IN VITRO IMPROVED PRODUCTION OF MONOCLONAL ANTIBODY AGAINST DOMOIC ACID IN SUPPLEMENTED CELL CULTURE MEDIA

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ABSTRACT: *A monoclonal antibody (mAb) specific to domoic acid which is a potent source of amnesic shellfish poisoning was produced from hybridoma cell line 3G9G1. It was generated by the fusion of Sp2/0 myeloma cells with spleen cells isolated from Balb/c mice with highest-titer 2.50. The mice were immunized with domoic acid-bovine serum albumin. Hybridoma cells were grown on cell culture media supplemented with carbohydrates (CHO) respectively. Monoclonal antibody produced by hybridoma in vitro belongs to the immunoglobulin subtype IgG3 with specific antibody titer 0.65. Culture medium supplemented with maltose was proved to be the most effective for hybridoma clones and achieved best titer at 1.485 as determined by icELISA. The use of supplemented cell culture media is an effective approach for enhanced monospecific-antibody production, better hybridoma growth and viability.*

Keywords: Domoic acid, Hybridoma, Monoclonal antibody, Supplemented media, ELISA

1. INTRODUCTION

Domoic acid (DA) is a naturally occurring neuroexcitatory toxin produced primarily by a dozen species of the marine diatome *Pseudo nitzschia* [1,2,3,4]. It was identified as responsible for human poisoning in Canada in 1987 [2], and characterized by a constellation of clinical symptoms and signs, most specific feature was memory impairment, which led to the name Amnesic Shellfish Poisoning (ASP) [5,6,7,8]. Hybridoma technology was first invented for the production of monoclonal antibodies in 1975 [9,10] (Figure 1). Cell culture involves isolation of cells from (in vivo) and their growth under controlled environment (in vitro) providing culture media and nutrients [11].

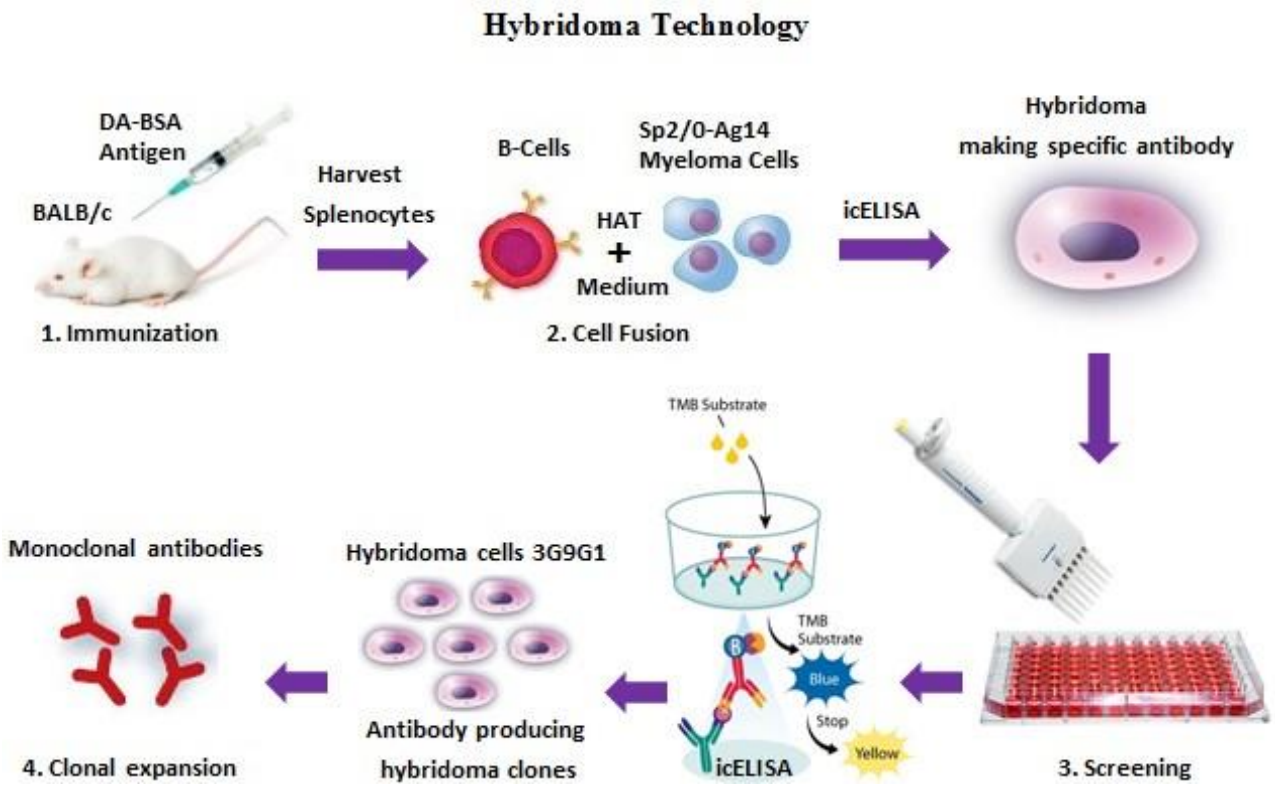

Figure 1. Technical route to hybridoma technology.

They have applications in cell differentiation, toxicology, pharmacology, cancer research and applied biological research [12]. CHO are supplied largely in the form of glucose as a carbon source for the hybridoma cells. In some cases, glucose is substituted with other sugars to improve cell growth, viability and protein production [13,14,15].

Enzyme-linked immunosorbent assay (ELISA) is a rapid, profound, and cost-effective immunoassay to detect various antigens and antibodies [16]. It was first reported in 1981 [17] and modified is 1997 [18].

The aims of this study were to prepare mAb against DA by CHO supplemented culture media, and establishment of an indirect competent enzyme-linked immunosorbent assay (icELISA) for such purposes.

2. MATERIALS AND METHODS

2.1 Ethics Statement

All animal experiments were carried out according to the rules by the Animal Welfare Committee of Fujian Agriculture and Forestry University, Fuzhou, China.

2.2 Materials

DA, bovine serum albumin (BSA), ovalbumin (OVA), Tween 20, dimethyl sulfoxide (DMSO), 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC), and N-hydroxysuccinimide (NHS), Goat anti-mouse-peroxidase conjugate, substrate solution 3, 3′,5,5′-tetramethylbenzidine (TMB), Polyethylene glycol (PEG 1500), hypoxanthine (H), aminopterin (A), and thymidine (T) were purchased from Sigma Chemical Co. (St. Louis, MO). 96-well immunoassay plates were purchased from (Maxisorp; Nunc). Freund's complete and incomplete adjuvant, Roswell Park Memorial Institute medium (RPMI-1640), fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Gibco Laboratories (Grand Island, NY). All other chemicals and organic solvents used were of reagent grade or better.

2.3 Mice and Cell Lines

Virus-free, 6-8 weeks old, three female BALB/c mice were obtained from Shanghai SLAC laboratory Animal Co., Ltd. (SLAC) China. The murine myeloma cell line Sp2/0 was obtained from Shanghai cell culture bank (SIBS), China. All cell lines were confirmed to be *Mycoplasma*-free by RT-PCR analysis.

2.4 Preparation of Conjugates

The hapten of 1mg of DA was dissolved in 100 μL DMSO and conjugated to the carrier proteins, (BSA; immunogen) and (OVA; coating antigen) in the presence of NHS and EDC according to the method as previously reported [19] with some modifications.

2.5 Immunizations and serum characterization

Three female Balb/c mice were immunized subcutaneously, at two week intervals, with 100 μg of DA-BSA in 0.01 M PBS in a total volume of 200μl. After 4 times injection, the titer and sensitivity of antibody in the serum was determined by improved icELISA [20].

2.6 CHO supplemented cell culture media

The four test media contained RPMI-1640, 10% FBS, and penicillin/streptomycin, supplemented with glucose (control), fructose, galactose and maltose at concentration of 15 mg/mL each as carbon sources. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

2.7 Fusion and Screening

Spleen cells were isolated from the immunized mice and mixed with Sp2/0 murine myeloma cells at ratio of 1:10 in presence of 1mL 50% PEG. Cell fusion and hybridoma culture procedures were carried out essentially as reported earlier [21] and by limiting dilution [22].

2.8 Cell density

Static cultures using several 25 cm^2 flasks (Corning, Sigma-Aldrich) were inoculated with an initial cell density of 10^5 cells/ml and then incubated at 37°C in 5% CO_2, one flask was sacrificed every time a cell count was performed after 3 d, 6 d and 9 d respectively to determine the cell density by the trypan blue exclusion method [23] with some modifications. The cells were successfully passaged each time into new flasks with fresh media.

2.9 Statistical analysis

Data are presented as mean SD. Appropriate statistical tests were performed using GraphPad Prism. For experiments with more than 3 groups, one-way ANOVA was used. If variances were the same among groups, one-way ANOVA, followed by Dunnett's multiple comparisons test, was used. P values of less than 0.05 were considered significant. All the experiments in the present study were performed in replicates and results were confirmed in at least three independent experiments.

3. RESULTS

3.1 Synthesis of Immunogen and Coating Antigen

As demonstrated in Figure 2, the gel results indicated that DA was successfully conjugated with carrier proteins. Their UV absorption spectra are shown in Figure 3(a) and (b). DA showed the same specific UV absorption peaks at 242 nm and of carrier proteins at 280 nm. The absorption spectra of DA in conjugates showed absorption peaks around 225 nm which indicated successful conjugation.

3.2 Serum anti-DA titer

After immunization, serum titers were determined by icELISA. The results showed that mouse 1 achieved highest titer 2.50 of anti-DA (Figure 4), and selected for cell fusion with Sp2/0 cells.

3.3 Production and characterization of mAb in selected CHO media

Supernatants of hybridoma cells were determined after 9 d post fusion for the reactivity with DA by icELISA, which resulted 15 positive clones. One clone was selected after limiting dilutions, and was characterized as 3G9G1 (Figure 5) with highest-titer (OD: 1.485) in maltose supplemented media, other CHO showed lower antibody titers i.e. fructose (OD: 1.285), galactose (OD: 0.88), sucrose (OD: 0.57) and lowest in glucose as a control (OD: 0.32) respectively (Figure 6), the differences were statistically significant (P<0.05). The isotype of 3G9G1 mAb was determined to be IgG3 (Figure 7).

3.4 Cell density

Hybridoma cells were harvested after 9 d culture *in vitro*. Viable cell densities were increased up to 1.5 million/mL when supplemented with maltose as compared to the control

0.7 million/mL, the difference was statistically significant (P<0.05) (Figure 8).

4. DISCUSSION

Hybridoma technology and fusion events revolutionized medical and biotech sciences, has made possible the higher productions of mono-specific antibodies and immortal hybridoma [24].

In this study, we produced and characterized mAb against a potent neurotoxin hapten. DA-BSA induces a strong immune response in mouse [25]. According to the results, the hapten-protein conjugation was successful in gel since DA bears a carboxyl group which is activated by the active ester method and been covalently attached to a carrier protein.

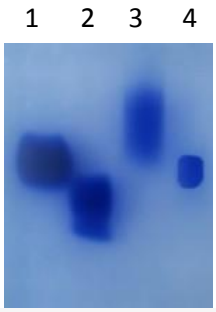

Figure 2. Coomassie blue-stained gel electrophoresis analysis revealed the successful cross-linking of domoic acid molecules with BSA and OVA. lane 1, BSA standard; lane 2, DA-BSA; lane 3, OVA standard; lane 4, DA-OVA.

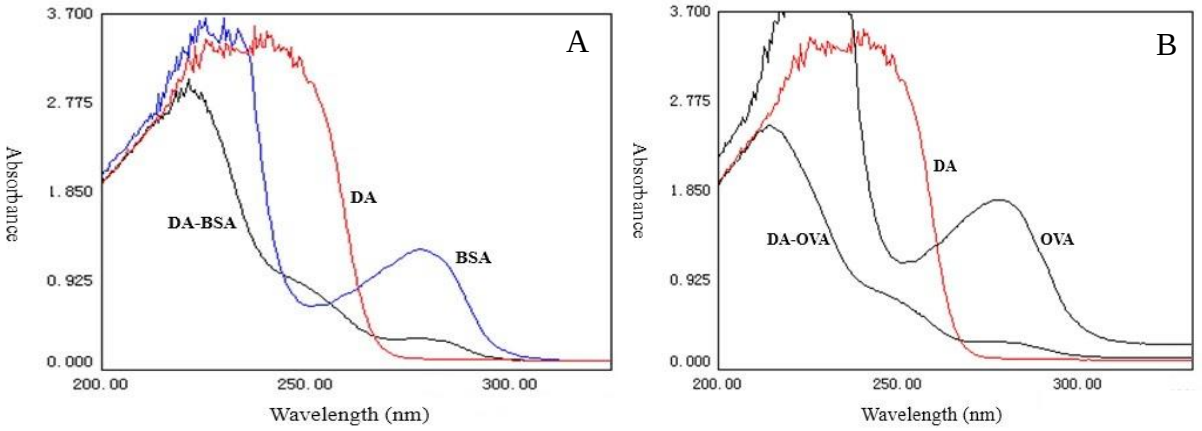

Figure 3. UV-Vis absorption spectra of (A) DA, BSA and DA-BSA conjugate and (B) DA, OVA and DA-OVA conjugate. Spectral scans from 200 nm to 300 nm in 1 nm increments were performed on the domoic acid, BSA, and OVA in 0.01 M PBS solution.

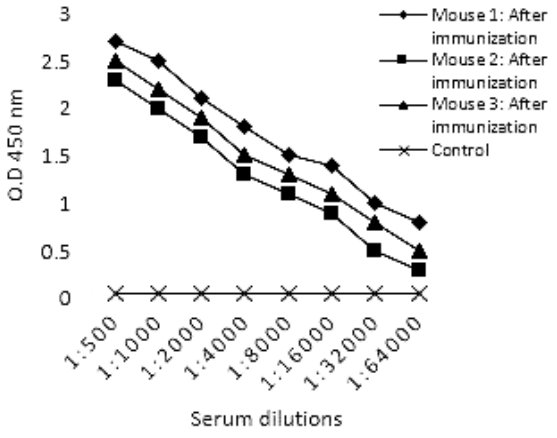

Figure 4. Serum titration of three immunized Balb/c mice by icELISA. Control, mouse before immunization; O.D, optical density.

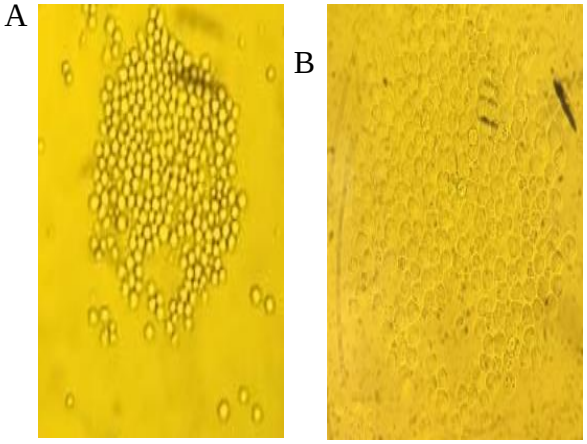

Figure 5. Post fusion light microscopic images of hybridoma colonies 5 (A) at magnification 200x and 9 (B) days at magnification 400x.

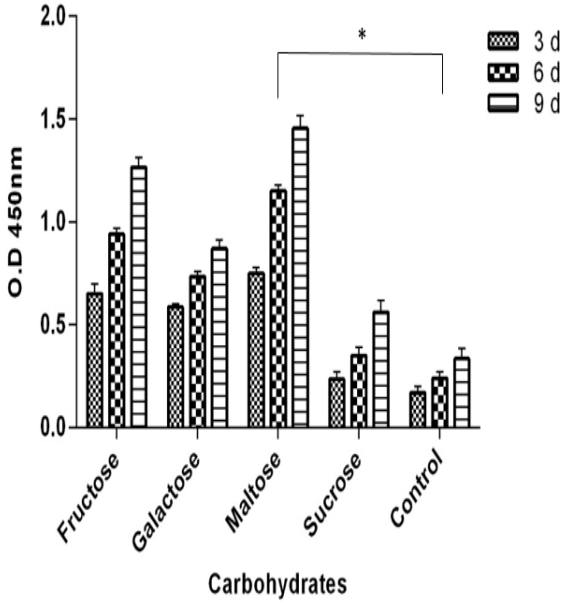

Figure 6. Effect of CHO supplemented cell culture media *in vitro* on the production of mAb. After 9 d of 3G9G1 growth, highest titer of 1.48 was obtained in maltose media. Control, glucose. *P < 0.05 between maltose and glucose control evaluated by one-way ANOVA followed by Dunnett's multiple comparisons test. Error bars indicate SD.

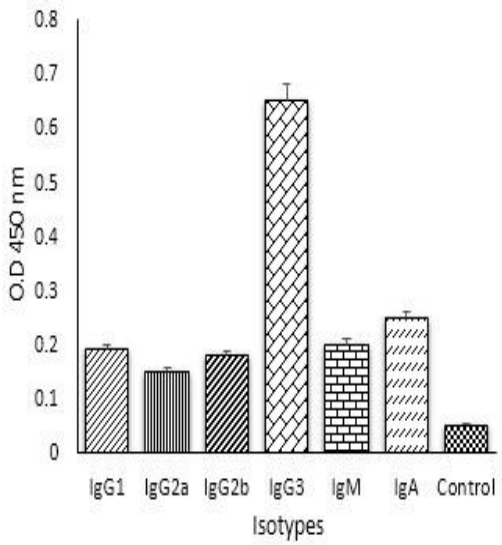

Figure 7. Isotype determination of 3G9G1 mAb by icELISA. Control, non-immune mouse. Error bars indicate SD.

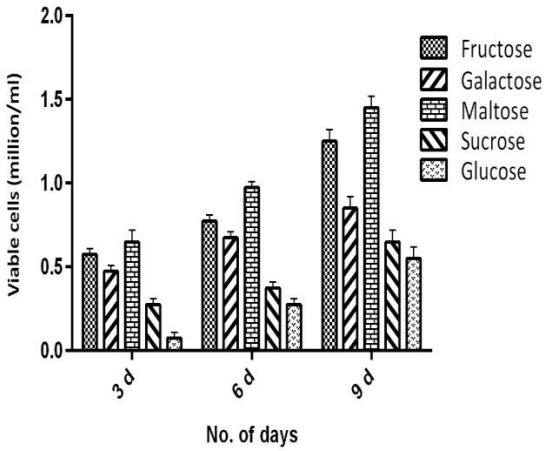

Figure 8. Effect of CHO supplemented media on 3G9G1 cell viability *in vitro*. The cells were harvested after 9 days. Glucose, control. P<0.05 using One-way ANOVA. Error bars indicate SD.

The UV-Vis spectra showed superposition properties to shift peak from 242 nm to new peak at 225 nm for successful DA conjugation [26]. In this study we used DA-OVA as a coating antigen, one DA-specific antibody-secreting hybridomas were obtained. Since the mAb reacted with DA in icELISA assay, the antibodies were non-conformational [27]. Monoclonal antibodies produced by the hybridomas were in the IgG3 class because in icELISA for mouse IgG, the mAbs were found to be positive. Since secondary antibodies were specific for mouse immunoglobulins, non-immune mouse serum was included in the assay in order to provide positive control for mouse IgG. These results were in agreement with the icELISA results, that 3G9G1 hybridoma secreted DA specific mouse IgG3 antibodies. In future studies, nanoparticle immunostrip and clinical applications of mAbs are planned.

In present study, maltose proved to be the best CHO substitute in cell medium which raised mAb titer to a significant level. Glucose served as a control, in general metabolizes to form lactic acid which hinders the cell growth or slows growth. The substitution of glucose with fructose, galactose and maltose favored the growth of 3G9G1 hybridoma and improved the growth conditions by best antibody titer in maltose media in agreement with previous studies [28]. It also enhanced cell density and viability [29,30,31,32,33].

5. CONCLUSION

Highest titers of anti-DA mAb were obtained with maltose and fructose supplemented media subsequently. So, it is concluded that, specific monoclonal antibody production, hybridoma density and viability could be enhanced by adding superior nutrients to the basal cell culture media in clinical, biological and pharmacological applications.

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Conflicts of Interest: The authors declare no conflict of interest.

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