7, 3A10, 6F6, 7B4, 5B7, 1E7 and 7E12; 8, Non-specific ascite; 9, Blank control.

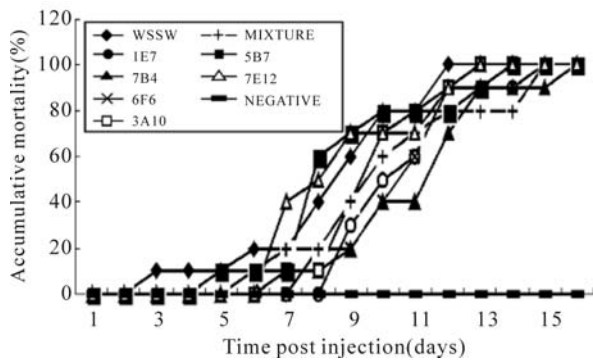


Fig.2.Neutralization assay. WSSV,Purified viral particles; NEGATIVE,TNE mixed with non-specific ascite; MIXTURE, Mixed monoclonal antibodies;Each point represents the mean value of triplicate assays within $\pm 1\%$ standard deviation.

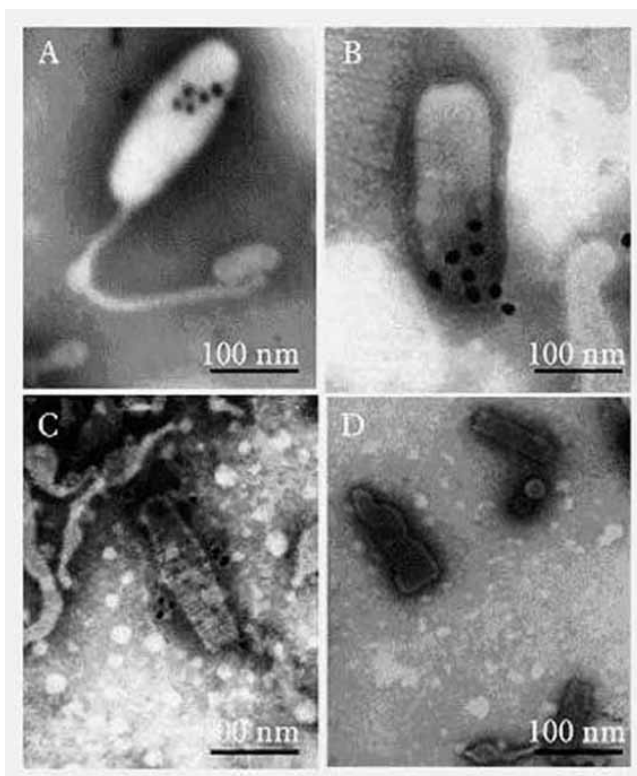


Fig.3.Immuogold labeling with Monoclonal antibody 7B4.A and B,Monoclonal antibody 7B4 localized VP28 on the membrane of WSSV particles. C,Monoclonal antibody 7B4 localized VP28 on the membrane of destroyed WSSV.

2.5 Localization of VP28 on virus

The results are shown in Fig 3. The mAb 7B4 labeled with colloidal gold particles can be localized to VP28 on the intact virus envelope and the collapsed envelope protein from the virus (Fig.3.A, B, C). The gold-labeled BALB/c mouse IgG did not localize on

the viral particles (Fig. 3.D).

3. Discussion

Since its detection, the prevention and control of WSSV has been an important issue in aquaculture. Unfortunately, no efficient methods have been found to control this viral disease. Thus, a prophylactic strategy is the only available option to minimize the economic losses of this disease. The six mAbs obtained in this study could be used to develop immunological diagnosis methods of WSSV infection based on techniques such as ELISA.

Envelope proteins usually play key role in infection, such as recognizing and attaching to the receptor, and fusing with cell membrane during assembly, etc. Previous studies showed that VP28 is involved in virus infection. VP28 expressed in vitro can protect crayfish from WSSV infection and induce a significant anti-virus response in crayfish (13, 15,16). Thus, further study of VP28 function is helpful to understand virus infection and to develop WSSV prevention and control methods. In 2002, Liu et al expressed recombinant VP28 protein(r-28) in *Escherichia coli* and used it as an antigen to obtain 3 monoclonal antibodies, and an antigen-capture ELISA(Ac-ELISA) was developed by virtue of these mAbs. The Ac-ELISA can differentiate WSSV-infected shrimp from uninfected shrimps and has the same sensitivity as the PCR assay(7). In our study, we used viral particles as antigen and screened mAbs against VP28. Western blot and dot blot demonstrated that all these monoclonal antibodies were against the conformational epitope of VP28. The in vivo neutralization assay using 6 mAbs in crayfish showed that mAbs 3A10, 1E7, 7B4, and 6F6 could significantly delay initial mortality compared with positive controls. It implies that the epitopes of these 4

mAbs may play an important role in the virus infection and can be used for further study of VP28 function, virus entry and development of anti-virus methods.

References

- Huang R, Chen X X, Zhang J H, *et al.* 2004. Experimental infection of red claw crayfish *Cherax quadricarinatus* with white spot Syndrome virus[J]. *J Wuhan Univ(Nat Sci Ed)*, 50 (S2): 79-82.(in Chinese)
- Huang R, Xie Y L, Zhang J H, *et al.* 2005. A novel envelope protein involved in white spot syndrome virus infection[J]. *J Gen Virol*, 86: 1357-1361.
- Inouye K, Miwa S, Oseko N, *et al.* 1994. Mass mortalities of cultured Kuruma shrimp *Penaeus japonicus* in Japan in 1993: Electron microscopic evidence of the causative virus[J]. *Fish Pathol*, 29: 149-158.
- Kanchanaphum P, Wongteerasupaya C, Sitidilokratana N, *et al.* 1998. Experimental transmission of white spot syndrome virus(WSSV) from crabs to shrimp *Penaeus monodon* [J]. *Dis Aquat Organ*, 34: 1-7.
- Li L J, Yuan J F, Cai C A, *et al.* 2006. Multiple envelope proteins are involved in white spot syndrome virus(WSSV) infection in crayfish[J]. *Arch Virol*, 151:1309-1317.
- Liang Y, Huang J, Song X L, *et al.* 2005. Four viral protein of white spot syndrome virus (WSSV) that attach to shrimp cell membranes[J]. *Dis Aquat Organ*, 66: 81-85.
- Liu W, Wang Y T, Tian D S, *et al.* 2002. Detection of white spot syndrome virus(WSSV) of shrimp by means of monoclonal antibodies (MAbs) specific to an envelope protein(28 kDa) [J]. *Dis Aquat Organ*, 49 (1): 11-18.
- Lo C F, Ho C H, Peng S E, *et al.* 1996. White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods[J]. *Dis Aquat Organ*, 27: 215-225.
- Mayo M A. 2002. A summary of taxonomic changes recently approved by ICTV[J]. *Arch Virol*, 147: 1655-1656.
- Tsai J M, Wang H C, Leu J H, *et al.* 2006. Identification of the nucleocapsid, tegument, and envelope proteins of the shrimp white spot syndrome virus virion[J]. *J Virol*, 80: 3021-3029.
- van Hulten M C, Westenberg M, Goodall S D, *et al.* 2000. Identification of two major virion protein genes of white spot syndrome virus of shrimp[J]. *Virology*, 266: 227-236.
- van Hulten M C, Witteveldt J, Peters S, *et al.* 2001a. The white spot syndrome virus DNA genome sequence[J]. *Virology*, 286: 7-22.
- van Hulten M C, Witteveldt J, Snippe M, *et al.* 2001b. White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp[J]. *Virology*, 285: 228-233.
- Wang B L, Scopsi L, Martvig M, *et al.* 1985. Simplified purification and testing of colloidal gold probes[J]. *Histochemistry*, 83:109-115.
- Wei K Q, Xu Z R. 2005. Effect of white spot syndrome virus envelope protein Vp28 expressed in silkworm-(*Bombyx mori*) pupae on disease resistance in *Procambarus clarkii*[J]. *Acta Biologica Experimentalis Sinica*, 38:190-198.(in Chinese)
- Witteveldt J, Vlak J M, van Hulten M C. 2004. Protection of *Penaeus monodon* against white spot syndrome virus using a WSSV subunit vaccine[J]. *Fish Shellfish Immunol*, 16: 571-579.
- Wu W, Wang L, Zhang X. 2005. Identification of white spot syndrome virus(WSSV) envelope proteins involved in shrimp infection[J]. *Virology*, 332: 578-583.
- Xie Y L, Zhang S Y, Huang R, *et al.* 2003. A modified technique for purifying white spot syndrome virus[J]. *Virologica Sinica*, 18 (4): 391-393. (in Chinese)
- Yang F, He J, Lin X, *et al.* 2001. Complete genome sequence of the shrimp white spot bacilliform virus [J]. *J Virology*, 75: 11811-11820.
- Yoganandhan K, Syed Musthaq S, Narayanan R B, *et al.* 2004. Production of polyclonal antiserum against recombinant VP28 protein and its application for the detection of white spot syndrome virus in crustaceans[J]. *J Fish Dis*, 27: 517-522.
- Zhang J H, Chen D H, Xiao L C, *et al.* 1994. Infection and morphogenesis of non-inclusion body baculovirus from *Penaeus orientalis kishinoye* in vivo[J]. *Virologica Sinica*, 9 (4): 364-366. (in Chinese)