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Characterization and Application of Monoclonal Antibodies against Turbot (*Scophthalmus maximus*) Rhabdovirus

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ABSTRACT

Five monoclonal antibodies (mAbs), 1G8, 1H9, 2D2, 2D3, and 2F5, against *Scophthalmus maximus* rhabdovirus (SMRV) were prepared. Characterization of the mAbs included indirect enzyme-linked immunosorbent assay, isotyping, viral inhibition assay, immunofluorescence staining of virus-infected cell cultures, and Western blot analysis. Isotyping revealed that 1G8 and 1H9 were of the IgG2b subclass and that the other three were IgM. 2D2, 2D3, and 2F5 partially inhibited SMRV infection in epithelioma papulosum cyprinid (EPC) cell culture. Western blotting showed that all five mAbs could react with two SMRV proteins with molecular masses of approximately 30 kDa (P) and 26 kDa (M). These two proteins were localized within the cytoplasm of SMRV-infected EPC cells by immunofluorescence assay. Also, progressive foci of viral replication in cell cultures were monitored from 6 to 24 h, using mAb 2D3 as the primary antibody. A flow cytometry procedure was used to detect and quantify SMRV-infected (0.01 PFU/cell) EPC cells with mAb 2D3, and 10.8% of cells could be distinguished as infected 36 h postinfection. Moreover, mAb 2D3 was successfully applied for the detection of viral antigen in cryosections from flounder tissues by immunohistochemistry tests.

INTRODUCTION

TURBOT (*Scophthalmus maximus*) RHABDOVIRUS (SMRV) was isolated from diseased turbot with lethal syndrome (5). Studies on the ultrastructural morphogenesis and induction of apoptosis in the carp leukocyte cell line (CLC) have demonstrated that the isolated virus was a strain of fish rhabdovirus. Rhabdoviruses are single-stranded RNA viruses (6,10). A significant pathogen, fish rhabdoviruses can infect common carp (*Cyprinus carpio*), flounder (*Paralichthys olivaceus*), rainbow trout (*Salmo gairdnerii*), and so on (1,8), causing high mortality in the wild and among cultured fish. Outbreaks of rhabdoviral diseases have caused great economic losses and became factors restricting aquaculture and the development of fisheries (11,33). Rapid detection and identification of infectious viruses are important for better management of diseases and to avoid the dissemination of pathogens, because vaccines or antiviral drugs are not available for the control of these diseases and avoidance of the pathogen is the only means of control (4). Many methods have been developed for rapid and sensitive detection and diagnosis of fish viruses according to immunological principles, such as the enzyme-liked immunosorbent assay (ELISA), immunofluorescence assay test (IFAT), immunohistochemistry, and so on (34,36), which all require abundant and specialized antibodies to antigens. Because they represent an effective and sensitive diagnostic tool, the development of specific monoclonal antibodies (mAbs) against viruses is

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thus highly desirable and might help implement both diagnostic assays for field application and methods for strain characterization (4,17,29).

Since the first report on mAbs specific to frog virus 3, a type species of fish *Ranavirus* (3) in aquaculture, many kinds of mAb against viral pathogens, such as fish viruses (17,18,30,31), shrimp viruses (15,20,25,32), and scallop virus (7), have been produced and characterized. Concerning fish rhabdoviruses, however, the literature on mAbs is limited. To date, mAbs specific to infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), and snakehead rhabdovirus (SHRV), and raised against IHNV glycoprotein, have been developed (14,22,26,27).

To further understand the pathogenesis and antigenesis of SMRV, to determine interactions between the virus and fish hosts, and ultimately to take measures to control viral infection, monoclonal antibodies against SMRV were prepared and characterized. In addition, the mAbs were applied to detect SMRV infection in fish cell lines and fish tissues by flow cytometry methods and immunohistochemistry, respectively.

MATERIALS AND METHODS

Cell culture and antigen preparation

Epithelioma papulosum cyprinid (EPC) cells were grown at 25°C in medium 199 (Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated fetal bovine serum (FBS). The virus used in this study was the turbot (*Scophthalmus maximus*) rhabdovirus (SMRV) isolated from diseased turbots in China (5). Purified SMRV was prepared as described previously by Zhang *et al.* (37). The purified virus suspension was prepared as antigen.

Mouse immunization and mAb production

Eight-week-old BALB/c mice were each inoculated intraperitoneally with 0.5 mL of a preparation of purified SMRV in the presence of Freund's adjuvant (Sigma, St. Louis, MO). After an interval of 2 wk, booster intraperitoneal injection with 0.5 mL of a mixture of purified virus and Freund's incomplete adjuvant was administered. Five weeks after the first immunization, serum samples were taken from immunized animals and tested for the presence of anti-SMRV antibodies by indirect enzyme-linked immunosorbent assay (iELISA), which is described below. A final boost of 0.5 mL was given (intraperitoneally) to each mouse after 6 wk.

Spleen cells from the immunized mice were fused with SP2/0 myeloma cells at a ratio of 5:1, using 50% polyethylene glycol (PEG 1000) as fusogen. The fused cells were resuspended in Dulbecco's modified Eagle's medium (GIBCO DMEM; Invitrogen) supplemented with hypoxanthine, aminopterin, and thymidine (DMEM–HAT) and plated into 96-well microplates. The hybridoma supernatants were screened for reactivity with SMRV by iELISA. Hybridomas giving positive results were cloned by limiting dilution with one cell distributed per well, and then tested again for mAb specificity by iELISA.

The iELISA principally comprised the following steps: microtiter ELISA plates were incubated overnight at 4°C with purified SMRV (100 μL/well) diluted 1:100 in bicarbonate coating buffer (pH9.6), and blocked at 37°C for 30 min with phosphate-buffered saline (PBS) solution containing 5% bovine serum albumin (BSA). The wells were emptied and hybridoma supernatants were added and incubated for 1 h at 37°C. After washing the plates three times for 5 min by immersion in PBST (0.05% Tween 20 in PBS), horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) (GAM–HRP, diluted 1:2000 with PBST) was added and incubated for 1 h at 37°C. After washing as described above, substrate solution (0.1 M citrate–phosphate buffer [pH 5.0], 0.05% *o*-phenylenediamine; 0.15% H_2O_2) was applied for 15 min at room temperature. The reaction was stopped with 2 M H_2SO_4 and the optical density (OD) of each well was read at 495 nm with a plate reader (model 550 microplate reader; Bio-Rad, Hercules, CA).

Determination of mAb isotype

Immunoglobulin isotypes of the mAbs were determined with a mouse monoclonal antibody isotyping kit (Sigma) in accordance with the instructions provided by the kit manufacturer.

Viral inhibition activities of mAbs

The viral inhibition assay was carried out on 96-well plates. Serial 10-fold dilutions (10^{-3} to 10^{-8}) of SMRV ($l0^6$ $TCID_{50}$ [50% tissue culture infective dose]) in growth medium with 5% FBS were added to 96-well plates at 0.1 mL/well. mAb supernatant was then added in equal volume to each viral dilution and the 96-well plates were placed in an incubator at 25°C for 1 h. After incubation, the mixtures were then inoculated into EPC cells in 96-well plates at 0.1 mL/well, and then the plates were incubated at 25°C for 6 d. Normal uninfected cells and infected cells served as positive and negative controls, respectively. Plates were examined by microscopy every day for evidence of a cytopathic effect (CPE).

Western blot analysis

Proteins of purified SMRV were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis

(SDS–PAGE) (37). The gels were then blotted onto polyvinylidene difluoride (PVDF) transfer membrane (Millipore, Bedford, MA) at 70 V for 45 min on ice. The blotted membrane was rinsed with distilled water and then saturated for 1 h with TBS (0.02 M Tris-HCl [pH 7.4], 154 mM NaCl) containing 5% albumin, and then washed three times with TBS containing 0.1% Tween 20. The membrane was then cut into several strips according to the sample lanes and these strips were put into supernatant of each mAb and mouse antiserum raised against SMRV (polyclonal antibody, pAb) diluted with TBS containing 1% skim milk, and incubated for 1 h at room temperature, followed by washing three times (5 min each) in TBS containing 0.1% Tween 20. mAb binding was detected with alkaline phosphatase-conjugated goat anti-mouse IgG (GAM–AP; Vector Laboratories, Burlingame, CA) for 1 h at room temperature, and washing three times as described above. The reaction was developed with substrate nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for 5 min, and stopped by rinsing the strips with distilled water.

Immunohistochemistry test

EPC cells grown on glass coverslips in a 6-well plate were infected with SMRV at a low multiplicity of infection (MOI; 0.5 plaque-forming units [PFU]/cell) and incubated at 25°C for 0, 6, 12, and 24 h postinfection. Monolayers were then rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature followed by permeabilization with absolute ethanol for 10 min at −20°C. After washing, two incubations were successively performed for 1 h each with one randomly selected mAb and GAM–AP. After antibody incubations, the slides were rinsed three times with PBS. The presence of viral replication foci was shown by incubation for 5 min with freshly prepared substrate solution (NBT–BCIP in AP buffer). The coverslips were then washed with PBS, mounted with glycerol–PBS (1:1) on glass slides, and examined under a microscope (Leica Microsystems, Wetzlar, Germany).

For indirect immunofluorescence staining, the slides were first incubated with mAbs and subsequently stained with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Pierce Biotechnology, Rockford, IL) diluted 1:2000 in PBS. After mounting in glycerol–PBS (1:1), monolayers were observed with a fluorescence microscope (Leica Microsystems).

To confirm the specificity of the mAbs, immunohistochemical tests were also performed on tissue sections from flounder, both uninfected and infected. Every flounder of the infection group was infected with 0.5 mL of SMRV (10^6 $TCID_{50}$) by intraperitoneal injection. Eleven kinds of tissue (gill, spleen, kidney, head kidney, liver, skin, ovary, spermary, intestine, heart, and muscle) were collected 3 d postinfection. The tissues were fixed in 4% paraformaldehyde overnight. After washing three times with PBS, the tissues were saturated with 30% sucrose overnight and then embedded in Tissue-Tek O.C.T. compound (Sakura Finetek USA, Torrance, CA) for freeze sectioning. Serial sections (thickness, 6 μm) were prepared and processed for indirect immunoperoxidase staining, using the selected mAb and GAM–HRP diluted 1:1000 with PBS. Preparations were also counterstained with Hoechst 33342, a kind of fluorescent dye, to detect cell nuclei. Peroxidase activity was revealed by incubation with 0.04% *o*-phenylenediamine and 0.15% H_2O_2 in citrate–phosphate buffer. Uninfected flounder tissues were treated in the same manner as infected tissues. After extensive washing, the slides were mounted in glycerol–PBS (1:1) and examined via light and fluorescence microscopy (Leica Microsystems).

Flow cytometry test

To quantify the virus-infected cells rapidly and sensitively, flow cytometry was used to distinguish SMRV-infected cells with the selected mAb. Infected EPC cells (0.01 PFU/cell) at 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 anti-SMRV mAb supernatant for 1 h at 37°C. The cells were washed as described above and then incubated with FITC–goat anti-mouse IgG (diluted 1:200) for 1 h at 37°C. Finally, the stained cells were washed again and resuspended in 1 mL of PBS supplemented with 0.5% BSA. The control experiments were

Table 1. Characterization of mAbs to SMRV

mAb	*Isotype*	*ELISA*	*Inhibition activity*	*Western blot*	*Immunofluorescence*
1G8	IgG2b	0.072	−	+	+
1H9	IgG2b	0.075	−	+	+
2D2	IgM	0.069	+	+	+
2D3	IgM	0.068	+	+	+
2F5	IgM	0.066	+	+	+

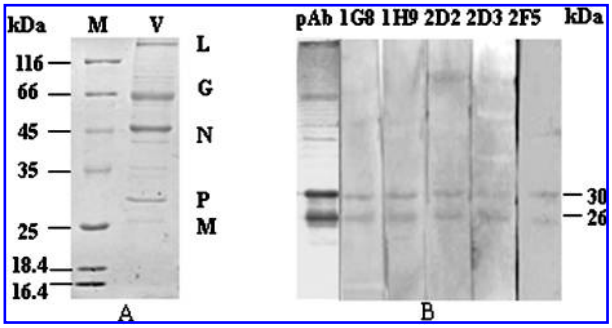

FIG. 1. SDS–PAGE and Western blot analysis of SMRV proteins. (**A**) Coomassie blue-stained SDS–polyacrylamide gel of SMRV proteins. M, molecular weight marker; V, purified SMRV. (**B**) Western blot with five mAbs and antiserum (pAb).

done in the same manner. The infection rate was determined by flow cytometry (Epics Altra; Beckman Coulter, Fullerton, CA).

RESULTS

Hybridoma production and screening

The fused cells were cultured in three 96-well plates. After cultivation in HAT medium for 2 wk, an approximately 46.6% fusion rate was achieved in seeded wells. There were 123 hybridomas producing SMRV-specific antibodies as screened by iELISA.

Characterization of mAbs

The hybridomas with higher absorbance were selected for screening mAbs by iELISA, and five mAbs (1G8, 1H9, 2D2, 2D3, and 2F5) were eventually isolated and cloned. The immunoglobulin classes of these antibodies were determined by ELISA isotyping according to the capture ELISA procedures. 1G8 and 1H9 are IgG2b in subclass, whereas the other three (2D2, 2D3, and 2F5) are IgM.

The optical densities (ODs) of ELISA titers were measured after ELISA at a wavelength of 450 nm, using grow-to-die mAb supernatants. The OD values for the IgG2b subclass mAbs were similar to those of the IgM mAbs, but 2D2, 2D3, and 2F5 exerted partial inhibitory effects on SMRV infection in inhibition tests. For the 10-fold serial dilutions of SMRV (10^{-3} to 10^{-8}), no CPE could be observed after 7 d in wells containing viral dilutions from 10^{-4} to 10^{-8} and treated with 2D2, 2D3, and 2F5. This suggested that the inhibitory abilities of the three mAbs could process to 10^{-4} virus dilution (10^{6} $TCID_{50}$). The results are shown in Table 1.

Recognition of SMRV proteins by mAbs

At least five viral bands were observed by SDS–PAGE after Coomassie staining of purified SMRV proteins (Fig. 1A). The results of Western blotting showed that all mAbs reacted with two viral protein bands of 30 and 26 kDa in the denatured form. According to the published data on fish rhabdovirus genomes and putative protein molecular weights (24), the two proteins should be phosphoprotein (P) and matrix protein (M). Another three positive viral bands were presented by mouse antiserum, which were defined as RNA-dependent RNA polymerase (L), glycoprotein (G), and nucleoprotein (N) on the basis of their map positions (Fig. 1B).

Immunohistochemistry of infected cells and flounder tissues

All the mAbs produced staining signals in the cytoplasm of SMRV-infected EPC cells. No staining signal

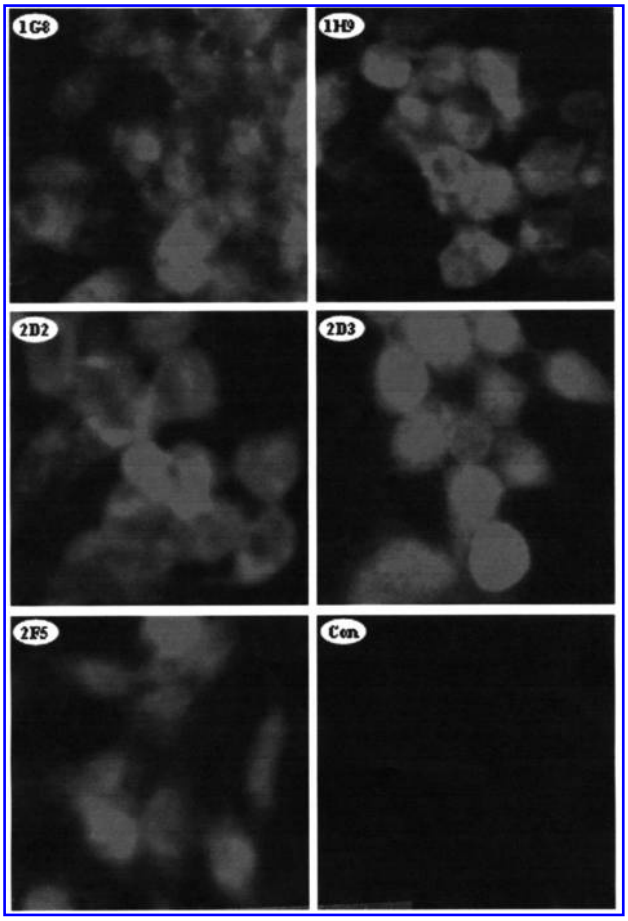

FIG. 2. Immunofluorescence of SMRV-infected EPC cells with mAbs (1G8, 1H9, 2D2, 2D3, and 2F5). Photographs were taken 24 h postinfection (original magnification, ×600). Con, control with PBS.

was observed in uninfected cells (Fig. 2). The data showed that the P and M proteins reacting with the five mAbs in the Western blot analysis were localized mainly within the cytoplasm.

With 2D3, immunostaining of monolayers at various postinfection time points showed the progressive development of distinct foci of viral replication within the layer of normal cells (Fig. 3), indicating that the antibody recognized proteins of viral origin that are present in large amounts inside cells as early as 6 h postinfection. The infected cells became rounded and detached from the cell sheet by 12 h postinfection. Until 24 h postinfection, more and more positive signals were observed in the infected cells (Fig. 3).

Immunohistochemistry with mAb 2D3 showed that positive reactions were detected in nine tissue section types (gill, liver, kidney, head kidney, spleen, skin, ovary, spermary, and intestine), but not in the sections of muscle and heart. The results for infected gill and liver tissues are shown in Fig. 4. Normal gill lamellae distributed symmetrically and no positive signal was seen (Fig. 4A), but infected gill lamellae swelled and conglutinated, and strong positive signals were observed in the blood vessels of the gills (Fig. 4C). Uninfected hepatocytes appeared to be arrayed compactly and normal in appearance, and no positive signal was seen (Fig. 4E), whereas experimental liver cells became hypertrophic and vacuolar, and infected hemocytes in liver veins were immunostained positively (Fig. 4G). When stained with Hoechst 33342, the nuclei of infected hemocytes condensed or were even undetectable (Fig. 4D and H), but the nuclei of uninfected tissues were normal (Fig. 4B and F). Thus, the results revealed that specific immunostaining was observed mostly in the hemocytes of tissues.

Flow cytometry test

The percentage of SMRV-infected cells 36 h postinfection was determined with mAb 2D3. Gated areas *A* and *B* in Fig. 5 represent uninfected and infected cell pop-

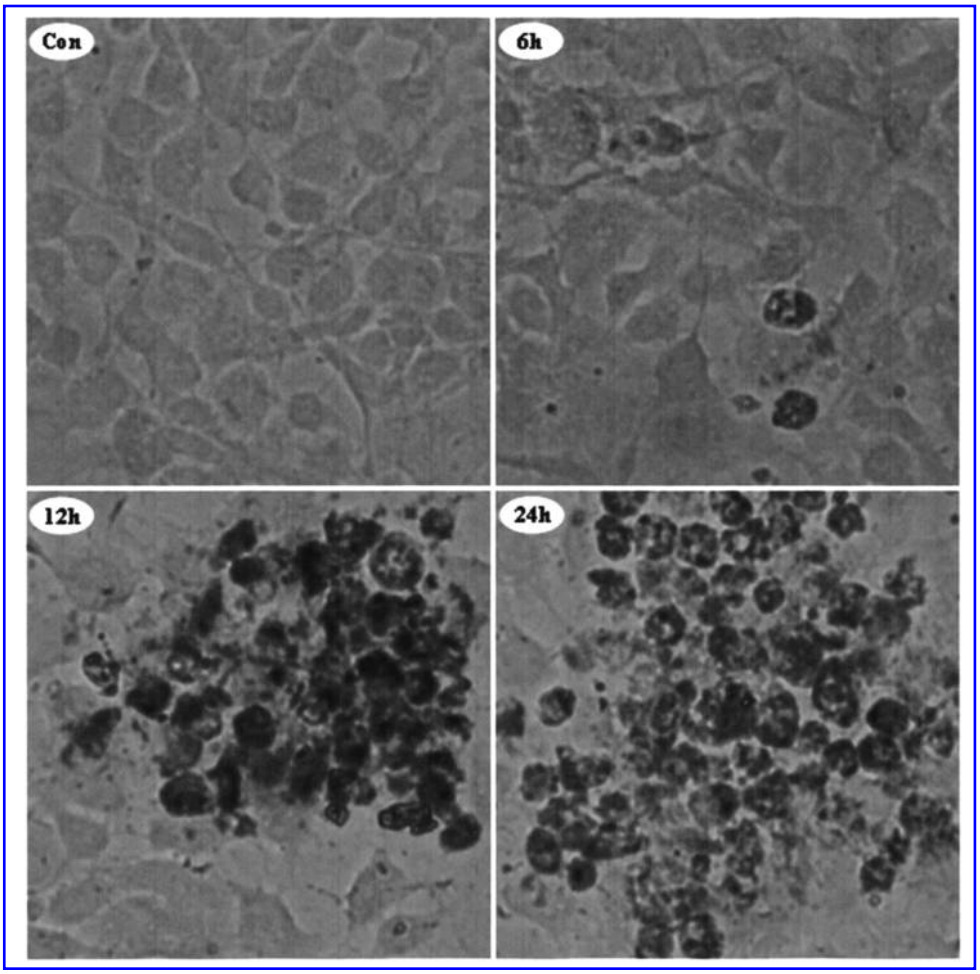

FIG. 3. Immunostaining of SMRV-infected EPC cells at various times postinfection. Photographs, showing the progressive development of foci of viral replication, were taken 6, 12, and 24 h postinfection (original magnification, ×600).

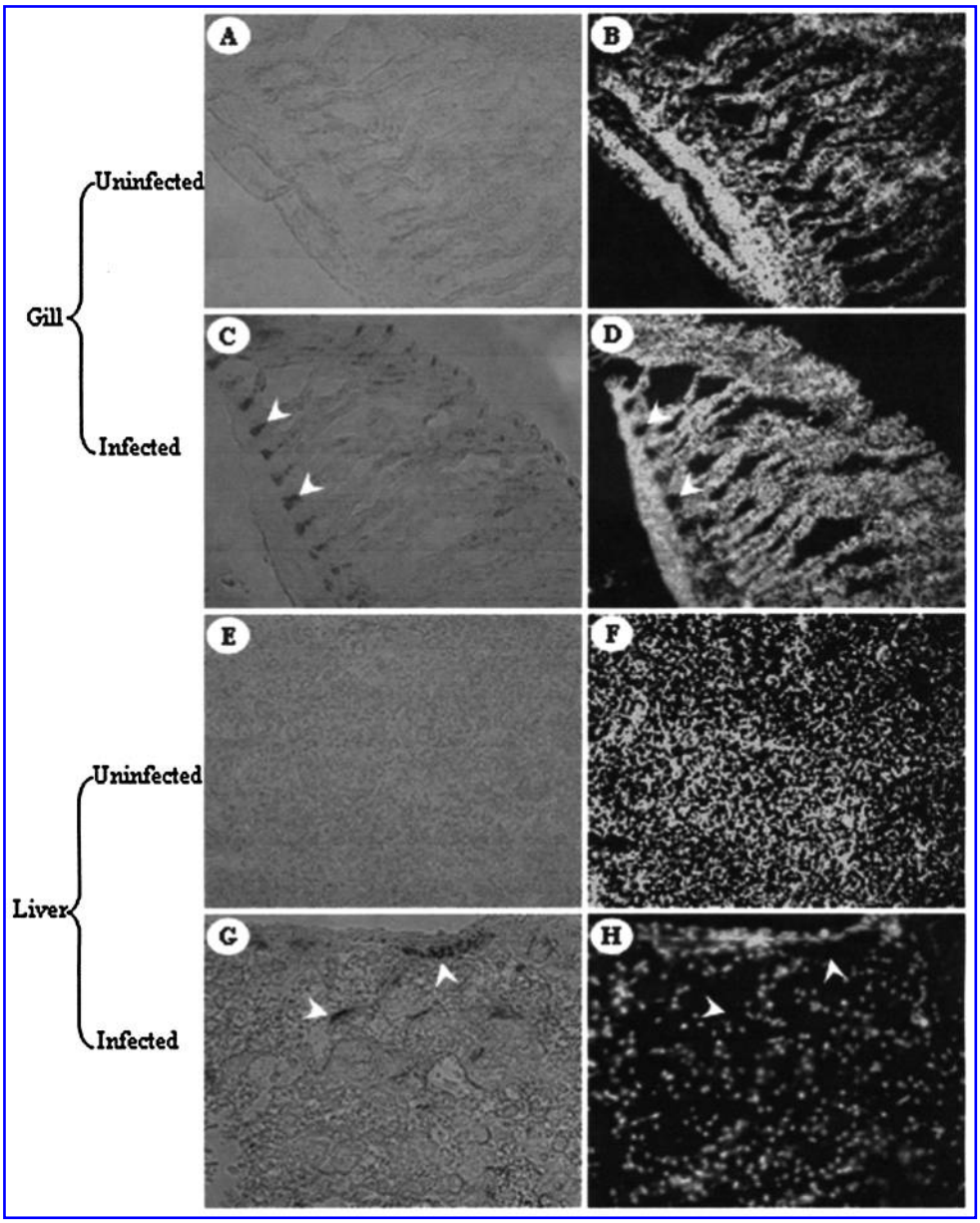

FIG. 4. Observation of frozen tissue sections by light microscopy (*left*) and fluorescence microscopy (*right*). Positive signals (arrowheads) appeared in the hemocytes of infected gill and liver (**C** and **G**, respectively), the nuclei of which could not be detected (**D** and **H**, respectively). No positive signal appeared in normal flounder gill and liver [(**A** and **B**) and (**E** and **F**), respectively]. Original magnification, ×60.

ulations, respectively, and the scale of histogram portions *C* and *D* denote the percentages of the two kinds of EPC cells, respectively. Results showed that 36 h postinfection, the virus-infected cell population (gated area *B*) could be distinguished from uninfected cells (gated area *A*), and 10.8% of EPC cells (scale of histogram portion *D* in Fig. 5, bottom) were infected with SMRV, where approximately 18,000 cells were counted. The corresponding value of control EPC cells came to only 1.2% in view of the unspecific reaction (scale of histogram portion *D* in Fig. 5, top).

DISCUSSION

Five monoclonal antibodies specific to SMRV were developed and identified as belonging to two isotypes (IgG2b and IgM) on the basis of structure, antigenicity,

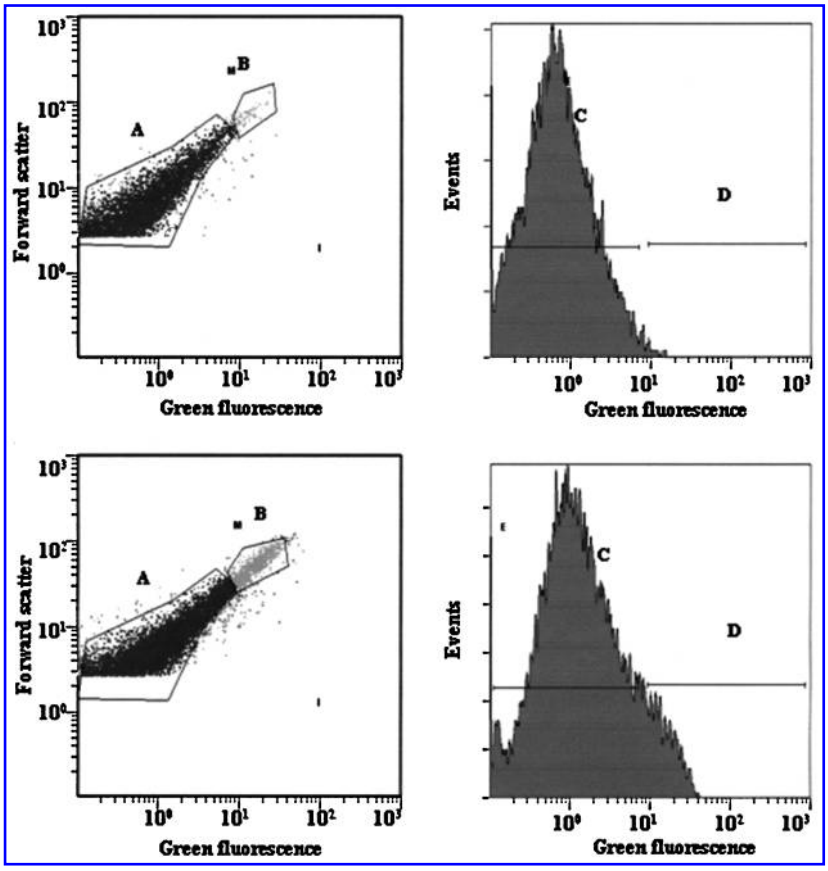

FIG. 5. Detection of uninfected and infected EPC cells with SMRV viral antigens 36 h postinfection by flow cytometry. *Top:* Normal cell culture control, uninfected with virus. *Bottom:* SMRV virus-infected cells 36 h postinfection. The virus-infected cell population (gated area *B*) could be distinguished from uninfected cells (gated area *A*), and it was found that 10.8% of EPC cells (scale of histogram portion *D*) were infected with SMRV. Each histogram is based on 100,000 events.

and amino acid sequence of constant areas of the immunoglobulin heavy chain.

The inhibition assay (constant mAb plus viral dilutions), the results of which were characterized by whether CPE was present, was used to determine the inhibitory effect of these mAbs on SMRV. Because of the low ELISA titers of the mAb supernatants relative to ascites, the inhibitory effects on SMRV infection were incomplete, although more CPE in negative controls was observed than in mAb-treated samples. The results seemed to be related to quantitative deficiencies in the generation of mAbs by hybridoma cells. Also, the anti-IHNV mAbs were not able to inhibit IHNV isolates completely, although they were reactive with the isolates by immunofluorescence assay, indicating that binding by an mAb does not guarantee neutralization by that mAb (23). Our inhibition experiment was consistent with the preceding conclusions.

The genomes of fish rhabdoviruses encode five proteins, named L, G, N, P, and M (9,12,28). SDS–PAGE showed the five putative protein bands of SMRV. Western blot analysis revealed that all the antibodies produced virus-specific positive reactions with two blotted protein bands at about 30 kDa (P) and 26 kDa (M). In general, one mAb reacted with one protein in Western blot. But in our study, the mAbs reacted with two bands. Similar results were reported in studies developing mAbs against a grouper iridovirus and hemocytes of scallop, respectively (30,35), in aquaculture research. The inconsistency is due to the complexity of the antigen, suggesting that mAbs may identify a common epitope present in more than one antigen. Although the Western blot assay is a valuable testing format to characterize the specificity of mAbs, it is also highly technical. Factors referred to previously may have led to the current results, that is, the fact that the five mAbs presented more than one band. Identification and further characterization of the two antigenic-related proteins are needed.

Twenty-four hours postinfection, infected EPC cells

were fixed and tested by the indirect fluorescent antibody technique, and all the EPC cell samples showed positive results with the five mAbs. It is characteristic of rhabdoviruses that their replication occurs in an area of the cytoplasm that acts as a virus "factory" and appears as a characteristic cytoplasmic inclusion body, for example, perinuclear Negri bodies of rabies virus (13,19). In the present study, mAbs produced cytoplasm-staining patterns consistent with these reports. By performing immunocytochemical analyses at various times, progressive development of distinct foci of viral replication was shown, which was detected as early as 6 h postinfection. These results suggested that the mAbs could be used for early viral diagnosis.

We applied 2D3 as the capture antibody to detect viral infection in the immunohistochemical tests; most of the positive signals were observed in the peripheral hemocytes of infected tissues. This suggested that the blood cells of flounder were the targets of SMRV. In addition, in 3 d, the virus did not have enough time to infect the basal portion of tissues; it did, however, have time to be sent all over the body via the blood. It has been demonstrated that SMRV could induce apoptosis in carp leukocyte cell (CLC) cultures (5). Chromatic condensation and nuclear fragmentation in the infected cells were investigated 9 h postinfection by fluorescence microscopy after staining with 4′,6-diamidino-2-phenylindole (DAPI). Similarly, an obvious morphological change of infected hemocytes is that the nuclei condensed or could not be observed by Hoechst 33342 staining in our experiments.

Flow cytometry has allowed sensitive and rapid detection and quantification of viral infection by immunofluorescence staining (16). For mammal rhabdovirus, the flow cytometry assay was used to detect intracellular rabies virus (2). A flow cytometry-based method for detection of a marine fish iridovirus in cell culture was also developed (21). In the present work, SMRV-infected EPC cells were tagged with FITC-conjugated GAM–IgG and differentiated according to fluorescence and light-scattering properties; virus-infected cells, representing 10.8% of cells, could be distinguished from uninfected cells 36 h postinfection. This is the first report on fish rhabdovirus detection in cell culture by flow cytometry. Although we detected virus-infected cells at just one time point, the result shows that the mAbs could be used for detection and quantification of cytoplasmic SMRV in cell culture.

In conclusion, a panel of monoclonal antibodies specific to SMRV was characterized and applied in cell culture and at the tissue level, and showed considerable potential as a tool for immunodiagnosis and for studying the biology of infection and pathogenesis of SMRV.

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