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Preparation and Identification of Monoclonal Antibody against Abrin-a

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ABSTRACT: BALB/c mice were immunized four times with formalin-prepared abrin-a. Using the polyethylene glycol method, immunized splenocytes were isolated and fused with SP2/0 cells. An indirect ELISA was established and used to detect positive clones secreting monoclonal antibodies (mAbs) against abrin-a. After analysis, three hybridoma clones secreting IgG-subtype mAbs were obtained. The antibodies were purified from the hybridoma growth medium using protein A or G affinity chromatography. Western blot analysis was used to analyze the antigenic epitopes on abrin-a recognized by the mAbs. The mAbs were specific for abrin-a, with no detectable cross-reactivity with several homologous toxins and associated agglutinins. Sandwich ELISA was then developed using these mAbs, which had a detection limit for abrin-a of 7.8 ng/mL.

KEYWORDS: abrin-a, mAb, ELISA, Western blot

INTRODUCTION

Abrin is a natural toxic protein isolated from the seeds of *Abrus precatorius* L. and is one of the most powerful phytotoxins ever discovered.[1] Abrin comprises four isotoxic proteins, abrin-a, -b, -c, and -d. Of these, abrin-a is the most toxic. Abrin-a comprises two polypeptide chains (A and B) linked by a single disulfide bond. The A-chain is an *N*-glycosidase, which inactivates eukaryotic ribosomes by cleaving the *N*-glycoside linkage of residue A4324 of 28S RNA, whereas the B-chain is a galactose-specific lectin, which binds to galactose-containing receptors on the cell surface.[2,3] The mechanism of action of abrin-a is identical to that of ricin; both are type II ribosome-inactivating proteins. However, abrin-a is 75 times more toxic to mice than ricin, and it has more potent antitumor activity.[4,5] A recent biological study of abrin-a and the abrin-a A chain focused mainly on their use as immunotoxins for cancer chemotherapy. Several immunotoxins have been made from abrin-a and have proved to be effective against both hematologic malignancies and solid tumors.[6] However, the extreme toxicity of abrin-a with a mouse intraperitoneal injection LD_{50} of 2.11 μg/kg,[7] coupled with its ease of production on a large scale from natural sources or modern biotechnology processes, makes it a potential weapon for use in chemical warfare or by terrorists. In both India and Sri Lanka, abrin has been used criminally to poison cattle and humans, and it is included in the Biological and Toxin Weapons Convention (BTWC) Procedural Report (2001). Therefore, it is vital that a sensitive detection method for abrin-a is developed. Detection of abrin has been reported through an aptamer-based abrin direct detection assay with a detection limit of 1 nM (64 ng/mL).[8] A Biacore-based abrin detection system using human monoclonal antibody displayed a detection limit of 35 ng/mL.[9] There was also a sandwich assay using polyclonal and monoclonal antibodies for the detection of abrin with a high detection limit from 0.5 to 10 ng/mL, but it had high cross-reactivities with abrin C and abrin agglutinin.[10] Simultaneous multiplex detection and confirmation of the proteinaceous toxins abrin, ricin, botulinum toxins, and *Staphylococcus* enterotoxins A, B, and C in food employing multiple antibodies and polystyrene microspheres displayed limits of detection in the original food samples ranging from 0.03 to 1.3 μg/mL, from 0.03 to 0.07 μg/mL, from 0.01 to 0.1 μg/mL, and from 0.01 to 0.03 μg/mL, respectively.[11] The aim of this study was to generate monoclonal antibodies (mAbs) against abrin-a and analyze them using a Mouse Monoclonal Antibody Isotyping kit, Western blotting, and other similar toxins and associated agglutinins. The generated mAbs were then incorporated into a sandwich ELISA for detecting abrin-a.

MATERIALS AND METHODS

Preparation of Immunogen. Abrin-a was purified from the seeds of *A. precatorius* L.[3,7] with a purity of ≥97% as measured by densitometric scanning of SDS-PAGE gels. The protein was treated with 1% formaldehyde in 10 mmol/L PBS (pH 8.0) for 4 days at 4 °C and dialyzed in 10 mmol/L PBS (pH 8.0) to remove the formaldehyde.[12] The prepared immunogen was stored at −20 °C until use.

Preparation of Immunized Mice. Female BALB/c mice (6−8 weeks old; $n = 3$) were immunized by hypodermic injection with 50 μg of immunogen emulsified in Freund's complete adjuvant, followed by 30 μg of immunogen in incomplete Freund's adjuvant on days 14 and 28.

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A booster injection (50 μg immunogen) was given via the tail vein on day 42.

Indirect ELISA. Indirect ELISA was used to determine the anti-abrin-a activity of the sera from the immunized mice. Abrin-a (100 μL; 100 ng/mL), diluted with 0.05 mol/L carbonate buffer (pH9.6), was added to each well of an ELISA plate (Costar, Corning Inc., Corning, NY) and incubated overnight at 4 °C. The wells were then washed three times with phosphate buffer solution containing 0.5% Tween-20 (PBS-T) and blocked with 100 μL of PBS containing 3% BSA for 1 h at 37 °C. After washing, 100 μL of diluted (from 1:1000 to 1:10^6) immunized mouse serum was added to each well and incubated for 1 h at 37 °C. The plates were washed, 100 μL of peroxidase-conjugated goat anti-mouse IgG (1:5000; Sigma, St. Louis, MO) was added to each well, and the plates were incubated for 1 h at 37 °C. After washing, the enzymatic reaction was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma) and stopped by adding 1 M H_2SO_4. The optical density was measured at 450 nm (OD_{450}) in a microtiter plate reader (ELX800, Bio-Ted Instruments Inc., Winooski, VT).[13,14]

Production of Monoclonal Antibodies. Hybridomas producing mAbs against abrin-a were generated as described by Kohler and Milstein.[15] Three days after the booster injection, the immunized mice were sacrificed. Mouse spleen cells were fused with myeloma cells (Sp2/0) at a ratio of 9:1 and hybridomas prepared using polyethylene glycol 1450 (50% w/v in 10% aqueous DMSO).[16] Hybridomas were selected for 10−14 days in RPMI-1640 medium (Gibco, Invitrogen Corp., Grand Island, NY) supplemented with 20% newborn calf serum (TBD, Tian Jing, China) and 1% HAT (Sigma). Supernatants were collected and analyzed using an indirect ELISA for abrin-a to identify positive clones as described under Indirect ELISA.

The hybridomas yielding positive results were cloned by limiting dilution in RPMI-1640 supplemented with 20% newborn calf serum and 1% HT (Sigma). Single clones were screened using abrin-a as described under Indirect ELISA. Positive clones were expanded in RPMI-1640 supplemented with 20% newborn calf serum and cryopreserved. To detect the subtype of the mAbs produced by the hybridomas, the antibodies in the hybridoma culture supernatants were analyzed using the Mouse Monoclonal Antibody Isotyping kit (ISO2-1KT, Sigma-Aldrich) in accordance with the manufacturer's protocol.

Purification and Activity of the Monoclonal Antibodies. To obtain purified mAbs, the positive clones were expanded, the hybridoma growth medium was collected, and the mAbs were salted-out in 50% ammonium sulfate.[17] After centrifugation at 10000*g* for 15 min at 4 °C, the precipitate was dissolved in loading buffer. IgG2b and IgG1 mAbs were purified by affinity chromatography using protein A and protein G (GE Healthcare), respectively, according to the manufacturer's recommendations. The concentration of the purified antibodies (mg/mL) was determined by spectrophotometry (K5500, Beijing Kaiao Technology Development Co., Ltd., China). To determine the activity of the purified mAbs, they were initially diluted to 1 mg/mL and then further diluted from 1:10^4 to 1:1.28 × 10^6 for analysis by indirect ELISA.

Western Blot Analysis. Western blotting was performed to determine which chain of abrin-a the mAbs were directed against. Briefly, abrin-a was boiled in reducing sample buffer (containing 10% 2-mercaptoethanol) and subjected to electrophoresis on 12.5% SDS-PAGE gels in SDS−Tris−glycine buffer. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) in a 10% formaldehyde ProtoBlot system. The membranes were then blocked with 3% BSA in PBS for 1 h. After washing, the membranes were incubated with the purified mAbs (10 μg/mL) at room temperature for 1 h. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma) was used to detect specific antibody binding. After washing, the membranes were developed with ready-to-use TMB blotting substrate (Sigma).[14]

mAb Reactivity. Indirect ELISA was performed to assess the reactivity of the selected mAbs. Samples (100 μL) containing 1 μg/mL of abrin-b, abrin-c, abrin-d, abrus agglutinin, ricin, or ricin agglutinin were added to the wells of ELISA plates (Costar) and incubated overnight at 4 °C. All tests were performed in duplicate. The wells were washed three times with PBS-T and blocked with 100 μL of 3% BSA for 1 h at 37 °C. After washing, 100 μL of purified mAb (1 μg/mL) was added to the wells and incubated for 1 h at 37 °C. After washing, 100 μL of peroxidase-conjugated goat anti-mouse IgG (1:5000) was added to the wells and the enzymatic reaction developed with TMB substrate and measured at 450 nm in a microtiter plate reader.

Sandwich ELISA for Abrin-a. To develop a sandwich ELISA for detecting abrin-a, mAb 4G1 was labeled with HRP as described by Wilson and Nakane.[18] The optimum dilution of HRP-labeled 4G1 mAb was then determined by indirect ELISA. Purified mAbs 3C2 and 1G5 were immobilized on Hi-bind microplates (Costar), respectively, by incubating 100 μL the antibodies (4 μg/mL) in 50 mM carbonate buffer (pH 9.6) at 4 °C overnight and used for antigen capture. The wells were then washed twice with PBS, followed by blocking with 3% BSA in PBS (pH 7.4) for 1 h at room temperature. After removal of the blocking reagent, the wells were dried and stored at 4 °C prior to use. Standard solutions of abrin-a were prepared by serially diluting abrin-a from 8000 to 0.43 ng/mL in PBS-T containing1% BSA (Figure 2); PBS-T containing 1% BSA was used as a negative control. The standard solutions were then added to the wells (100 μL/well) and incubated for 1 h at 37 °C. After washing, 100 μL of HRP-labeled 4G1 mAb (diluted 1:1000 in PBST containing 1% BSA) was added to each well and incubated for 1 h at 37 °C. After washing, the enzymatic reaction was developed with TMB substrate and stopped by adding 1 M H_2SO_4. The optical density was measured at 450 nm in a microtiter plate reader.

RESULTS AND DISCUSSION

Production of Monoclonal Antibodies. Abrin-a is the most toxic phytotoxin derived from *A. precatorius* L. Abrin-a (MW 64.3 kDa) shows good immunogenicity, but its immune dose is limited by high toxicity when used for direct immunization.[3] Thus, in the present study, the immunogen was prepared in formaldehyde, and the immunogenic response induced by high-titer antibodies in immunized BALB/c mice was examined by indirect ELISA. Sera from immunized BALB/c mice produced a positive result in the indirect ELISA test at a dilution of 1:10^5. Three days after the booster injection, splenocytes from the immunized BALB/c mice were fused with SP2/0 murine myeloma cells to generate hybridomas. After 14 days, the undiluted culture supernatants were analyzed by indirect ELISA. Nine hybridomas showing ELISA reactivity against abrin-a were subcloned. After three rounds of subcloning, seven stable hybridomas producing mAbs against abrin-a were identified. The Mouse Monoclonal Antibody Isotyping kit was then used to identify hybridomas producing IgG mAbs. These hybridoma clones were named 1G5, 3C2, and 4G1. Of these, 1G5 belonged to the IgG1 subtype and 3C2 and 4G1 to the IgG 2b subtype.

There are numerous methods for purifying mAbs. In this study, the ammonium sulfate method was chosen to isolate the mAbs from the primary cell culture fluid. Affinity chromatography was then used to yield mAbs of high purity. The resulting 1G5, 3C2, and 4G1 mAbs showed activity in indirect ELISA at dilutions of 1:2.0 × 10^4, 1:3.2 × 10^5, and 1:3.2 × 10^5, respectively. These results confirmed the successful production of three hybridoma cell lines with high reactivity against abrin-a.

Western Blotting. Abrin-a comprises two nonidentical polypeptide chains (A and B) cross-linked by a single disulfide bond.

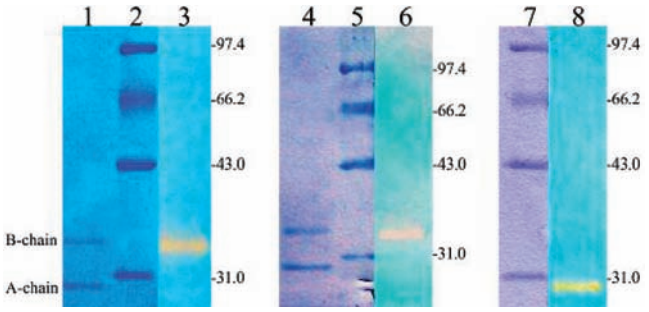

Figure 1. Reactivity of mAbs to denatured abrin-a protein determined by immunoblot analysis. Lanes: 1, 2, 4, 5, and 7, proteins transferred to PVDF membranes and stained with Coomassie brilliant blue R-250; 1 and 4, abrin-a in reducing sample buffer; 2, 5, and 7, MW markers; 3, reaction with mAb 3C2; 6, reaction with mAb 1G5; 8, reaction with mAb 4G1.

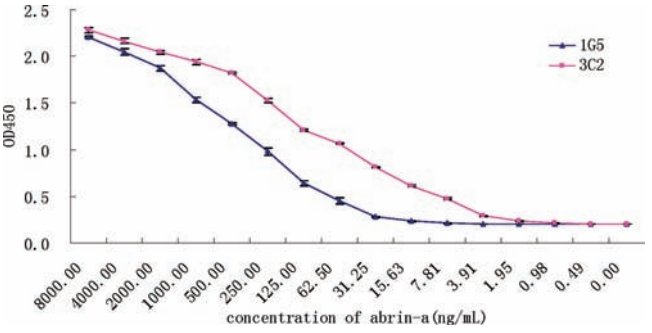

Figure 2. Sandwich ELISA for abrin-a. Results represent the average ± standard error of three replicates.

The A chain inhibits protein synthesis, and the lectin B chain binds to D-galactose moieties on the cell membrane. The A chain is transferred across the plasma membrane (via the B chain) by endocytosis. To determine the epitopes recognized by the mAbs, abrin-a was first treated with 2-mercaptoethanol to destroy the single disulfide bond. It was then subjected to electrophoresis on 12.5% SDS-PAGE gels. The proteins were transferred to a PVDF membrane and incubated with mAbs 1G5, 3C2, and 4G1. As shown in Figure 1, mAb 4G1 reacted with abrin-a A chain, and mAbs 1G5 and 3C2 reacted with abrin-a B chain. This knowledge was vital to the successful development of an abrin-a testing kit.

Determining Specific mAb Binding Activity. To examine the cross-reactivity of the mAbs, several toxins and associated agglutinins (abrin-b, abrin-c, abrin-d, abrus agglutinin, ricin, and ricin agglutinin) were used. Indirect ELISA tests showed no evidence of cross-reactivity between the mAbs and any of the test proteins. This suggests that the three hybridomas produce IgG mAbs highly specific for abrin-a. Thus, the three hybridomas were suitable for developing an ELISA for abrin-a.

Sandwich ELISA for Abrin-a. mAb 4G1 was the only antibody specific for the abrin-a A chain. Therefore, it was conjugated to HRP and tested for immunoreactivity in an indirect ELISA (data no shown). mAbs 1G5 and 3C2 (which recognize the abrin-a B chain) were coated onto ELISA plates respectively, and then serial dilutions of abrin-a were added to the wells. After the unbound reagent had been washed away, HRP-labeled mAb 4G1 was added (which recognizes the abrin-a A chain). After washing, the plates were developed with TMB substrate and the absorbance was measured at 450 nm. The detection limits of the sandwich ELISA were determined as the ratio of OD_{450} values of samples and negative control to 2.1 (Figure2). The results showed that 3C2 was more sensitive than 1G5 with a lower detection limit of 7.8 ng/mL. This may be due to steric hindrance; that is, mAb 1G5 may partially block mAb 4G1 from accessing its epitope on the abrin-a A chain.

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Author Contributions

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