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Production of a Monoclonal Antibody against the Sea Nettle Venom Mouse Lethal Factor[1] (41182)

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Abstract. A monoclonal antibody to the sea nettle, *Chrysaora quinquecirrha*, venom mouse lethal factor was prepared using the crude venom as the antigen. Cloned hybridomas were selected by an enzyme-linked immunosorbent assay (ELISA) using partially purified lethal factor as the antigen. Ascites fluid from the cloned hybridoma-bearing mouse had an ELISA titer of 12.800 and the capability to neutralize the lethal activity (2 LD_{50}) of the intravenous injection of crude venom into mice after overnight incubation.

The sea nettle, *Chrysaora quinquecirrha*, is a venomous pelagic coelenterate found in large numbers in the Chesapeake Bay. The cardiotoxic, dermonecrotic, musculotoxic, and neurotoxic properties of sea nettle venom have been reviewed (1). The agent responsible for the immediate mouse lethal action of the venom has not been purified, but it appears to be independent of the numerous deleterious enzymes present in the venom (2). The mouse lethal factor exerts its pharmacological action by altering the transport of calcium across the cardiac conduction system (3). The purification of this lethal factor has been hampered by the lack of a simple and sensitive assay. Animal lethality has been the standard test used to analyze column fractions, but the thermolabile nature of the lethal factor, coupled with the large volume of eluate required to identify and confirm activity, frustrated the total purification of the lethal factor (1). Recently an enzyme-linked immunosorbent assay (ELISA) has been developed by this laboratory which is capable of detecting anti-sea-nettle venom antibodies by using either crude or partially purified venom as antigen (4).

The monoclonal antibody technique developed by Kohler and Milstein (5, 6) has become an important biological research tool. By using suitable screening methods, it is now possible to isolate rare hybridomas producing antibody of desired specificity. The individual hybridoma cell can be cloned and large amounts of antibody to a single antigenic determinant can be produced.

In this paper we describe the details of isolation of hybridomas that secreted specific antibody to the mouse lethal factor of sea nettle venom and discuss the practical importance of this antibody as a diagnostic, as well as preparative tool.

Materials and Methods. The materials used in these investigations were as follows: All inorganic and organic chemicals were reagent grade quality or better. All tissue culture media were obtained from M. A. Bioproducts (Walkersville, Md.). Freund's complete and incomplete adjuvants were products of Grand Island Biological Company (Grand Island, N.Y.). Male Balb/C and ICR mice were purchased from Flow Laboratories, Inc. (McLean, Va.). SP-Sephadex C-50 was obtained from Pharmacia Fine Chemicals (Piscataway, N.J.). Control ascites fluid (BRL Lot No. 0111) was obtained from Bethesda Research Laboratories, Inc. (Rockville, Md.). Pristane (2,6,10,14-tetramethylpentadecane) was a product of Aldrich Chemical Co. (Milwaukee, Wisc.).

Preparation of the venom. Sea nettle nematocyst suspensions (NS) were obtained by homogenization and centrifuga-

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tion of mixed fishing and mesenteric tentacles (7). Active venom (SU) was prepared by sonic treatment and ultracentrifugation of the NS preparations as previously described (8). SU preparations were also obtained in the same manner from fishing and mesenteric tentacles which were manually separated prior to preparation of the NS. All NS and SU preparations were stored at −60° until use.

Column chromatography. Two-milliliter columns of SP-Sephadex C-50 were used as previously described (2) for partial purification of lethal factor from SU preparations of fishing and mesenteric (or mixed) tentacles. After equilibration of the columns with 0.001 M phosphate buffer, pH 7.0, 0.2 ml of SU was layered onto the gels. The columns were eluted stepwise with 4 ml each of 0.001, 0.01, 0.03, 0.05, 0.075, and 0.10 M phosphate buffer, pH 7.0, and fractions were collected. Comparisons were made between color production by ELISA and mouse lethality for each fraction.

ELISA. An ELISA method, recently described by this laboratory for detecting antibodies against sea nettle venom (4), was used to monitor antibody production by the hybridomas and for titration of monoclonal antibodies purified from the ascites fluid. In brief, 25 μg of SU was air dried onto wells of polystyrene microtiter plates which had been treated with bovine serum albumin and glutaraldehyde. The plates were washed and 50-μl aliquots of the medium from the hybridomas were added to each well. After 2 hr incubation at 37°, the wells were washed and 50 μl of alkaline phosphatase-labeled anti-mouse immunoglobulin (7S) was added to each well. After an additional 1 hr incubation at 37°, the plates were washed and reacted for 15 min with 100 μl of *p*-nitrophenyl phosphate substrate solution. The reaction was terminated by the addition of 25 μl of 3 M sodium hydroxide solution and absorbance at 407 nm was determined in a Titertek Multiskan microplate reader (Flow Laboratories, Inc.).

Immunization of mice. Balb/C mice, weighing approximately 10 g, were primed by footpad injection of 0.4 mg of crude venom (SU) that had been emulsified in Freund's complete adjuvant (1:1). Booster injections were given, intraperitoneally, with 0.4 mg SU in incomplete adjuvant (1:1) on Days 4, 9, and 16. The mice were bled and tested by ELISA to insure they possessed anti-lethal factor antibodies at titers of 1:500 or greater. The spleen was removed from the immunized mouse 5 days after the last injection.

Hybridoma production. Immunocytes (1×10^8) from the spleen of an immunized Balb/C mouse were fused with 1×10^7 plasmacytoma cells (P3X63Ag8) in the presence of 30% polyethylene glycol 1000. Detailed protocols of this fusion procedure and formulations of the maintenance medium (HT) and the selective medium (HAT) have been previously reported by others (9, 10).

Immediately after the fusion (Day 0), the cells were diluted in 30 ml HT medium and were distributed, in 50 μl volumes, into wells of 96-well culture dishes (Costar No. 3596). After 24 hr of incubation (Day 1) at 37° in a humidified atmosphere of 7% CO_2 in air, 50 μl of HAT medium, which selectively permits the growth of hybridomas, was added to each well (10). An additional 100 μl of HAT medium was added to all wells on Day 5. After 10 days, all wells containing hybridomas were identified microscopically. At that time 50 μl of medium was removed from each culture and assayed by ELISA, using the 0.1 M fraction of the SP-Sephadex column as antigen for the selection of antilethal factor antibody-producing hybridomas. The control antigens for this ELISA were the nonlethal fractions of SP-Sephadex eluant. ELISA-positive hybridomas were expanded initially to 60 × 15-mm dishes and subsequently to 75-cm^2 flasks.

Cells from antibody producing hybridomas were cloned in medium comprised of 0.25% agarose in HT medium. Feeder cell layers were not used. After 10 days, macroscopic clones were isolated and seeded into 96-well plates. The clones were expanded subsequently to the 75-cm^2 flasks.

To produce large quantities of monoclonal antibodies, Balb/C mice were primed with Pristane (11) and were injected intraperitoneally with 2×10^6 cells of clone.

After 2 weeks, the tumors became visible and the ascites fluid was aspirated. After centrifugation at 1000 rpm for 10 min, the cell pellet which contained hybrid cells was resuspended in HT medium and 2 × 10^6 cells were injected into new Pristane-primed Balb/C mice. The monoclonal antibodies were precipitated from the ascites fluid supernatant with 50% ammonium sulphate, pH 7.2, and dialyzed extensively with 0.1 M phosphate-buffered saline pH 7.2.

Neutralization test. The ability of the ascites fluid recovered from monoclonal hybridoma tumor-bearing Balb/C mice to counteract the lethal activity of sea nettle venom was tested after overnight incubation of 30 (2 LD_{50}) or 120 μl (8 LD_{50}) with 50 μl of ascites fluid at 4°. In the control experiments, 50 μl of saline or control ascites fluid (BRL Lot No. 0111) were incubated with similar volumes of the venom. The final volume of the two reaction mixtures was adjusted to 0.5 ml with normal saline before being injected, intravenously, into ICR mice. Each mixture was tested in triplicate. The mice were observed daily up to 5 days after injection.

Results. The fusion of spleen cells from immunized Balb/C mice with plasmacytoma cells (P3X63Ag8) led to the formation of 110 (16%) hybridomas. Of these hybridomas, only 4 secreted antibodies (IgG) that could be bound specifically to partially purified sea nettle mouse lethal factor. A cloned hybrid C6D3/c, isolated from hybrid C6D3 by agar plate technique, was used in the monoclonal antibody experiments.

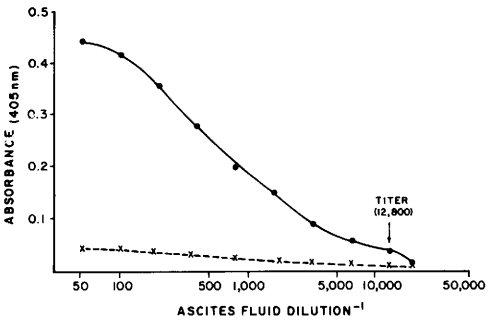

FIG. 1. ELISA titration of anti-sea-nettle venom antibody. Titration was performed as described under Materials and Methods. Symbols are defined as follows: (●) Ascites fluid removed from the C6D3/c hybridoma-bearing Balb/C mouse, (X) control ascites fluid (BRL Lot No. 0111).

The cloned hybrid C6D3/c was successfully transplanted into the peritoneum of Pristane-primed Balb/c mice. A comparison of the colorimetric results of an ELISA titration of ascites fluid taken from a mouse carrying clone C6D3/c with control ascites fluid (BRL Lot No. 0111) is shown in Fig. 1. The monoclonal antibody was detected in ascites fluid of tumor-bearing mice at dilutions of 12,800.

There was a direct correlation between the potency of mouse lethality and the amount of substrate hydrolyzed by ELISA in the supernatant fractions of both fishing and mesenteric tentacles (Table 1). Most of

TABLE I. MOUSE LETHALITY AND ELISA[a] OF FISHING AND MESENTERIC TENTACLE VENOM FRACTIONS BEFORE AND AFTER SP-SEPHADEX CHROMATOGRAPHY

Column fractions (M)	Fishing tentacles		Mesenteric tentacles	
	Mouse lethality (LD_{50}/ml)	ELISA (OD, 405 nm)	Mouse lethality (LD_{50}/ml)	ELISA (OD, 405 nm)
Precolumn	400	0.521	66	0.820
0.001	14	0.055	ND[b]	0.002
0.01	ND[b]	0.005	ND	0.016
0.03	ND	0.021	ND	0.151
0.05	ND	0.019	20	0.660
0.075	120	0.136	10	0.646
0.1	132	0.329	10	0.528

[a] 50 μl of each fraction was air dried on ELISA plates and tested, as described under Materials and Methods, against 50 μl of monoclonal hybridoma (C6D3/c) supernatant.

[b] Activity not detected within the limits of the assay.

the lethal and ELISA-positive activity of fishing tentacle supernatant (FSU) was recovered in the 0.1 M phosphate buffer wash of the SP-Sephadex column, whereas, the correspondingly active fractions from mesenteric tentacle supernatant (MSU) were the 0.05–0.1 M eluates (Table I).

Ascites fluid from a Balb/C mouse previously inoculated with clone C6D3/c contained 5 mg/ml immunoglobulins (as determined by Lowry's assay after 50% ammonium sulfate precipitation), an ELISA titer of 1:12,800 and an ability to completely neutralize an intravenous challenge of 2 LD_{50} in three of three mice. Immunoglobulins purified by ammonium sulfate precipitation of the ascites fluid neutralized an intravenous challenge of 2 LD_{50} in two of three additional mice. This ascites fluid did not protect normal mice against a challenge with 8 LD_{50} of crude venom. Similar volumes of saline or control ascites fluid (BRL Lot No. 0111) had no ELISA nor mouse protecting activities.

Discussion. The experiments in this paper describe the production of a monoclonal antibody active against sea nettle mouse lethal factor. The complex mixture of physiologically active materials in crude venom should complicate the isolation of hybridomas for a single specific antigenic compound. The disruption of proteinaceous materials by the numerous degradative enzymes present in the venom should further lower the expected yield of hybridomas producing antibodies to the lethal factor. However, the relatively high yield of ELISA-detectable hybridomas active against this lethal factor (3.6%) seen in our experiments indicated that the lethal factor is highly antigenic.

Sea nettle mouse lethal factor antibody is detectable by an ELISA, thereby obviating the need for a mouse assay system. The ELISA titer of the mouse ascites fluid of 1:12,800 correlates well with its lethal antibody titer (1:2) measured in mouse neutralization tests since ELISA is capable of detecting antibody in the picogram range (4), whereas, the amount of venom necessary for killing an adult mouse is in the low milligram range (1). With the establishment of a standard curve for competitive binding ELISA (4), it is possible to quantitate the amount of lethal factor presented in the venom.

Further experiments using this antibody as a preparative tool in the immunochromatographic purification of sea nettle mouse lethal factor are now possible and currently being undertaken in this laboratory.

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