Development of Murine Monoclonal Antibodies against an Enterotoxin Produced by Bacillus cereus

Kunihiro SHINAGAWA, Ryoji YOKOI, Naonori MATSUSAKA, and Shunji SUGII

Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, Ueda, Morioka, Iwate 020 and Department of Serology and Immunology, School of Medical Technology, Kitasato University, Kitasato, Sagamihara, Kanagawa 228, Japan

(Received 14 November 1990/Accepted 23 January 1991)

ABSTRACT. Three murine monoclonal antibodies (MAbs) were prepared against an enterotoxin (ET) produced by Bacillus cereus. Although these MAbs were found to react with the ET, their specificities appeared to be different in competitive binding assays. One of the MAbs (D-8), which was highly reactive with the ET, will be useful in developing immunological methods to detect crude ET and to isolate the ET in high yield.—KEY WORDS: Bacillus cereus, enterotoxin, monoclonal antibody.


Diarrheal-type food poisoning outbreak caused by Bacillus cereus is known to attributable to a diarrheagenic enterotoxin produced by B. cereus [6, 8, 10, 14]. Although attempts have been made to isolate and/or purify the enterotoxin (ET) [4, 13, 16, 18], pure ET has not been obtained [11]. Thus, immunological methods have not yet been developed to detect ET produced by B. cereus although biological assays have been commonly used to determine the enterotoxic activity [6, 8, 13, 14]. However, we have recently succeeded in isolating ET in high purity from culture filtrates [11]. We have prepared rabbit anti-ET serum to the purified ET [11]. Monoclonal antibodies (MAbs) have been prepared against other bacterial toxins and used to develop immunological methods for their detection [1, 2, 9, 14]. Thus, the present study was undertaken to prepare murine MAbs against an ET produced by B. cereus and to characterize their specificities.

MATERIALS AND METHODS

Purification of ET: For production of ET, B. cereus strain FM-1 was cultured for 5–6 hr at 32°C in brain heart infusion broth containing 1% glucose as reported previously [11]. The ET in culture supernatant was isolated by the following methods such as ammonium sulfate precipitation at 70% saturation, ion-exchange chromatography on DE-32 cellulose at pH 8.6, gel filtration on Sephadex G-100 at pH 8.6, and gel filtration on Sephadex G-75 superfine at pH 8.6 as described previously [11].

Immunization and cell hybridization: Male BALB/c mice (6 weeks old) were immunized as follows: (1) an intraperitoneal injection of 20 μg of purified ET with Freund’s incomplete adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.) was given on day one; (2) two weeks later a second intraperitoneal injection of 20 μg of purified ET with adjuvant was given; (3) three weeks later 50 μg of purified ET with adjuvant injected intraperitoneally. Spleen cells were removed 3 days after the final injection and fused with myeloma cells SP2/0 Ag14 maintained in Dulbecco’s modified Eagle’s medium (Gibco Laboratories, New York, U.S.A.) supplemented with 10% fetal calf serum (Flow Laboratories Inc., U.S.A.) by use of 50% polyethylene-glycol 4,000 (Wako Pure Chem. Indst. Ltd., Osaka, Japan) according to the methods described previously [3, 5, 7]. Hybrid cells were selected in HAT medium and hybridomas producing antibodies were cloned by the limiting dilution methods.

Isolation of MAbs: Hybridomas producing anti-ET were intraperitoneally injected into BALB/c mice to obtain ascitic fluids by the methods described previously [3, 5, 7]. MAbs in the ascitic fluids were purified with a Affi-Gel Protein A column (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The subclasses of the purified MAbs were determined by use of the Mouse-Typer™Sub-Isotyping kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Enzyme-linked immunosorbent assay (ELISA): The binding assays were carried out in triplicate by ELISA. Each well of polystyrene microtiter plate (Dynatech Laboratories Inc., VA, U.S.A.) was coated with 100 μl of purified ET (10 μg/ml) in 0.1 M...
carbonate buffer, pH 9.5) and the microtiter plate was incubated at 37°C for 3 hr. After coating, the wells were blocked with 1% bovine serum albumin in 0.02 M phosphate buffered saline (PBS, pH 7.2) at 4°C overnight. After washing the plate 5 times with PBS containing 0.05% Tween 20, 100 μl of purified MAb were added to each well and incubated at 37°C for 2 hr. After washing, 100 μl of horseradish peroxidase-coupled goat anti-mouse IgG (diluted to 1:1,000) were added to each well and incubated at 37°C for 1 hr. After washing, 200 μl of the substrate solution containing 0.02% H₂O₂ and 3 mg/ml o-phenylenediamine (Nakarai Chemical Ltd., Kyoto, Japan) were added to each well; the plate was held in the dark at room temperature for 30 min for color development. Two hundred microliters of the reaction mixture from each well was transferred to test tubes, to which 3 ml of 2 N H₂SO₄ was added to stop the reaction. The enzyme-substrate reaction was determined spectrophotometrically with a Shimadzu double-beam spectrophotometer UV-150 (Shimadzu Co. Ltd., Kyoto, Japan).

For determination of reactivities of MAbs to ET, 100 μl of the purified MAbs at 100 ng/ml were incubated at 37°C for 60 min with 100 μl of ET at 0 to 1 μg/ml. After incubation, 100 μl of the mixture were added to each well coated with 100 μl of ET at 10 μg/ml to determine the remaining antibody activity in the mixture.

**Immunoblotting:** The specificity of each purified MAb was tested by Western blotting [17]. Purified ET was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose paper (Toyo Roshi Co. Ltd., Tokyo, Japan). The nitrocellulose paper was treated with a purified MAb followed by treatment with horseradish peroxidase-conjugated goat anti-mouse IgG (diluted: 1:1,000). After washing the nitrocellulose paper 5 times, the ET was detected by use of 4-chloronaphthol (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

**RESULTS AND DISCUSSION**

Fusion of mouse spleen cells from mice immunized with ET by Western blotting (Fig. 1). The antibody titer of D-8 in culture supernatant fluids was found to be slightly higher than those of B-10 and H-1 in ELISA (Fig. 2). One to ten ng of purified MAbs D-8, B-10, and H-1 were reactive with the ET in ELISA (Fig. 3).

To study reactivities of the MAbs obtained, 10 ng of purified MAb was treated with different amounts (0–100 ng) of purified ET for 1 hr at 37°C. After incubation, any remaining antibody activity was determined by ELISA. With 50 ng of purified ET, no antibody activity was detectable by ELISA. However, slight differences in the remaining antibody activities was observed with 5 ng of purified ET (Fig. 4), indicating differences in the specificities of the MAbs. Although the characterization of the three MAbs has been completed as yet, the MAbs should be useful in the development of immunological methods similar to those developed for other bacterial toxins [1, 2, 9, 15]. Of particular impor-
Fig. 2. Titration curves of MAbs in culture supernatant reactive with enterotoxin at 10 μg/ml. 
D-8 (●●●), B-10 (○○○), H-1 (▲▲▲).

Fig. 3. Titration curves of purified MAbs reactive with enterotoxin at 10 μg/ml. 
D-8 (●●●), B-10 (○○○), H-1 (▲▲▲).

Fig. 4. Reactivities of purified MAbs after incubation with different amounts of B. cereus enterotoxin. 
D-8 (●●●), B-10 (○○○), H-1 (▲▲▲).

Importance will be their use in the development of an ELISA method to detect ET in incriminated foods and clinical specimens obtained from outbreaks of B. cereus food poisoning. Because ET produced by B. cereus has been reported to be labile to low pH, proteolytic enzymes, and long storage at 4°C, the purification of ET should be completed in a relatively short time. It should be possible to purify the ET in one step utilizing the MAbs in affinity chromatography as has been done for the staphylococcal enterotoxin(s) [12]. High yields of the purified enterotoxins were obtained relatively easily [12].

ACKNOWLEDGEMENTS. The authors would like to express a gratitude to Dr. M. S. Bergdoll at University of Wisconsin for his valuable advice and comments in preparing the manuscript. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

3. de St. Groth, S. F. and Scheidegger, D. 1980. Production of