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Neutralizing Monoclonal Antibodies to Striped Jack Nervous Necrosis Virus (SJNNV)

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Viral nervous necrosis (VNN) caused by a nodavirus (striped jack nervous necrosis virus: SJNNV) is a serious problem in seed production process of striped jack *Pseudocaranx dentex* in Japan. Mouse monoclonal antibodies (MAbs) to SJNNV were produced by using a homogenate of infected larval striped jack. Among 8 MAbs reacting with the homogenate of infected larvae in enzyme-linked immunosorbent assay (ELISA), three MAbs (SJ-102B, SJ-204D, SJ-207C) were proved to recognize the 42 kDa coat protein of SJNNV by Western blot analysis. An *in vivo* neutralization test, where larval striped jack were exposed to purified SJNNV (100 ng/m*l*) or the virus treated with MAbs (1 mg) and the resultant infection with SJNNV in larvae was monitored by ELISA using an anti-SJNNV rabbit serum, exhibited that two MAbs SJ-102B and SJ-204D have neutralizing activity against SJNNV.

Key words: monoclonal antibody, nodavirus, SJNNV, neutralization, striped jack, viral nervous necrosis, VNN

Striped jack nervous necrosis virus (SJNNV), the causative agent of viral nervous necrosis (VNN) of larval striped jack *Pseudocaranx dentex*, belongs to the family Nodaviridae. The virus contains a major coat protein with molecular weight of 42,000, and two single-stranded, positive-sense, non-polyadenylated RNAs (RNA1 and RNA2). The coat protein is encoded in RNA2 (Mori *et al.*, 1992). SJNNV is highly virulent to larval striped jack in natural and experimental conditions (Arimoto *et al.*, 1993, 1994). The disease is characterized by necrosis in the central nervous system with numerous virus particles in the cytoplasm of affected nerve cells. Detection of the virus from gonads of striped jack spawners by ELISA (enzyme-linked immunosorbent assay) using an anti-SJNNV rabbit serum indicated that vertical transmission is the most important infection route of the disease (Arimoto *et al.*, 1992). Based on the sequence data of RNA2 (Nishizawa *et al.*, 1995), a PCR (polymerase chain reaction) system was developed to amplify the target regions of SJNNV gene in the total nucleic acids extracted from affected striped jack larvae (Nishizawa *et al.*, 1994). This PCR amplification was also effective for detecting the virus from asymptomatic spawners of striped jack and PCR-based elimination of the virus-positive spawners was proved to be a useful control measure of the disease (Mushiake *et al.*, 1994).

As compared with these detailed studies based on the nucleic acid analysis, fewer studies have been made from the aspect of protein analysis, mainly due to the lack of cell lines which can support multiplication of the virus. For example, there have been no detailed informations on the epitopes concerning virus neutralization on the coat protein. In the present study, mouse monoclonal antibodies (MAbs) against SJNNV were produced and the neutralizing activity was examined by an *in vivo* neutralization test using larval striped jack.

Materials and Methods

Virus

Larvae of striped jack naturally infected with SJNNV were homogenized with 9 volumes of Dulbecco's PBS (pH 7.4) and centrifuged at 10,000 $\times g$ for 5 min. The supernatant was used as an immunogen for the production of MAbs against the virus. The virus concentration in the supernatant was calculated as approximately 700 μg/m*l* by indi-

rect ELISA using an anti-SJNNV rabbit serum (Arimoto *et al.*, 1992). SJNNV was purified from infected larvae by the method described previously (Mori *et al.*, 1992) and the purified virus was used for Western blot analysis and *in vivo* virus-neutralization test.

Monoclonal antibodies

One month old female mice (BALB/c) were immunized by intraperitoneal injection with 200 μl of the above-mentioned supernatant preparation from infected striped jack larvae which was emulsified with an equal part of Freund's complete adjuvant. Two booster injections were done intravenously with 100 μl of the supernatant at 10 day intervals. The immunized spleen cells were harvested 3 days after the final immunization and fused with mouse myeloma cells (SP2/0-Ag14) at a ratio of 10 : 1 by using polyethylene glycol (MW 1500). Fused cells were resuspended at a concentration of 3.5×10^6 cells/m*l* in E-RDF medium (Kyokuto Co., LTD.) supplemented with 10% fetal bovine serum (FBS), dispensed at 100 μl/well each in 96-well micro culture plates, and incubated under 5% CO_2 at 37°C. After one day culture, 100 μl of E-RDF medium containing hypoxanthine, aminopterin and thymidine (HAT) was added into each well. After 2 weeks of HAT-selection, hybridoma colonies were selected by indirect ELISA as follows. Microplates were coated with the supernatant from infected striped jack larvae or the supernatant from normal larvae as a control. Culture fluid from each hybridoma was added to the plates and alkaline-phosphatase conjugated rabbit Ig to mouse Ig (Dako) was used to develop the reaction. The hybridoma colonies reacting only with the supernatant from infected larvae were cloned by limited dilution and cultured in E-RDF medium supplemented with 10% FBS. Supernatant of each cultured hybridoma producing MAb was precipitated with 50% (V/V) of a saturated solution of ammonium sulfate and centrifuged at $8{,}000 \times g$ for 25 min. The precipitate was resuspended in a small amount of Dulbecco's PBS and dialyzed overnight. The protein concentration was adjusted at 1 mg/m*l*.

Western blot analysis

The reactivity of MAbs to viral polypeptide was examined by Western blot technique. After SDS-polyacrylamide gel electrophoresis (PAGE) of the supernatant from infected striped jack larvae or the purified SJNNV (0.25 mg/m*l*), proteins were electrophoretically transferred to a nitrocellulose membrane filter (0.45 μm) by the method of Towbin *et al.* (1979). The membrane was washed in TBS buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl) and blocked with TBS buffer containing 2% skim milk for 30 min. After washing, the membrane was reacted with partially purified MAbs or the anti-SJNNV rabbit serum as a positive control, and then with alkaline-phosphatase conjugated anti-mouse Ig rabbit antibody or anti-rabbit Ig swine antibody (Dako). Finally, the protein bands were visualized by 5-bromo-4-chloro-3-indoryl phosphate and 4-nitroblue tetrazolium chloride (NBT).

Neutralization assay

Two m*l* of a partially purified MAb and 100 ng of purified SJNNV were mixed and incubated at 20°C for 1 h. Five hundred striped jack larvae (0 day old) were exposed to the virus, non-treated or treated with MAbs, and kept in 1 *l* of sea water at 25°C for 4 days without aeration and feeding. Ten fish were daily collected from each group to detect SJNNV antigens by indirect ELISA. Each sample containing 10 fish was homogenized with 80 volumes of PBS and centrifuged at $12{,}000 \times g$ for 5 min. A 100 μl of the supernatant was poured into two wells of a microplate and incubated at 4°C overnight. After blocking with 2% skim milk, they were treated with the anti-SJNNV rabbit serum (1 : 1,000 diluted with PBS) at 37°C for 2 h and then with alkaline-phosphatase conjugated anti-rabbit Ig swine antibody at 37°C for 2 h. After coloring by *p*-nitrophenyl phosphate disodium salt solution (1 mg/m*l*) in diethanolamine (pH 9.8), the absorbance was measured at 405 nm using a microtiter plate reader (Toso, MPR-A4).

Results and Discussion

One of the advantageous properties of monoclonal antibodies (MAb) against polyclonal antibodies (PAb) is to be generated by using an impure antigen. In the present study, MAbs were successfully raised by using infected larvae as immunogen.

MAbs from eight cloned hybridoma cells, SJ-102A, SJ-102B, SJ-204D, SJ-204F, SJ-206C, SJ-207C, SJ-305C, and SJ-306C, reacted in ELISA test with the homogenate from infected striped jack larvae. In Western blot analysis using purified

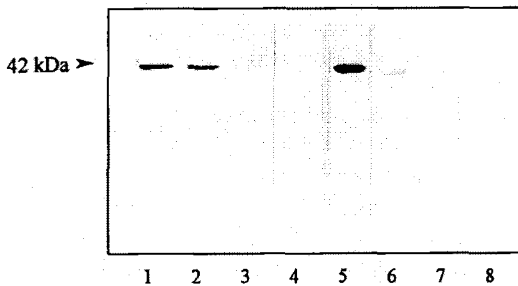

Fig. 1. Reactivity of rabbit polyclonal (PAb) and mouse monoclonal (MAb) antibodies against SJNNV coat protein. Purified virus (lanes 1–4) and homogenate from diseased striped jack larvae (lanes 5–8) were used for staining with PAb (lanes 1, 5), MAb SJ-102B (lanes 2, 6), MAb SJ-204D (lanes 3, 7), and MAb SJ-207C (lanes 4, 8).

SJNNV, the rabbit PAb against SJNNV, which was used as a positive control, strongly reacted with the coat protein (42 kDa) of SJNNV, while among the eight MAbs only three MAbs SJ-102B, SJ-204D, and SJ-207C reacted with the coat protein (Fig. 1). From these results, it is considered that the three MAbs, SJ-102B, SJ-204D, and SJ-207C, recognize the epitopes on the viral coat protein, while other MAbs might recognize the 100 kDa polypeptide coded for in RNA1 (Mori *et al.*, 1992) or the secondary or tertiary structure of the coat protein.

The result of the *in vivo* neutralization test, where viral antigens were monitored by indirect ELISA in experimentally infected striped jack larvae, was shown in Fig. 2. In a positive control group (Control 1), which was exposed to SJNNV without antibody treatment, the SJNNV antigens were detected from fish on the 3rd and 4th days. Almost the same patterns of antigen detection were shown in groups exposed to SJNNV treated with MAbs SJ-207C, SJ-204F, SJ-306C, SJ-305C, SJ-206C, and SJ-102A. Thus these 6 MAbs did not neutralize the infectivity of the virus. On the other hand, the viral antigens were not detected during the experiment (4 days post exposure) from fish of the negative control group (Control 2) which was not exposed to the virus or from fish exposed to SJNNV treated with the rabbit PAb. ELISA values of fish exposed to SJNNV treated with MAbs SJ-204D and SJ-102B were below a detectable level on the 3rd day and were quite low, less than 0.1, on the 4th day post exposure. From these results, MAbs SJ-204D and SJ-102B as well as

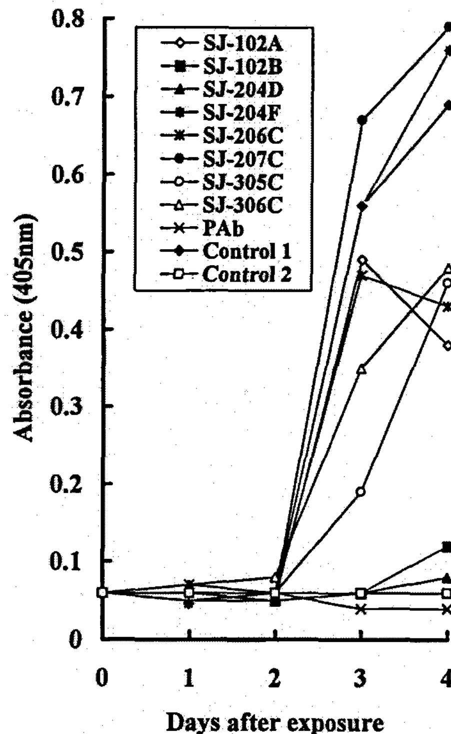

Fig. 2. Detection of viral antigens by ELISA in experimentally infected striped jack larvae. Striped jack larvae were exposed to purified SJNNV (100 ng/*l*) (Control 1), or to the virus treated with 8 mouse monoclonal antibodies (SJ-102A, SJ-102B, SJ-204D, SJ-204F, SJ-206C, SJ-207C, SJ-305C, SJ-306C) or a rabbit polyclonal antibody (PAb). Control 2 was not exposed to the virus.

the rabbit PAb proved to have neutralizing activity against SJNNV.

An ELISA competition assay was made between MAbs SJ-204D and SJ-102B, but resultant cross-inhibition was not observed between the two MAbs (data not shown). This result suggests that these two MAbs recognize different epitopes on the coat protein.

VNN occurred not only in striped jack but also in various marine fishes (Muroga, 1995), and our previous works using fluorescent antibody technique with anti-SJNNV rabbit serum indicated the presence of antigen(s) common to VNN-agents (Nguyen *et al.*, 1994; Nakai *et al.*, 1994). In Western blot analysis, VNN-agents other than SJNNV did not react with any of the neutralizing MAbs for SJNNV (data not

shown). Although there was a fault that the concentration of the antigens could not be unified in that experiment, the result may indicate the difference of neutralizing epitopes on the coat protein between SJNNV and other VNN agents.

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