**Note**

An Established Hybridoma Clone Producing a Monoclonal Antibody against *Vibrio anguillarum*

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*Vibrio anguillarum* is a pathogenic microorganism of vibriosis, an infectious disease found in various fish species. A mouse hybridoma clone, named C5, that produced a monoclonal antibody to *V. anguillarum* was established. The specific reaction of C5 antibody with *V. anguillarum* was confirmed by the pre-adsorption effect of the *V. anguillarum* cells in ELISA and a cell immunoprecipitation experiment. Western blotting analysis indicated that the C5 antibody recognized a high molecular weight substance extracted from cells with detergents.

**Key words:** monoclonal antibody; *Vibrio anguillarum*

The large mortality of cultured marine fishes caused by infectious diseases has been a big problem to the fish aquaculture business.1) *Vibrio anguillarum* is the etiologic agent of vibriosis, one of the most serious infectious diseases found in various fish species.1) Monoclonal antibodies to fish infectious pathogens such as bacteria and virus are promising as useful tools for diagnosis and prevention of infectious diseases, and identification of the antigenic substance for development of vaccines. Hybridoma clones producing the monoclonal antibodies also can be good sources of mRNA for the construction of the recombinant antibody expression systems.2,3) In this study, we have established a hybridoma clone that produces a monoclonal antibody against *V. anguillarum* and analyzed its antigenic substance.

The following materials were obtained from the sources indicated: Dulbecco's modified Eagle's medium powder (GIBCO BRL, Gaithersburg, MD), brain heart infusion medium powder (DIFCO Lab., Detroit, MI). *Vibrio anguillarum* (National Research Institute of Aquaculture, Mie, Japan), a hybridoma cloning factor, ORIGEN (Igen Inc., Rockville, MD), polystyrene microtiter plates used for enzyme-linked immunosorbent assay (ELISA) (Nunc Inc., Roskilde, Denmark), goat anti-mouse IgG and anti-mouse IgM antibodies conjugated with peroxidase (Cappel, West Chester, PA). an ELISA color reagent kit for peroxidase reaction (Type-T (Sumitomo Bakelite Co., Ltd., Tokyo, Japan), and a mouse monoclonal antibody isotyping kit and ECL-Western blotting detection system (Amersham Japan, Tokyo, Japan).

*V. anguillarum* cells cultured in brain heart infusion medium containing 2% NaCl were washed with 2% NaCl and inactivated by immersion in 0.5% formaldehyde. The inactivated cells were lyophilized and suspended in phosphate buffered saline (PBS: 10 mM Na+-phosphate, pH 7.4 containing 0.15 M NaCl) to 1 mg/ml. This cell suspension was used as the antigen solution. Female BALB/c mice (8 weeks old) were immunized by intraperitoneal injection with 0.2 ml of the antigen solution. Two booster injections were given in the same way at 2-week intervals. The second booster injection was given 3 days before cell fusion. The fusion of spleen cells and mouse myeloma cells (SP2/O-Ag14) by use of polyethylene glycol-1500 and hybridoma selection culture using a medium containing hypoxanthine, aminopterine, and thymidine were done as described previously,4) except that Dulbecco's modified Eagle's medium was used as a base medium instead of RPMI-1640 medium. Hybridoma cultures producing antibodies that bound to *V. anguillarum* were selected by ELISA. Hybridoma cells from cultures positive for the antibody production were cloned by the limiting dilution method as described previously,5) except that a hybridoma cloning factor, ORIGEN, was added to the culture to 10% instead of mouse thymocytes used as feeder cells. ELISA was done at room temperature as follows. To each well of microtiter plates with 96 wells was added 0.05 ml of 0.005% polysine solution. The plates were incubated for 1 h and wells were washed with water. The antigen cell suspension (0.1 mg/ml, 0.05 ml) was put into each well, and the plates were incubated for 15 min and centrifuged at 200 x g for 10 min. After removing the supernatant, 0.05% glutaraldehyde (0.05 ml) was added to each well, and the plates were incubated for 3 min and washed with PBS. To each well 0.1 ml glycine (0.1 ml) was added, and the plates were incubated for 1 h and washed with water. Test sample (54 μl) was mixed with 0.5% Tween-20 (6 μl) and added to each well and the plates were incubated for 2 h. After the wells were washed with PBS containing 0.05% Tween-20, 0.05 ml of goat anti-mouse IgG antibody conjugated with peroxidase (1 μg/ml in PBS containing 0.05% Tween-20) was added to each well and the plates were incubated for 1 h. After the wells were washed with the PBS containing 0.05% Tween-20, 0.05 ml of the peroxidase reaction mixture was added to each well. After incubation for 30 min, the peroxidase reaction was stopped by addition of the stopper solution (0.05 ml) of the kit reagents, and absorbance of each well was measured at 450 nm.

The ascitic fluid of BALB/c mice containing the monoclonal antibody to *V. anguillarum* was obtained as described previously.6) The monoclonal antibody was partially purified from the ascitic fluid by ammonium sulfate fractionation; the antibody fraction was precipitated by the addition of 0.9 volume of a saturated ammonium sulfate solution and by subsequent centrifugation. The precipitate was dissolved in and dialyzed against PBS. The obtained antibody sample had a purity of about 80% on SDS-PAGE.

SDS-PAGE was done by the method of Laemmli.7) Western blotting was done essentially by the method of Burnet.8) In brief,
the proteins separated in the SDS-PAGE gel were transferred electrophoretically to nitrocellulose paper. The paper was immersed in a blocking solution containing milk proteins (Block Ace) for 2h. Then the paper was dipped into PBS containing 0.05% Tween-20 and a monoclonal antibody sample (10 µg/ml) and incubated for 2h at room temperature. The paper was rinsed in PBS containing 0.05% Tween-20 and immersed in the same PBS-Tween-20 solution containing the goat anti-mouse IgM antibody conjugated with peroxidase (2 µg/ml) for 1h at room temperature. After the paper had been rinsed by the procedure described above, the antigen on the paper was detected by a chemiluminescence reaction with an ECL-Western blotting detection system by the manufacturer's protocol.

One hybridoma clone, named C5, which produced a monoclonal antibody that recognized the *V. anguillarum* cells in ELISA was established. The isotyping assay showed that the class of this immunoglobulin was IgM and its light chain was κ. To confirm the reactivity of the monoclonal antibody with *V. anguillarum*, we did two kinds of experiments; a pre-adsorption ELISA, in which the inhibitory effect of incubation with the antigen cells on the activity of the monoclonal antibody sample in ELISA was tested, and a cell-immunoprecipitation assay, in which the accelerating effect of the antibody sample on the precipitation of the suspended antigen cells was tested by measuring a decrease in the turbidity of the cell suspension. Figure 1 shows that *V. anguillarum* cells lowered the activity in ELISA by adsorbing the C5 monoclonal antibody. A negative control sample, *Enterococcus seriolicida* cells, did not affect the activity of C5 monoclonal antibody in the pre-adsorption ELISA. In the cell-immunoprecipitation assay, the addition of C5 monoclonal antibody to the *V. anguillarum* cell suspension immediately increased the turbidity for the first 10min and then accelerated the precipitation of the suspended cells in a time-dependent manner (Fig. 2). This indicated that the *V. anguillarum* cells were aggregated through the antibody-mediated cross-linking. Negative controls such as PBS and mouse monoclonal antibody (IgM/κ) to bovine casein did not cause the cell aggregation. These findings supported the specific reactivity of C5 monoclonal antibody with *V. anguillarum* cells.

To examine the antigenic substance probably protruding from the cell surface, *V. anguillarum* cells were treated with several detergents (SDS, octyl glucoside, sodium deoxycholate, and Triton X-100) and the solubilized extracts were analyzed by SDS–PAGE and Western blotting analysis (Fig. 3). In the SDS–PAGE, all the extracts obtained with the detergents showed one broad band with a molecular mass of more than 200 kDa together with many other bands of low molecular mass (Fig. 3A). The band of more than 200 kDa was reactive with C5 monoclonal antibody in the Western blotting analysis (Fig. 3B). This reactive band was not detected in the extract prepared with Tris-buffered saline alone.

We succeeded in establishing one hybridoma clone (C5) which produces the monoclonal antibody to *V. anguillarum*, a pathogen.

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**Fig. 1.** Pre-adsorption ELISA Showing Reactivity of C5 Monoclonal Antibody with *V. anguillarum*.

Pre-adsorption ELISA was done in the same way as the standard ELISA described in the text, except that the sample antibody solutions were prepared by incubating (25°C, 1h) a constant amount of C5 monoclonal antibody (1.3 µg/ml) together with different concentrations (from 0.5 µg/ml to 0.5 mg/ml) of cells in PBS (●). *V. anguillarum* or (●) *E. seriolicida*. Cells were removed by centrifugation. The abscissa indicates the logarithmic values of the dilution of the incubated cells (log dilution=0 means the cell concentration of 0.5 mg/ml). The relative absorbance at 450 nm was based on the absorbance value (0.560) obtained for the antibody sample incubated in the absence of the cells.

**Fig. 2.** Immunoprecipitation of *V. anguillarum* Cells.

*V. anguillarum* cells cultured in brain heart infusion medium were washed with and suspended in PBS. The cell suspension (1.5 ml, absorbance at 660 nm was 1.2) was mixed with an equal volume of (●) C5 monoclonal antibody (1 mg/ml in PBS), (○) mouse monoclonal antibody (IgM/κ) to bovine casein (1 mg/ml in PBS), or (▲) PBS in a cuvette (10 x 10 x 40 mm) of spectrophotometer and allowed to stand at 25°C. Absorbance at 660 nm of each mixture was measured at indicated times.

**Fig. 3.** SDS–PAGE and Western Blotting Analysis Patterns of Detergent Extracts of *V. anguillarum* Cells.

*V. anguillarum* cells washed with 10 mM Tris-HCl, pH 7.5 containing 0.15 M NaCl were mixed with five volumes of detergent solution (1.2% in the same buffer) and incubated at room temperature for 1h with continuous gentle mixing. The incubated mixture was centrifuged (7000 x g for 10 min) and the supernatant obtained was dialyzed against 10 mM Tris HCl, pH 7.5. The dialysate was used as the extract solubilized with the detergent. (A) SDS-PAGE gel stained with Coomassie brilliant blue. Lane 1, sample extracted with SDS; lane 2, octyl glucoside; lane 3, sodium deoxycholate; lane 4, Triton X-100; lane 5, 10 mM Tris–HCl, pH 7.5 containing 0.15 M NaCl. Two microliters of each extract were put in. (B) Western blotting analysis using C5 monoclonal antibody. Samples were the same as those of (A). The arrows indicate the bands reactive with C5 monoclonal antibody. Numbers on the left-hand side show the positions of molecular mass marker proteins (from top: myosin, β-galactosidase, phosphorylase-b, bovine serum albumin, and ovalbumin).
of vibriosis, an infectious disease found in various fishes. This antibody is expected to be useful for developing systems for diagnosis and prevention of vibriosis in the field of fish aquaculture. The class of C5 monoclonal antibody was IgM, although we used the anti-mouse IgG antibody for ELISA in the course of its screening. This is probably due to the cross-reactivity to the mouse IgM of the anti-mouse IgG antibody used. Our findings indicated that the antigenic substance recognized by the C5 monoclonal antibody can be solubilized from *V. anguillarum* cells with several detergents. This antigenic substance of high molecular mass may be a substance containing protein because it was stained with Coomassie brilliant blue. For the precise identification of the antigenic substance, further studies are needed. Hitherto, various monoclonal antibodies to *V. anguillarum* cells have been generated,\(^9\)–\(^{12}\) but their epitope structures were not analyzed precisely. Some of them recognize the lipopolysaccharide extract and the lipopolysaccharide O-antigens of cell wall,\(^9,\)\(^{11}\) and two ones react differently with 30 and 33 kDa proteins extracted with SDS from cells.\(^{12}\) To our knowledge, monoclonal antibodies which, like C5, recognize specifically the protein-like substance with a molecular mass of more than 200 kDa have never been reported. At present it is unclear as to whether the epitope recognized by C5 monoclonal antibody belongs to the epitopes reactive with the reported monoclonal antibodies to *V. anguillarum* cells.

**References**