Generation and characterization of monoclonal antibodies against the flounder *Paralichthys olivaceus* rhabdovirus

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Abstract

Two MAbs (3C7 and 3C9) against flounder *Paralichthys olivaceus* rhabdovirus (PORV) were generated with hybridoma cell fusion technology and characterized by an indirect enzyme-linked immunosorbent assay, isotype test, Western blot and immunodot analysis and immunofluorescence assay. Isotyping tests demonstrated that both of the two MAbs belonged to IgM subclass. Western blot analysis showed the MAbs reacted with 42, 30, and 22 kDa viral proteins, which were localized within the cytoplasm of PORV-infected grass carp ovary (GCO) cells analyzed by indirect immunofluorescence tests. The MAb 3C7 was also selected at random for detecting virus antigens in the inoculated grass carp tissues by immunohistochemistry assay. Flow cytometry tests showed that at the 36 h postinfection (0.25 PFU/cell), the 23% PORV-infected GCO cells could be distinguished from the uninfected cells with the MAb 3C7. Such MAbs could be useful for diagnosis and potential treatment of viral infection. © 2007 Elsevier B.V. All rights reserved.

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1. Introduction

Rhabdoviruses are some of the most important virus pathogens in aquaculture infecting both fresh water and marine fish. In recent years, the Chinese sucker rhabdovirus (CSRV) (Zhang and Li, 2001), spring viremia of carp virus (SVCV) (Chen et al., 2006), *Siniperca chuatsi* rhabdovirus (SCRV) (Tao et al., 2007), *Scophthalmus maximus* rhabdovirus (SMRV) (Zhang et al., 2007) were isolated from the Chinese sucker, common carp, mandarin fish, and turbot, respectively. More recently, the *Paralichthys olivaceus* rhabdovirus (PORV) has been isolated from diseased flounders (Gui et al., 2007). Like other fish rhabdoviruses, ultrastructure observation showed that PORV are bullet-like particles. SDS-PAGE analysis of the purified PORV particles indicated that the structural proteins of PORV were composed mainly of five polypeptides. All rhabdoviruses (SMRV, SCRV, and SVCV) isolated so far were propagated efficiently at 25 °C in fish cell lines maintained in the laboratory. However, the infectivity of PORV in the same conditions was very low, although it replicated well at 20 °C. This indicated that PORV may be different from isolated rhabdoviruses during the infectivity and virus replication kinetics. It would be very helpful to establish specific diagnostic methods.

Although DNA vaccines have been proven to be efficient in protecting the fish from rhabdoviruses infection experimentally (Kurath, 2005; Lorenzen and LaPutra, 2005; Kanellos et al., 2006), the nonavailability of vaccines or antiviral drugs for the control of viral diseases is still the major difficulty for avoiding the dissemination of the pathogens in fish farms. At present, the only means of control of viral infectious diseases in aquaculture is early diagnosis of virus pathogens and timely segregation. To date, fish rhabdovirus diseases were diagnosed mainly following observation of clinical signs in fish suspected to be infected and by the implementation of various methods such as virological isolation in cell culture or RT-PCR techniques at the molecular level (Miller et al., 1998; Mork et al., 2004; López-Vázquez et al., 2006; Zhang et al., 2007). Immunoassays used routinely for laboratory diagnosis of many viral infections could be very helpful due to their sensitivity and specificity, low cost, reagent stability, and ease of procedures (Monini and Ruggeri, 2002). The use of methods based on specific antibodies against fish rhabdoviruses could help to improve and to reduce the time needed for diagnosis. This paper describes the generation and characterization of two monoclonal antibodies (MAbs) directed

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2.1. Virus purification

Virus purification was successfully used to detect the virus antigens in the cell cultures and fish tissues with immunocytochemistry and flow cytometry tests.

2. Materials and methods

2.1. Virus purification

Grass carp ovary (GCO) cells were cultured at 20 °C in medium 199 supplemented with 5% heat-inactivated fetal bovine serum (FBS). The procedures described by Zhang et al. (2007) were used to purify the PORV. The virus purity was checked by a transmission electron microscope using 2% phosphotungstic acid (PTA) as the negative stain.

2.2. Mouse immunization and MAb production

Balb/c mice were each immunized intraperitoneally (i.p.) with 0.5 ml of a preparation of purified PORV in the presence of Freund’s adjuvant. Subsequent boosters with antigen in incomplete adjuvant were given on day 14, 28 and 42. Splenic lymphocytes were fused with SP2/0 cells as described by Zhou et al. (2006). The supernatant fluids from all wells were tested by the indirect enzyme-linked immunosorbent assay (iELISA) according to reported procedures (Zhou et al., 2006). The supernatants with high antibody titers were collected and applied directly. Antibodies were also produced by injecting hybridomas into the peritoneal cavity of 8-week-old Balb/c mice. Ascitic fluids with high antibody titers were obtained within 2 weeks.

2.3. Determination of MAb isotype

The immunoglobulin (Ig) isotype of the MAbs was determined using a Mouse Monoclonal Antibody Isotyping Kit (Sigma) according to the manufacturer’s instructions.

2.4. SDS-PAGE and Western blot analysis

Proteins of purified PORV were separated by 12% SDS-PAGE according to the method described previously (Zhang et al., 2006). The gels were stained using Coomassie Brilliant Blue R-250. For Western blot, the samples separated by SDS-PAGE were blotted onto polyvinylidene fluoride (PVDF) membrane. The saturated membrane was then incubated successively with different MAB ascites (both diluted in 1:100 with PBS) and alkaline phosphatase-conjugated goat anti-mouse IgG (GAM-AP, Vector) for 2 h at room temperature (RT) as outlined by Zhou et al. (2006). Polyclonal antiserum (1:1000) was used as the positive control. After the final wash, membrane strips were put into substrate solution containing tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for 5 min, and stopped by rinsing the strips with distilled water.

2.5. Immunodot test

PVDF membrane was cut according to the number of antigens and samples to be tested and labeled by making a grid of 0.8 cm × 0.8 cm squares with a lead pencil. Negative (PBS) and positive control samples were also included. After washing with PBST (0.05% Tween-20 in PBS) for 15 min, the membrane was then soaked in transfer buffer (25 mM Tris, 192 mM glycine) for 30 min and air dried on filter papers. 1 μl purified PORV antigen or PORV-infected GCO cell supernatants was pipetted into the center of each square and incubated at RT overnight. Diluted ascites (1:50) and polyclonal serum (1:1000) were then placed at the center of each square (1 μl), and incubated at 37 °C for 2 h. The membrane was washed in PBST three times for 5 min, and incubated in the second antibody (GAM-AP) by putting 1 μl of the conjugate (1:1000) over each square. Reactions were revealed with NBT-BCIP solutions for 5 min at RT.

2.6. Immunoperoxidase staining and immunofluorescence of PORV-infected cells

GCO cells cultured on coverslips were infected with PORV at one m.o.i. (1 PFU/cell) and incubated at 20 °C for the 0, 6, 12 and 24 h postinfection (h.p.i.). Monolayers were then rinsed with PBS and fixed with 4% paraformaldehyde for 15 min at RT followed by permeabilization with absolute ethanol for 10 min at −20 °C. After washing, monolayers were incubated with a 1:50 dilution of 3C7 MAb ascites in PBS. A replicate aliquot of each dilution was placed onto uninfected control cells. After 1 h at 37 °C, the ascites dilutions were removed and the coverslips were rinsed three times with PBS. A 1:1000 dilution of GAM-AP in PBS was added, and the presence of virus replication foci was shown by reaction with NBT-BCIP solutions.

For indirect immunofluorescence staining, paraformaldehyde-treated GCO cells on coverslips were incubated with ascites dilutions and fluorescein isothiocyanate (FITC)-conjugated antimouse IgG antibody (Pierce), respectively. Hoechst 33342, a kind of fluorescence dye, was used to detect nuclei. After mounting in 50% glycerol/PBS, monolayers were observed with a fluorescence microscope (Leica).

2.7. Immunohistochemistry assay

The total of 4 healthy grass carp Ctenopharyngodon idellus, with mean weight of about 7 g, were infected experimentally each with 0.5 ml of PORV (10^7 TCID50/ml) by intraperitoneal (i.p.) injection. Six kinds of tissue (gill, spleen, kidney, liver, heart, and intestine) were collected 10 days post infection. The immunohistochemistry tests were carried out as outlined by Zhou et al. (2006). Briefly, after fixing in 4% paraformaldehyde and saturating with 30% sucrose, the tissues embedded in Tissue-Tek® O.C.T. compound (Sakura Finetek USA, Torrance, CA) were sectioned to produce slices of 6 μm thickness, and incubated successively with 3C7 ascites diluted solutions (1:100 in PBS) and GAM-HRP IgG (1:2000 in PBS). Sections were also counterstained with Hoechst 33342 to detect nuclei. Peroxidase activity was revealed by incubation with 0.04% o-
phenylenediamine and 0.15% H₂O₂ in citrate–phosphate buffer. Finally, the slides were examined via light and fluorescence microscope.

2.8. Flow cytometry test

To target PORV-infected cells rapidly and sensitively, flow cytometry was used to detect PORV-infected cells with one of the MAbs produced 3C7. Infected GCO cells (0.25 PFU/cell) at the 36 h postinfection were fixed in 1% paraformaldehyde with 0.2% Tween-20, and incubated overnight at 4 °C. After being washed with PBS and blocked with PBS containing 1% BSA, the permeabilized cells were incubated with 3C7 MAb ascites (1:100) for 2 h at 37 °C. The cells were washed as described above and then incubated with FITC-GAM IgG (1:200) for 2 h at 37 °C. Finally, the GCO cells were washed again and resuspended in 1 ml of PBS supplemented with 0.5% BSA. The control experiments were done in the same way. The infection rate was determined by flow cytometry (Epics Altra).

3. Results

3.1. Production and screening of MAbs

An approximately 48.5% fusion rate was obtained in tested wells. Among the fused cells, the hybridomas with higher absorbance were selected for screening, and two MAbs (3C7 and 3C9) were finally isolated and cloned. Isotype determination showed that both of them were IgM subclass. After 2 weeks of injecting hybridomas, the MAb ascites were collected and their titers came to 1:5000 (3C7) and 1:4000 (3C9) according to the ELISA tests.

3.2. Western blotting

Five viral bands could be observed by SDS-PAGE after Coomassie staining of purified PORV proteins (Fig. 1A). In general, fish rhabdoviruses have five structural proteins (Ruan and Zhang, 2003), which were the polymerase (L), glycoprotein (G), nucleoprotein (N), phosphoprotein (P), matrix protein (M) (Schütze et al., 1999; Hoffmann et al., 2002; Kim et al., 2005). The Western blot analysis indicated that both 3C7 and 3C9 MAbs reacted with three viral protein bands of 42, 30, 22 kDa in the denatured form. According to the genome encoding characteristics and the molecular weights, the three bands should stand for N, P, and M (Fig. 1B). In addition to this, glycoprotein (G) was also identified by antiserum (Fig. 1B).

3.3. Immunodot

3C7 and 3C9 MAbs (diluted in 1:100) were used to capture the PORV from the purified virus and virus-infected GCO cell supernatants, respectively. Infected cell supernatants were pipetted into the center of the A2 and A3 grids (Fig. 2), while purified virus were dotted in the other 6 wells. Both showed positive signals though the results obtained with infected cell supernatants were weaker that those yielded by purified virus (Fig. 2). A1 and A4 indicated the results of negative and positive controls with PBS and polyclonal antisera as primary antibody, respectively.

3.4. Immunocytochemistry tests

Using immunofluorescence screening, both antibodies showed positive immunostaining in the PORV-infected GCO cells. The specific fluorescence signals were granular and observed at the peripheral zone of hypertrophied cells cytoplasm where the cytoplasmic inclusion bodies were located. The control cells did not show any signals with PBS as primary antibody (Fig. 3). In addition, results obtained with Hoechst staining indicated that the nuclei of PORV-infected cells became condensed and small in comparison with the uninfected cell nuclei.

At different postinfection times, the GCO cell monolayers were immunostained with 3C7 as primary antibodies, which showed a progressive development of distinct foci of virus replication, indicating that the antibodies recognized proteins of viral origin that are present in large amounts inside cells as early as 6 h.p.i. The infected cells became rounded and detached from the cell monolayer by 12 h postinfection in current experiment system. Until the 24 h postinfection, more and more positive signals were observed in the infected cells (Fig. 4).
3.5. Immunohistochemistry assay

Six kinds of carp tissues were examined. With the exception of the intestine, the remaining 5 tissues were found positively with some variation. The results for infected spleen and kidney tissues are shown in Fig. 5. Hematopoietic cells of the interstitial stroma of kidney or spleen were positive and showed early histomorphologic signs of degeneration and necrosis, typically as indistinct cells borders and condensation of the infected cell nucleus (Fig. 5B and F), while the normal tissues did not get immunostained, and the cell nucleus kept intact (Fig. 5D and H). Above results indicated that specific immunostaining were detected in the infected tissues.

3.6. Flow cytometry analysis

The percentage of PORV-infected GCO cells 36 h.p.i. was determined with MAb 3C7. PORV-infected GCO cells were tagged with FITC-conjugated GAM-IgG and differentiated according to fluorescence and light scattering properties. The virus-infected cell population could be distinguished from uninfected cell population by flow cytometry. Results showed that 36 h postinfection, 23% of GCO cells were infected with PORV, where approximately 10,000 cells were counted. The corresponding value of control GCO cells came to only 3.28% in view of the unspecific reaction.

4. Discussion

The _P. olivaceus_ rhabdovirus (PORV) has shown to be different from previous fish rhabdovirus isolates analyzed so far in the laboratory (Gui et al., 2007). Based on MAbs production experience (Zhou et al., 2006), two strains of hybridomas cell line (3C7 and 3C9) specific to PORV were obtained and identified. Western blot analysis confirmed that the molecular weights of PORV protein bands recognized by the two MAbs were indeed different from previous isolates. The two MAbs presented more than one band and this could be due to the complexity of the antigens, leading to the identification of a common epitope presenting in more than one protein. Similar results were also reported in studies developing MAbs against a grouper iridovirus (Shi et al., 2003), haemocytes of scallop (Xing and Zhan, 2005), lymphocystis disease virus (Cheng et al., 2006), _S. maximus_ rhabdovirus (Zhou et al., 2006) in aquaculture research, respectively. Concerning fish rhabdoviruses, previous reports on MAbs focused mainly on virus detection using the antigen capture ELISA, sandwich ELISA and immunoperoxidase techniques (Schultz et al., 1985; Mourton et al., 1992; Sanz et al., 1992; Reno and Lannan, 1992; Reschova et al., 2007). With the development of modern immunological techniques, many new diagnosis and characterization methods of virus pathogens have been established in virtue of MAbs.

Immunocytochemistry tests were used to localize the PORV antigens in infected cells and fish tissues. For mammal rhabdoviruses, e.g. the rabies virus, replication occurs in an area of the cytoplasm which acts as a virus “factory” and appears as a characteristic cytoplasmic inclusion body (Kristensson et al.,...
Fig. 5. Observation of frozen tissues by light microscope and fluorescence microscope. Positive signals (arrows) appeared in the hematopoietic cells of infected kidney and spleen (A and E), whose nuclei could not be detected (B and F). No positive signal appeared in the normal flounder kidney and spleen (C, D, G and H) (original amplification, ×400).

In present study, indirect fluorescent antibody analysis showed viral assembly sites staining patterns in the infected-GCO cells, which were consistent with those previous reports. Furthermore, analyzed by the immunoperoxidase tests at different times, a progressive development of well distinct foci of virus replication was showed and detected as early as 6 h.p.i. Besides that, the viral antigens in the infected grass carp tissues were also detected with the immunohistochemistry tests. After 10 days postinfection, positive immunostainings were found in five tissues. The spleen and kidney showed a higher level of immunostainings in comparison with liver, heart and gill, suggesting that haematopoietic organs of fish might be the major infection targets of fish rhabdovirus as reported previously (Romero et al., 2005). Another obvious morphological change of infected tissue cells was the condensed or invisible nuclei after the Hoechst 33342 staining, which could be related to cell apoptosis induced by fish rhabdovirus (Du et al., 2004).

Besides specificity, another characteristic of MAbs is their diagnosis sensitivity. It is known that immunodot test is based on the same principles than the Western blot. The difference between both assays is that there is no need for the former to transfer the antigens to the PVDF membrane with electrophoresis, so it requires little amounts of antibodies, antigens and it also permits the use of non-purified antigens (Domingues et al., 2002). Hence, with 1 μl PORV-infected GCO cell supernatants, the immunodot analysis can give positive signals. Moreover, in order to test further the MAbs sensitivity, one quantitative procedure was developed to differentiate infected cells from uninfected cells using flow cytometry. Flow cytometry has been applied widely for cell apoptosis, cell cycle, immune cells, cell receptor... in the basic biological research (Geng et al., 2005). In the present study, according to fluorescence and light scattering properties, flow cytometry tests showed that 23% of cells were infected and distinguishable clearly from uninfected cells at 36 h.p.i. Similar results had been obtained with the intracellular rabies virus (Bordignon et al., 2002). The result showed that the MAbs could be used for the sensitive detection and quantification of PORV-infected cells by flow cytometry.

In conclusion, two MAbs specific to PORV were produced and characterized. The results using different immunocytochemistry as well as flow cytometry indicated that these MAbs can be used for early diagnosis of viral infection.
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