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Development of a novel antibody probe useful for domoic acid detection

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ABSTRACT

The generation of monoclonal antibody (mAb) against marine toxins can serve as a valuable probe to detect this kind of compounds by immunological methods. However, traditional approaches to mAb generation usually need a comparative large quantity of standard substance (more than 400 μg mouse^{-1}), and a comparative long immunization period (more than 6 weeks). Here we report a simple, inexpensive and fast protocol for the generation of monoclonal antibody probe specific for domoic acid (DA). In the method, lymph node cells were harvested from the Balb/C mice of hind footpad injection and fused with murine myeloma cells SP2/0 for hybridoma generation. This method for the preparation of mAb for DA has two main advantages: (a) there is no need for large-scale expensive antigen (only 70 μg antigen for one mouse); (b) immunization protocol can be accomplished within 16 days.

Some characteristics of the mAb were studied and a direct competitive ELISA for the detection of DA using the mAb as a probe was developed. The detection limit was 0.41 ng well^{-1} in phosphate buffered saline (PBS) and 0.53 ng well^{-1} in blue mussel *Mytilus edulis*. The recoveries of DA from mussel and PBS buffer were from 94.8% to 105.1% and from 96.2% to 103.7%, respectively. Thus, the newly developed direct competitive ELISA using the mAb appears to be a reliable and useful method for monitoring of DA in shellfish (228 words).

1. Introduction

Monoclonal antibodies (mAbs) have proven to be an indispensable probe in the study of clinical chemistry, food analysis, and environmental monitoring, and successful therapeutic applications to cancer and inflammatory disease (Adams and Weiner, 2005). Immunization of animals, mainly mice, in combination with hybridoma technology is still the most common method for the generation of mAbs (Cervino et al., 2008). The efficient immune procedure are crucial steps that usually needs a considerable amount of antigen (200–475 μg mouse^{-1}) (Glass et al., 2004; Li et al., 2007; Jua et al., 2008; Chen et al., 2008; Ren et al., 2008; Hirasawa et al., 2008; You et al., 2008) and have to be accomplished within about 2 months (Kawatsua et al., 1999; Naar et al., 2001; Miyano et al., 2006; Sheng et al., 2007; Cheong et al., 2007; Mesci and Carlyle, 2007; Xu et al., 2008). However, for most of marine toxins, it is very expensive to get enough amount of standard substance to generate mAb. Thus, the ideal immunological method should be fast, reliable, and easy to accomplish, especially if the hapten such as saxitoxin (STX), brevetoxin (BTX), okadaic acid (OA), microcystin (MC), domoic acid (DA) and ciguratoxin (CTX), etc., are expensive.

DA, which is produced by algae *Pseudo-nitzschia* and ingested by phytoplankton shellfish is the principal neurotoxin responsible for amnesic shellfish poisoning (ASP) in humans (Marios et al., 2008). ASP was first recorded in Canada in 1987, when over one hundred people became ill and three people died after consuming mussels *Mytilus edulis* contaminated with DA (Bogan et al., 2007). Since this incident, global awareness of DA has been raised, and contaminated shellfish or seaweed have been found in marine environments in various regions of the world (Sierra Beltran et al., 1997; Campbell et al., 2001; Wekell et al., 2002; Costa et al., 2005; Wang et al., 2007). In this report, we describe a simple, inexpensive, and fast method for the generation of mAb specific for DA and its application to a direct competitive enzyme-linked immunosorbent assay (ELISA).

2. Materials and methods

2.1. Reagents

Domoic acid, agarose, complete and incomplete Freund's adjuvant (CFA, IFA), polythylene glycol-4000 (PEG), RPMI 1640, fetal bovine serum (FBS), horseradish peroxidase labeled goat anti-rabbit IgG antibody (HRP-anti-IgG), dialysis pocket, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), dimethyl sulfoxide (DMSO), dimethylformamide (DMF), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), human

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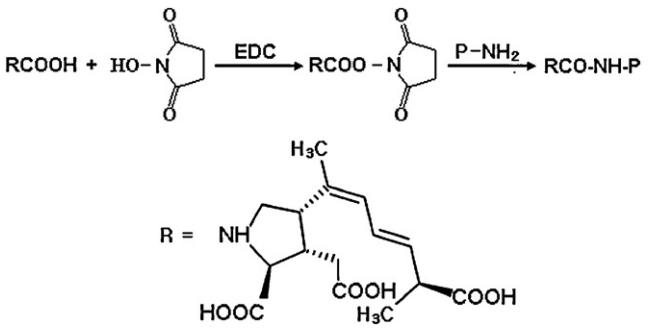

Fig. 1. Reaction scheme of synthesis for DA-protein conjugates. P-NH_2: carrier protein. 50 μL of DMSO containing 1 mg of DA, 20 μL of EDC solution and 10 μL of N-hydroxysuccinimide solution were added. After stirring for 1.5 h at room temperature, 0.25 mL of protein (BSA or HGG) solution was added to the mixture, respectively. Details are described in the text.

gamma globulin (HGG), HT and HAT were purchased from Sigma (Changchun, China). All other reagents were of analytical grade. Myeloma Cells SP2/0 were conserved by our laboratory. The ELISA was carried out in 96-well polystyrene microtiter plates (Stripwell plate 2592, Costar, Changchun, China). Well absorbencies were read with a MK3 microplate reader (Thermo, Shanghai, China).

2.2. Buffers and solutions

The buffers used regularly were coating buffer, 50 mmol L^{-1} carbonate buffer (pH 9.5); phosphate buffered saline (PBS), 10 mmol L^{-1} sodium phosphate buffer (pH 7.4) containing 140 mmol L^{-1} NaCl; dilution buffer, PBS containing 0.1% (w/v) gelatin; washing buffer (PBST), 10 mmol L^{-1} sodium phosphate buffer (pH 7.4) containing 140 mmol L^{-1} NaCl and 0.05% (v/v) Tween 20; and TMB solution, 70 μL of 0.65% H_2O_2, 250 μL of 10 mg mL^{-1} 3,3′5,5′-tetramethylbenzidine (TMB) in dimethylsulfoxide (DMSO) per 25 mL of phosphate citrate buffer, pH 5.4.

2.3. Preparation and analysis of DA-protein conjugates

DA-BSA (plate coating antigen) and DA-HGG (immunogen) were prepared using a modified carbodiimide method (Fig. 1) (Kawatsua et al., 1999). Briefly, 50 μL of DMSO containing 1 mg of DA, 20 μL of EDC solution (25 mg mL^{-1} in DMSO) and 10 μL of N-hydroxysuccinimide solution (30 mg mL^{-1} in DMSO) were added. After stirring for 1.5 h at room temperature, 0.25 mL of protein (BSA or HGG) solution (20 mg mL^{-1} in 0.085 M borate buffer containing 0.06 M NaCl, pH 8.2) was added to the mixture, respectively. After incubation for 1.5 h at room temperature, the conjugation was dialyzed against 800 mL of 0.01 M PBS (pH 7.4) at 4 °C for 72 h with 4 changes of PBS to remove residual free DA. The DA-protein conjugates were then stored at −20 °C until used.

The conjugates were analyzed by nondenaturing agarose gel electrophoresis using a modification of the methods of Kamps et al. (1993). Briefly, TAE buffer (pH 8.0) was employed for electrophoresis buffer and the mixture solution of 0.04% bromophenoland blue and 6.67% sucrose was used as loading buffer. Each sample (5.0 μg protein) mixed with an equal volume of loading buffer was applied to the gel and samples were separated at 120 V for 50 min. The gel was fixed with 20% trichloroacetic acid for 30 min, then stained with Coomassie blue R-250 for more than 2 h and destained with ethanol–acetic acid (250 mL 95% ethanol + 80 mL acetic acid, distilled water to 1000 mL) with several changes until clear. The pictures of the gels were taken by UVI gel auto-imaging system.

2.4. Making the emulsion

The antigen-CFA (or antigen-IFA) emulsions were prepared using a modification of the methods of Kamala (2007). Briefly, the antigen solution with the CFA (or IFA) suspension were mixed together at a ratio of 1:1 (v/v), using two glass syringes, one loaded with the adjuvant, and the other with the antigen solution in PBS, connecting them with a 3 way stopcock. The approximately 200–250 μL dead volume in the 3-way stopcock was eliminated by carefully and slowly pushing the antigen solution through the stopcock right until the other edge, at which point the syringe containing the adjuvant was attached. Care was taken to first slowly introduce the antigen solution into the adjuvant suspension drop by drop before mixing thoroughly. Bubbles were carefully eliminated from the syringe and the needle removed.

The antigen-CFA (or antigen-IFA) emulsion was tested for readiness by putting a drop of emulsion onto distilled water. When the emulsion is done, it will stay intact as a tight drop on the distilled water. If the emulsion is not done, then it will start to spread out on the distilled water with globs of oil radiating out from the center.

2.5. Immunization protocol

Male Balb/C mice, 8-week-old, and female Balb/C mice, 9–10-week-old, were obtained from Changchun Institute of Biological Products, Jilin province China. Two protocols of immunization were carried out. In protocol 1 (Fig. 2e), the male mice were immunized by subcutaneous injection in a hind footpad with 20 μL of the CFA

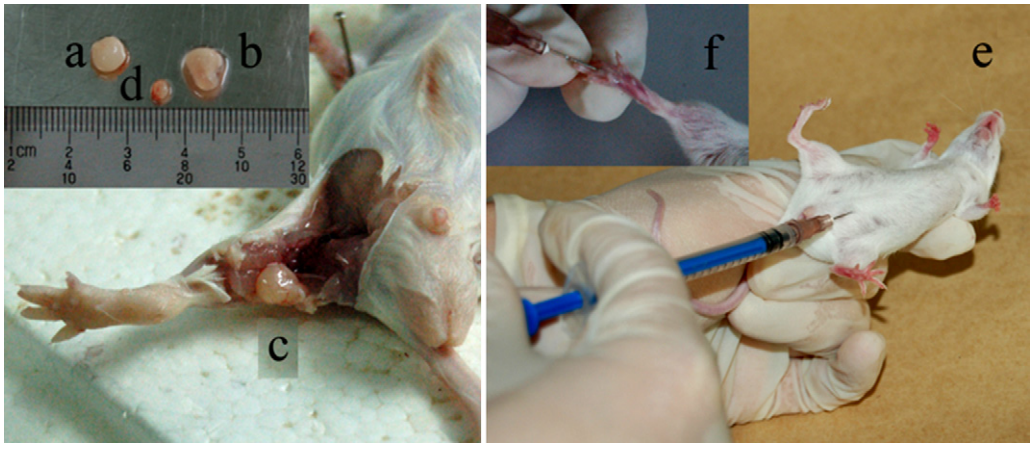

Fig. 2. Footpad immunization result. (a–c) Tumescent popliteal lymph nodes, (d) iliac muscle lymphoid node. The size of popliteal lymph nodes in normal (not immunized) mouse is very small, it is not easy to be observed by naked eyes. (e) i.p. injection immunization. (f) Footpad immunization. Protocol details are described in the text.