

Development and Characterization of Monoclonal Antibodies against the Snakehead Rhabdovirus

J. KASORNCHANDRA

Department of Microbiology, Oregon State University
Nash Hall 220, Corvallis, Oregon 97331-3804, USA

P. CASWELL-RENO AND C. N. LANNAN

Laboratory for Fish Disease Research, Department of Microbiology
Oregon State University, Mark O. Hatfield Marine Science Center
Newport, Oregon 97365-5296, USA

J. L. FRYER¹

Department of Microbiology, Oregon State University, Corvallis

Abstract.—Monoclonal antibodies (MAbs) directed against the snakehead rhabdovirus (SHRV) were produced. These MAbs were characterized by immunofluorescence and neutralization tests, and by their ability to immunoprecipitate viral proteins. Of 15 MAbs developed, 9 were isotypized as IgG1 and 6 were IgG2a. Eight of the MAbs recognized the viral glycoprotein in an immunoprecipitation assay. Three of these, designated E1-9A, P10C, and O10F, had neutralizing activity. By immunofluorescence, 12 MAbs showed good binding activity in SHRV-infected epithelioma papulosum cyprini cells. In an indirect fluorescence assay, the MAbs gave varied staining patterns depending upon the viral structural proteins recognized.

A rhabdovirus that is serologically different from other fish rhabdoviruses (Ahne et al. 1988; Kasornchandra et al. 1991) was isolated (Wattanavijarn et al. 1986) from chevron snakehead *Channa (=Ophicephalus) striata* during an outbreak of ulcerative disease in southeast Asia. The snakehead rhabdovirus (SHRV) is one of the several potential pathogenic organisms isolated from animals with this disease, but the etiologic agent has not been established (Boonyaratpalin 1989; Frerichs et al. 1989). Kasornchandra et al. (1991) found that SHRV contained five structural proteins similar to those of infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV). Replication of the SHRV and production of cytopathic effect is rapid in susceptible cell lines. In the epithelioma papulosum cyprini (EPC) cell line (Fijan et al. 1983) and the snakehead fin cell line (Kasornchandra et al. 1988), cytopathic effect is apparent within 14 h and complete in 24–48 h at 27°C (Kasornchandra et al. 1991). Although polyclonal antibodies were produced in rabbits and used for identification of SHRV by serum neutralization assay, the rabbit anti-SHRV sera had low titers and were toxic to the cells. In immunofluorescence assays, the rab-

bit antisera had extensive nonspecific binding with high levels of background fluorescence both in the control and the infected cell lines tested. To improve methods for identification of this virus, monoclonal antibodies (MAbs) against SHRV were developed.

The purpose of this study was to produce MAbs against SHRV for use in immunodiagnosis. These MAbs were characterized by immunoprecipitation, immunofluorescence, and serum neutralization.

Methods

Production and screening of hybridomas.—For antigen preparation, SHRV was propagated in EPC cells. Spent medium was harvested from infected cultures when cytopathic effect reached completion, and this sample was clarified by low-speed centrifugation. Virus was concentrated from the fluids by high-speed centrifugation and further purified on discontinuous and continuous sucrose gradients as described by Engelking and Leong (1989).

Splenocytes from BALB/c strain mice that had been immunized with the purified SHRV were fused with SP2/0-Ag-14 mouse myeloma cells by the methods of Galfre et al. (1977) and Caswell-Reno et al. (1986). Hybridomas that were positive for production of an antibody specific for SHRV

¹ To whom correspondence should be addressed.

were identified by dot-immunoblotting assay (Hawkes et al. 1982) and cloned at least twice. The immunoglobulin (Ig) class and subclass of the MAb produced by each hybridoma were determined by an enzyme-linked immunosorbent assay (ELISA; HyClone Laboratories, Logan, Utah). The MAb preparations used in the following studies were unpurified cell culture supernatants from the hybridomas.

Specificity of MAbs.—The identity of the SHRV polypeptides recognized by the MAbs was determined by immunoprecipitation and electrophoretic separation of radiolabeled viral proteins. Cell cultures inoculated with SHRV, or uninoculated control cultures, were labeled with ^{35}S -methionine (Calbiochem, San Diego, California) as described by Engelking (1988) and Ristow and de Avila (1991). After an 8-h incubation, the cells were harvested, resuspended in lysis buffer (0.867 g NaCl, 1.17 g EDTA, 0.48 g tris, and 0.5 mL Nonidet P40 [polyoxyethylene ether] in 100 mL distilled water; pH 7.0), and sonicated for three 5-s pulses at 4°C.

After clarification by centrifugation, the solid phase immunoisolation technique of Tamura et al. (1984) was used to immunoprecipitate the labeled viral proteins from the lysate. For each MAb, 100 μL of undiluted hybridoma culture fluid was added to each of three wells of a 96-well ELISA plate previously coated with goat antimouse IgG. After a 5-h incubation at 4°C, the wells were washed, and 100 μL of the labeled cell lysate were added to each well. The preparations were incubated overnight at 4°C, then the lysate was removed, the wells were washed, the bound proteins were eluted in a denaturing sample buffer, and the resulting contents of the three wells were combined.

The proteins were dissociated by heating and separated by discontinuous denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli 1970) with a 4.75% acrylamide stacking gel and a 10% acrylamide separating gel. The labeled proteins bound by the MAbs were visualized on Hyperfilm-MP (Amersham Corp., Chicago), which was developed after 5–7 d of incubation on the fixed and dried separating gels.

Titration of antibody.—Neutralizing antibody titers were determined by the alpha procedure of Rovozzo and Burke (1973). The \log_{10} neutralization index was computed as the difference between the \log_{10} titer (TCID₅₀/mL) of virus incubated without antibody and the \log_{10} titer of virus incubated with antibody; TCID₅₀ is tissue culture

infective dose, the dose causing cytopathic effect in 50% of the inoculated cultures. Three SHRV neutralizing MAbs were tested for cross-neutralization with seven heterologous fish rhabdoviruses: IHNV, VHSV, hiram rhabdovirus (HRV), spring viremia of carp virus (SVCV), pike fry rhabdovirus (PFRV), and ulcerative disease rhabdovirus (UDRV-BP and UDRV-19).

Immunofluorescence assays.—Binding affinities of the MAbs were tested with a modified version of the indirect fluorescent antibody technique (IFAT) described by LaPatra et al. (1989). Briefly, monolayers of EPC cells plated on coverslips were inoculated with 2.8×10^3 TCID₅₀ SHRV, allowed to adsorb for 1 h, overlaid with growth medium, incubated for 10 h at 27°C, then rinsed in phosphate-buffered saline, and fixed in cold acetone (–70°C). The MAbs were applied to the coverslips, and these samples were incubated for 1 h at ambient temperature then rinsed. Fluorescein isothiocyanate-conjugated goat anti-mouse serum (Sigma Chemical Co., St. Louis, Missouri) then was applied. After 1 h, the coverslip samples were again rinsed and counterstained with 0.01% Evans blue, mounted in glycerol (pH 9.0), and examined with a Zeiss fluorescence microscope.

Results

Production and Screening of Hybridomas

Twelve days after fusion, approximately 21% of the 672 seeded wells contained hybridomas. In the initial dot-immunoblotting assay, about 46% of the hybridomas produced detectable antibodies against SHRV. Subcultivation and subsequent repeated screening yielded 15 positive hybridomas. Three MAbs possessed neutralizing activity against homologous virus, and 12 MAbs had binding activity detected by immunofluorescence with SHRV-infected cells. Two different subclasses were observed among 15 MAbs produced against SHRV. Nine MAbs were of the IgG1 subclass, and six were determined to be of the IgG2a subclass.

Specificity of MAbs

To determine the specific SHRV polypeptides recognized by the MAbs, the antibodies were bound to an ELISA plate via an anti-immunoglobulin reagent. Labeled cell lysates were reacted with the specific antibody, eluted with a denaturing sample buffer, and then electrophoresed. Autoradiograms of the viral protein immunoprecipitations by the MAbs are shown in Figures 1–3.

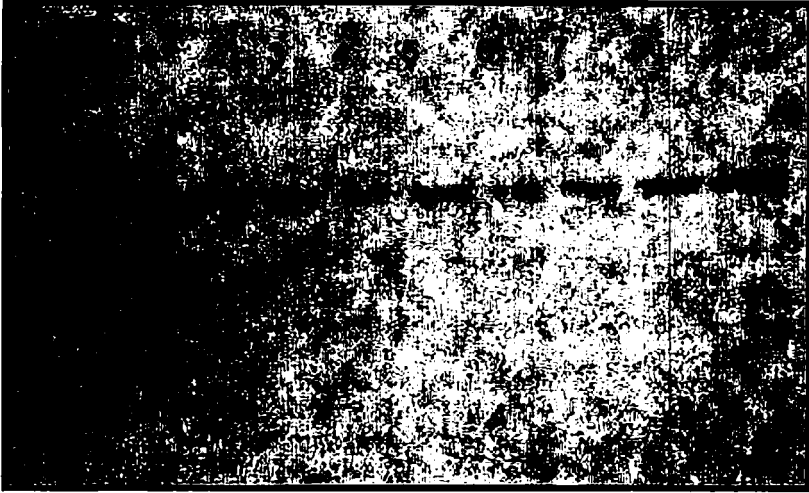


FIGURE 1.—Immunoprecipitation of SHR V proteins with monoclonal antibodies (MABs), shown by electrophoresis on a 10% acrylamide gel. Lane 1 contains purified SHR V; lanes 2–9 contain MABs against the G protein.

Of the 15 MABs tested, 8 recognized the G protein (glycoprotein), 5 recognized the N protein (nucleocapsid protein), and 2 recognized the M₁ protein (a matrix protein).

Titration of Antibody

Hybridoma culture fluid containing MAB was diluted 1:5 and reacted against SHR V. No toxicity was observed at this dilution. Three MABs (E1-9A, P10C, and O10F), all of which recognized the

G protein, gave log₁₀ neutralization indices ranging from 1.7 to 1.8. Twelve MABs did not neutralize SHR V, although 5 of these 12 bound to the G protein. In cross-neutralization tests, the neutralizing MABs did not react with any heterologous fish rhabdoviruses tested.

Immunofluorescence Assays

The IFAT reaction of the MABs with SHR V-infected EPC cells 10 h postinoculation is illus-

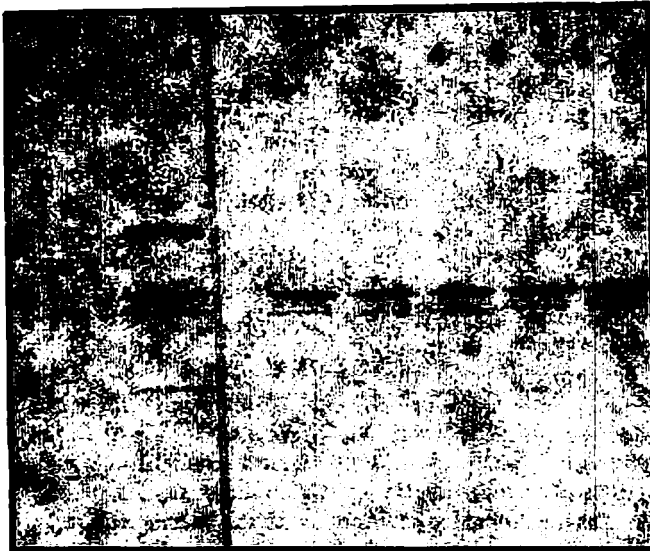


FIGURE 2.—Immunoprecipitation of SHR V proteins with monoclonal antibodies (MABs), shown by electrophoresis on a 10% acrylamide gel. Lane 1 contains purified SHR V; lanes 2–6 contain MABs against the N protein.

trated in Figure 4. Only the nonneutralizing MAbs displayed strong binding to SHRV-infected EPC cells. Antibody against the G protein stained most of the cell structures and provided uniform staining throughout the cell cytoplasm (Figure 4a). Antibody against the N and M₁ proteins provided a coarse granular staining in the cytoplasm of the infected cells (Figure 4b, c). In addition, the M₁-specific antibody stained the cell membranes (Figure 4d). No background fluorescence of EPC cells was observed. At a 1:500 dilution of the MAbs, they still displayed strong binding to the SHRV-infected EPC cells. Cell cultures infected with one of seven other rhabdoviruses (IHNV, VHSV, SVCV, PFRV, HRV, UDRV-BP, or UDRV-19) were tested by IFAT for cross-reactivity with selected MAbs against the G protein of SHRV. Cross-reactivity was detected in these preparations only at high antibody concentrations. The reaction was especially strong with cells infected with SVCV, PFRV, UDRV-BP, or UDRV-19. However, at a 1:100 dilution of the MAbs no cross-reactivity was observed.

Discussion

Monoclonal antibodies were successfully developed against SHRV. Two groups of MAbs, one neutralizing and one nonneutralizing, were characterized. Three neutralizing MAbs were directed against the G protein as determined by radioimmunoprecipitation. None of the MAbs that recognized the N and M₁ viral proteins had neutralizing activity. Like the G protein of IHNV and of the mammalian rhabdoviruses, vesicular stomatitis virus (VSV) and rabies virus (Kelley et al. 1972; Cox et al. 1977; Engelking and Leong 1989), the G protein of SHRV appeared to be responsible for inducing neutralizing antibody.

Although the rhabdoviral G protein elicits neutralizing antibody, not all of the MAbs specific for the G protein produce neutralization. In MAB competition assays, Dietzschold et al. (1983) demonstrated that the glycoprotein of the CVS strain of rabies virus had nine distinct epitopes involved in immunogenic activity. Three of these epitopes bind neutralizing MAbs (Seif et al. 1985). On the G protein of VSV, five nonneutralizing and four neutralizing epitopes were defined by MAB studies (Vandepol et al. 1986). Among our MAbs developed against SHRV, five that recognized the viral G protein in immunoprecipitation were nonneutralizing.

In immunoprecipitation assays, the N protein

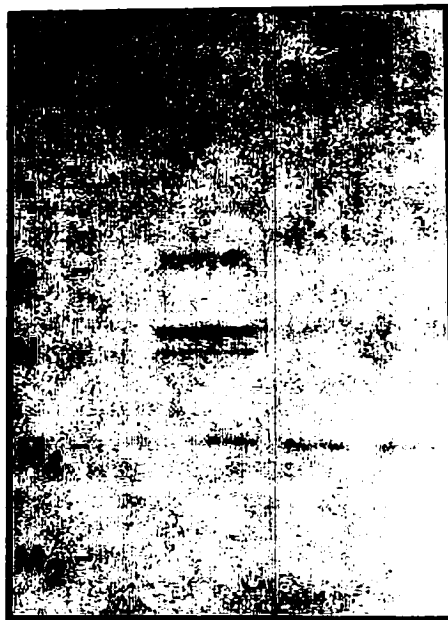


FIGURE 3.—Immunoprecipitation of SHRV proteins with monoclonal antibodies (MAbs), shown by electrophoresis on a 10% acrylamide gel. Lane 1 contains purified SHRV; lanes 2–3 contain MAbs against the M₁ protein.

of SHRV displayed two bands when reacted with the MAbs. The molecular mass of the lower band was 38 kilodaltons and that of the upper band was 42 kilodaltons. The two forms of the N protein may have a product-precursor relationship, or one may be a breakdown product of endonuclease cleavage.

The MAbs, recognizing different viral proteins, gave distinct immunofluorescent staining patterns. Similar results have been reported by Lorenzen et al. (1988) for VHSV, which has structural proteins similar to those of SHRV. These researchers discovered that MAbs specific for the G protein of VHSV gave two distinct staining patterns: one type stained reticular structures and the other stained cell membranes. Both stained juxtanuclear cisternae-like structures. With the G-specific MAbs used in our study only the characteristic reticular pattern was observed, although Lorenzen et al. (1988) suggested that staining of the reticular structures is an unusual observation. Using electron microscopy and immunofluorescence, Wehland et al. (1981) and Bergmann et al. (1982) found that, in VSV-infected cells, the G protein of VSV was present in the endoplasmic reticulum and the Golgi apparatus, and on the

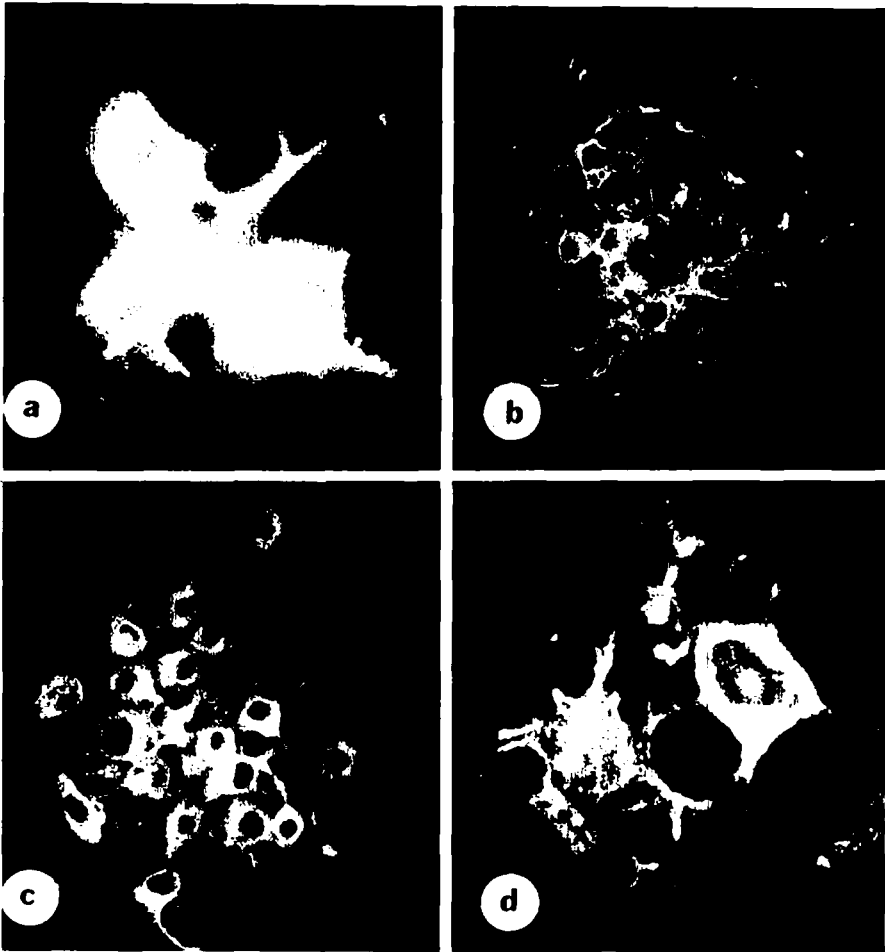


FIGURE 4.—Monolayer cultures of EPC cells that were infected with SHRV, fixed 10 h postinfection, and tested by the indirect fluorescent antibody technique for specific immunofluorescence. (a) Infected cells with monoclonal antibody (MAb) against the G protein. (b) Infected EPC cells with MAb against the N protein. (c) Infected EPC cells with MAb against the M₁ protein. (d) Higher magnification of infected EPC cells stained with MAb specific for the M₁ protein. Note staining of cellular membranes.

plasmalemma, and the cell surface. Therefore, the epitope of the G protein recognized by MAbs against SHRV may be present in cytoplasmic reticulum, as is the case with VSV.

The MAbs developed against SHRV have potential as tools for immunodiagnosis and studies of pathogenesis. The standard method for viral identification requires the use of serum neutralization tests, but rabbit anti-SHRV sera have low titers and are highly toxic to the cells. However, the SHRV-neutralizing MAbs provide adequate neutralizing titers and displayed no toxicity. The nonneutralizing MAbs have strong binding activity in the IFAT and will be useful in diagnosis and studies of pathogenesis.

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