Development of Monoclonal Hybridoma Cell Lines and Extracting Antibody Against Fummonisin B_1

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Abstract: *Objective*: To acquire monoclonal hybridoma cell lines against fummonisin B_1(FB_1) and extract monoclonal antibody against FB_1.

Methods: Coupling antigens of FB_1-KLH and FB_1-BSA with chemical methods and immune 6-8 weeks old female BALB/c mice with FB_1-KLH. Integrating spleen cells with sp2/0 myeloma cells to acquire hybridoma cell lines secreting McAb against FB_1. The method of multiple subclones was used to select cell lines stably secreting McAb. McAbs was got from ascites and purified. The subclass of antibody was measured and the molecular weight was identified. The specificity and sensitivity of McAb were identified with indirect competitive inhibition ELISA.

Results: The results of serum from immuned mice showed that after five times of immunization the titer stables at 1×10^{-6}, and the McAb belongs to IgG1 subclass, the light chain was κ, the molecular weight of heavy and light chain were 55kDa and 32kDa, respectively. ELISA results showed that McAb could react with FB_1. The linear range indirect competitive inhibition ELISA is 10-500ng/ml.

Conclusion: The monoclonal hybridoma cell lines and the high specificity,high sensitivity of FB_1-McAb was got.

Keywords: Fummonisin B_1, hybridoma cell lines, monoclonal antibody.

INTRODUCTION

Fumonisins are a class of Fusarium moniliform Sheldon, F. verticillioides, F. proliferatum and some other species of Fusarium contaminated corn and other grains, including food and feed [1-3]. Toxins produced by fungi are widely found in cereal and cereal products, such as noodles, beer, condiments, asparagus, etc., while there is some concentration in rice, wheat, barley, sorghum, milk and other food and feed. FB, separated firstly by Gelderbfom in 1988, has been classified to eleven types so far, however, the mainly and the most toxic type are fumonisin B races (FBs): FB_1, FB_2 and FB_3. Epidemic data showed that there are some association between fumonisins and the esophageal cancer [4-6]. FB_1 widely contaminated in Africa, Asia, North America, Latin America and Europe, FB_1 was detected in corn and its products. The international agency for research on cancer (IARC) classified it into the group of 2B, namely, human possible carcinogen [7, 8]. Experiments showed that FB_1 is the most toxic in the FB family. The tumor promotion and carcinogenicity have been demonstrated in rats, and FB_1 can also cause leukoencephalomalacia (ELEM) in horse [9], pulmonary edema (PPE) in swine [10]. There was renal and liver toxicity about FB_1 for most animal species and fumonisins, especially FB_1, are having more and more attention in animal and human health.

To detect the existence of FB_1, immunochemical method is often the ideal technique because of its high sensitivity as well as the specificity of the antibody, therefore, this research work is to develop anti FB_1 monoclonal antibody with hybridoma technique and then create the sensitive immunological method based on time-resolved fluoroimmunoassay (TRFIA) [11] used for FB_1 detection in food, so as to provide credible monitoring tools for the food safety risk monitoring.

1. MATERIALS AND METHODS

1.1. Materials

The FB_1 antigen was purchased from Fermentek (Jerusalem, Israel). Monoclonal mouse antihuman IgA was obtained from the Wuhan Institute of Virology (Wuhan, China). An Eu^{3+}-labeled kit and enhancement solution were purchased from PerkinElmer Wallac (Turku, Finland). Bovine serum albumin (BSA) and Keyhole limpet hemocyanin (KLH) was obtained from LKT Lab. (St. Paul, USA). Ninety-six-well polystyrene microtiter plates were purchased from Thermo Labsystems (Milford, MA). The commercial ELISA kits for FB_1-IgA detection were purchased from Zhongshan Bio-tech Co. Ltd (Zhongshan, China).

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ISSN: 1814-8085 / E-ISSN: 1927-5129/15

1.2. Animals and Cell Lines

Eight seven-weeks-old, SPF grade female BALB/c mice were supplied by Guangdong Medical Experimental Animal Center (certification No.0059665) with the temperature of 22 ± 1 °C and humidity of 70 ± 5 %. Mice were kept individually with one per cage. Animals were free to eat and drink water and weighed once a week. The experimental protocol followed the Guiding Principles for Research Involving Animals and Human Beings described in the Declaration of Helsinki and were approved by the Ethical Committee for Experimental Animals of Guangzhou Center for Disease Control and Prevention.

Mouse myeloma cell line sp2/0 was gifted by Dr. Yingsong Wu of Southern Medical University. Following controlled rate freezing in the presence of DMSO as cryoprotectants, sp2/0 cell lines and the cell lines of monoclonal hybridoma got in the process of research work were cryopreserved in a suspended state for indefinite periods with the maintaining of immersing in liquid nitrogen.

1.3. Preparation of Artificial Antigen and Immunization

Each mouse was intraperitoneally injected with 0.5 ml of emulsified antigen (with a protein content of approximately 200 μg). The first injection consisted of the antigen emulsified with complete Freund's adjuvant, and the subsequent injections consisted of the antigen emulsified with incomplete adjuvant. After the first injection, the injections were conducted every three weeks. Three days before the fusion experiment, direct injection of the antigen solution to spleen was performed. Antibody titers were detected during the immunization process. The FB_1-BSA antigen was added to a 96-well plate (100 μl in each well). After incubation in a 4 °C refrigerator overnight, the blocking solution was added, followed by incubation at 37 °C for 2 hours. Antibody dilution solution was added to dilute the antiserum, followed again by incubation at 37 °C. Next, HRP sheep anti-mouse IgG diluted to a certain concentration was added, followed by incubation at 37 °C for 60 min; 100 μl of TMB reagent was then added to each well, and the reaction was allowed to occur in the dark for 30 min, followed by the addition of 50 μl of 2M H_2SO_4 to stop the reaction. The absorbance was read using a microplate reader within 30 min.

1.4. Cell Fusion

The spleen cells of the immunized mice were mixed with the mouse sp2/0 myeloma cells at a ratio of 5-8:1, followed by fusion with 35% polyethylene glycol (PEG). Fusion was performed in a 50-ml centrifuge tube with a ratio of spleen cells:sp2/0 cells = 5-8:1 in the mixture, followed by centrifugation at 1000 rpm at room temperature for 5 min. The residual culture medium on the wall of the tube was used to break up the cell pellet evenly. The centrifuge tube was then placed in a beaker of water at 37 °C, and 1 ml of PEG was added while shaking, after which 2 ml of RPMI 1640 was slowly added dropwise, with shaking for mixing, to gradually dilute the PEG. Using the same method, dropwise addition of 8 ml of RPMI 1640 was performed within 2 min. After centrifugation, the supernatant was discarded. The entire process was controlled so that it was completed within 8 min. Next, 30 ml of complete HAT-RPMI 1640 medium was added and gently mixed to prepare a cell suspension, followed by incubation in a 37 °C incubator for 2 h. Finally, the evenly mixed cell suspension was equally applied to three 96-well cell culture plates (100 μl containing 4×10^5 cells in each well), and the plates were placed in a CO_2 incubator for culture, which was considered day 0 of fusion (Day 0, D0). In approximately 7-10 days, the supernatant was screened.

1.5. Screening of Hybridomas and Antibodies *via* Indirect ELISA

After approximately 10 days of the fusion, positive wells were screened through an indirect non-competitive ELISA. The FB_1-BSA antigen was applied to 96-well plates. After blocking, 100 μl of the supernatant obtained from the last step was added to each well. HRP goat anti-mouse IgG (100 μl/well) and the TMB reagent were added subsequently, and the absorbance was read by a microplate reader. Next, the positive wells were confirmed through an indirect competitive inhibitory ELISA. After the FB_1-BSA antigen was adsorbed in the 96-well plates and blocking was performed, the FB_1 standard (100 ng/ml) and the cell supernatant were simultaneously added to the coated 96-well plates. The remaining procedures were the same as above. The serum of the immunized mice served as a positive control, while the supernatant of sp2/0 cell culture served as the blank control, and the cell culture supernatant from cell culture plates without growing clones served as the negative control.

1.6. Production of a Monoclonal Hybridoma

Using the method of accurate cell counting in culture flasks, the wells with positive colonies were marked, and the cell density was counted in some of

the wells. According to the counting results, the cells were adjusted to a density of 10^4 cells/ml and seeded into a 96-well cell culture plate with culture medium, followed by culture in an incubator. From the 7th day to the 9th day, the size of the colonies was approximately 1-2 mm, at which time the supernatant of the wells with hybridomas was aspirated. When the hybridoma colony had grown to fill 1/3-1/4 of the field of a microscope, ELISA screening was carried out to detect the presence of antibodies. The wells with single colonies that were producing antibodies were determined. The cells in the antibody-positive wells were then transferred to a 24-well culture plate to amplify the culture for 2-4 days. The positive wells were re-cloned. When 3-5 consecutive tests for all wells were positive, the cell culture could be amplified, and the cells in the positive wells were promptly frozen or used for antibody production.

1.7. Mass Production of the Monoclonal Antibody Using the Method of Induction in Animals

The hybridoma culture was pipetted up and down and then centrifuged at 1000 r/min for 7 min. After discarding the supernatant, the cells were resuspended in complete medium, and the cell number was adjusted to 2×10^6-1×10^7/ml. Each BALB/C mouse was injected with 0.5 ml of hybridoma, and an ascites preparation of myeloma cells was injected as the negative control. At 7-10 days before cell injection, the mice were treated *via* intraperitoneal injection of Freund's complete adjuvant, and the ascites was collected after 10-14 days.

1.8. Purification and Identification of the Monoclonal Antibody

The ascites was isolated and purified through protein A affinity chromatography. The class and subclass of the immunoglobulin of the antibody were identified using an antibody classification assay kit. The protein content of the antibody was detected using a BCA protein assay kit. The molecular weight of the antibody was determined *via* SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Using FB_1-BSA as the sample, the specificity of the monoclonal antibody was assessed by Western blotting. The specificity and sensitivity were further determined in an indirect competitive inhibitory ELISA, using the specific procedures described above.

2. RESULTS

2.1. Evaluation of the Immune Effect in Mice

The ELISA test results for the serum of the mice after immunization showed that the amount of antibody secretion in the four groups of animals after immunization three times was in the order 150 ng>300 ng>200 ng>100 ng, with the antibody titers in all groups showing a low level. With an increase in the number of immunizations, the antibody titer in the serum continuously increased. After four immunizations, the 150 ng and 300 ng groups began to show a significant difference from the negative serum, and large differences were also observed compared with the titers in the other two groups. Antibody secretion was greatest in the 150 ng group. The titers detected in the mouse serum after the fourth immunization and the fifth immunization were basically the same, with a serum titer of 1×10^{-6} being observed after the last booster immunization. These findings indirectly suggested that the coupling of anti-fumonisin FB_1 with the carrier protein keyhole limpet hemocyanin (KLH) and BSA was successful.

2.2. Cell Fusion

On the tenth day of selective culture, a colony of pinpoint size could be observed (Figure **1**). The fusion

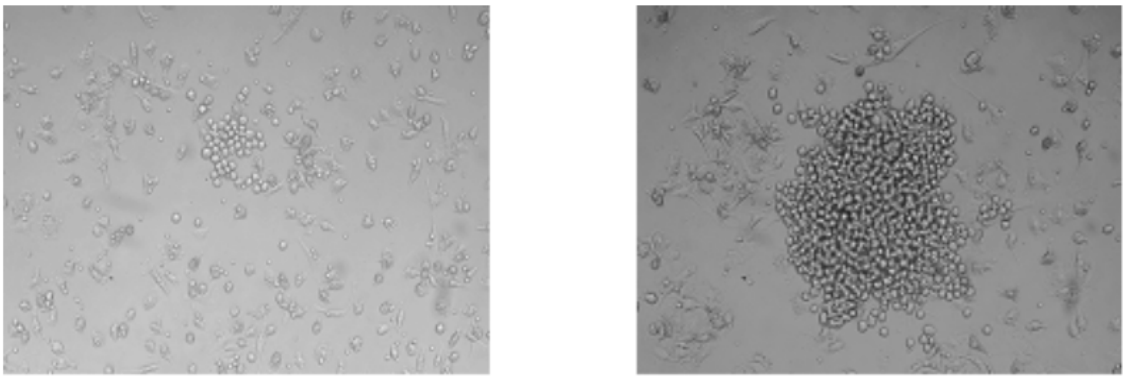
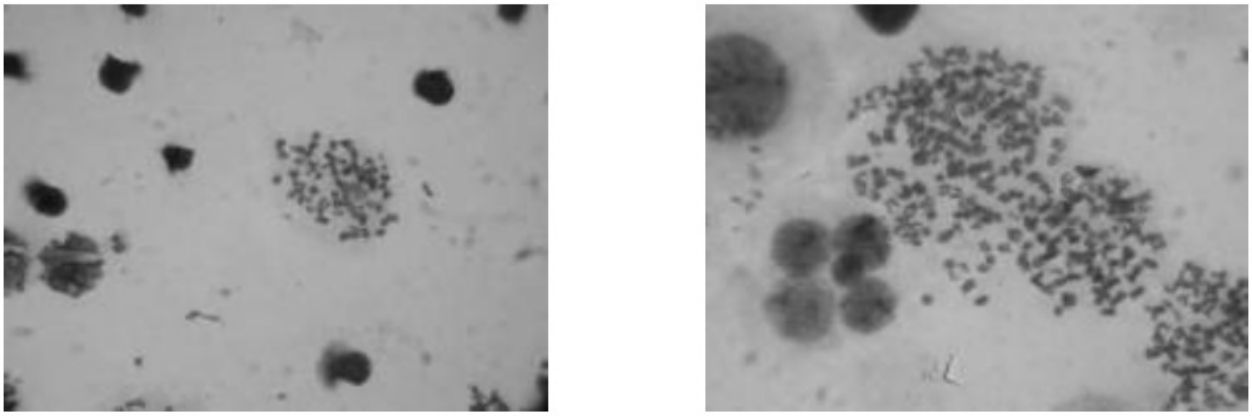


Figure 1: the colony on the tenth day of selective culture.



(a)sp2/0 cells (b) the hybridoma cells

Figure 2: Chromosomal analysis of the cells.

rate was calculated as follows: fusion rate = the number of all hybridomas in one fusion / the number of wells showing fusion. In this experiment, the cell fusion rate reached 70%.

2.3. Chromosomal Analysis of the Hybridoma Cells

Karyotype analysis was performed on the cell lines using the method of colchicine inhibition. The results showed that the number of chromosomes in the hybridoma cells producing the anti-FB_1 monoclonal antibody was 103-108, indicating a hybrid of two parent cells after fusion (Figure **2**).

2.4. Establishment of Hybridoma Cell Lines

Conventional cell fusion was conducted, with antibody screening *via* ELISA. The hybridoma cells in the wells with a strongly positive single colony were selected for subcloning, with four cell lines with strong positive expression being obtained.

2.5. Production of the Ascites Monoclonal Antibody

Approximately one week before hybridoma cell injection, the mice were injected with incomplete Freund's adjuvant. The adjuvant was able to stimulate rapid cell growth, resulting in high production of a specific antibody. At 14 days after injection, a large ascites tumor had formed in the mice.

2.6. Purification and Identification of the Antibody through Affinity Chromatography

After protein A purification and dialysis, a series of antibody characterization analyses were performed for the ascites antibody. SDS-PAGE was conducted to identify two cell lines presenting two protein bands at positions of 55 kD and 28 kD, which is consistent with the actual sizes of the IgG heavy and light chains, as shown in Figure **3**. Western blot analysis was performed to determine the specificity of three hybridoma cell lines, using sp2/0 and BSA as the control, as shown in Figure **4**. The results showed that FB_1 did not cross-react with the carrier proteins KLH and BSA, indicating that the antibody is specific against FB_1, showing specific binding to the FB_1-BSA antigen. After protein A purification and dialysis, antibody characterization was performed for the ascites antibody. The molecular weight of the antibody, the IgG content of the purified ascites antibody, the recommended working concentration, and the affinity constant are shown in Table **1**.

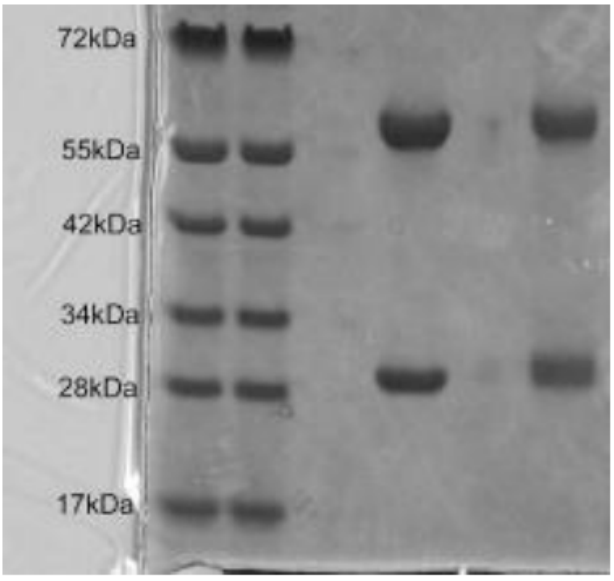


Figure 3: SDS-PAGE result of FB_1 antibody.

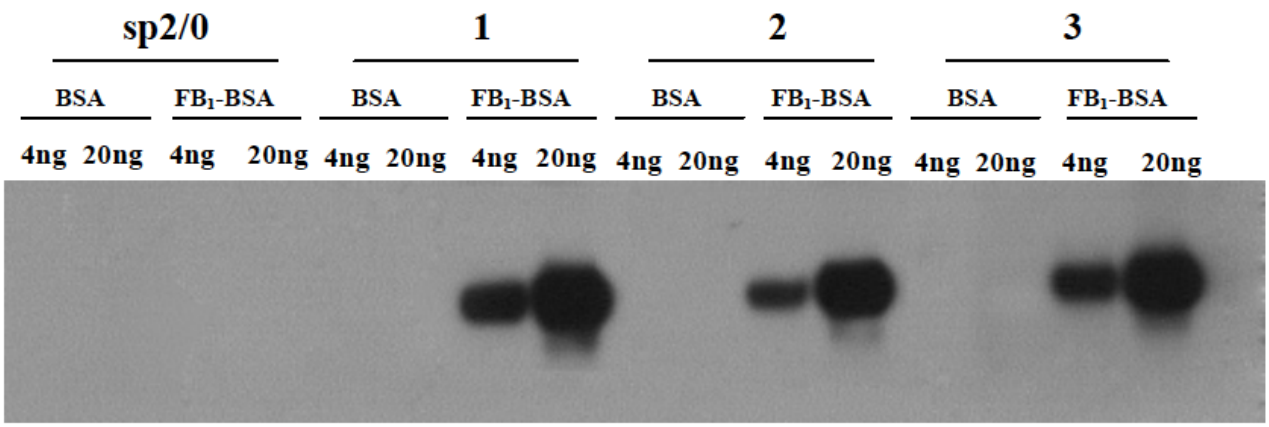


Figure 4: Western blot analysis of FB_1 antibody.

Table 1: General Characteristics of the FB_1 Antibody

antibody	subtype	Molecular weight (kD)	IgG content (ug/ml)	Working con.	afinity constant (mol/L)
E5G6	IgG2a	Heavy chains 55kD Light chains 28kD	103	$1:1.5\text{-}3\times10^3$	5.6×10^9

2.7. Sensitivity and Specificity of the Antibody

The results of ELISA demonstrated that the antibody did not cross-react with vomitoxin (also known as deoxynivalenol, DON), aflatoxin (AFB1) or the carrier protein BSA. The linear range of the ELISA curve of indirect competitive inhibition was 2-500 ng/ml, and the linear equation was Y = -0.6437X + 12.872 (R^2 = 0.9976), with a minimum detectable FB_1 concentration of 2 ng/ml and a 50% inhibitory concentration of 10.07 ng/ml.

3. DISCUSSION

FB_1 is a typical hapten with a molecular weight of 721.8, and it shows no immunogenicity in the immune response and cannot induce the generation of antibodies in mice [12]. To exhibit immunogenicity, the formation of macromolecules after binding with an artificial antigen is required. To prepare the corresponding antibody, it must be coupled with a particular macromolecule as the carrier protein to form a complete antigen. Additionally, the antibody produced by the antigen requires screening using the solid-phase antigen, and because FB_1 is a small molecule compound, it cannot easily be directly adsorbed to a solid-phase plate. Therefore, the antigen for detection must be prepared for screening of the specific antibodies. In this study, the classical glutaraldehyde method was applied to couple FB_1 and keyhole limpet hemocyanin (KLH), which is a common carrier protein with a high molecular weight and good immunogenicity, to produce a macromolecule with both the toxin structure and the immunogenicity [13]. Furthermore, to avoid cross-reaction of the obtained antibody with KLH after immunizing animals, FB_1 was also coupled with BSA as the detection antigen to preliminarily assess coupling by detecting the serum titer in mice and screen the target antibody. The basic procedure used for the preparation of antibody hybridomas was fusion of immune B cells and myeloma cells. On this basis, the myeloma cells were selectively cultured with selection medium, using their biochemical metabolic defect to obtain the hybridoma fusion. Therefore, cell fusion and screening of the fused cells are the technical foundation of hybridoma antibody technology, and the establishment of an ideal myeloma cell line with a biochemical defect is an essential prerequisite for the screening of the fused cells.

Mouse myeloma cell lines that do not secrete immunoglobulin have previously been established (such as sp2/0). The fact that all mouse IgG can bind to staphylococcus protein A facilitates the purification of the antibody. In this study, the myeloma sp2/0 cell line from BALB/c mice was selected for the cell fusion, and 8-AG (8-aza-guanine) was used to induce a hypoxanthine guanine phosphoribosyl transferase (HGPRT)-deficient cell line, which showed neither immunoglobulin synthesis nor immunoglobulin

secretion, thus ensuring the uniformity of antibody generation after the fusion of B cells and myeloma cells. This cell line cannot synthesize DNA through the salvage pathway (i.e., DNA is synthesized only through the primary pathway). In the primary synthetic pathway, a folic acid derivative is an essential intermediate that is involved in purine ring and thymine methyl biosynthesis. The folic acid antagonist aminopterin (A) can block this pathway, forcing the cells to synthesize DNA through the salvage pathway using H (hypoxanthine) and T (thymidine) in the culture medium based on HGPRT. However, myeloma cells lacking HGPRT would not survive because DNA synthesis is not achievable *via* the salvage pathway. Long-term *in vitro* culture of spleen cells is not possible, but fused cells carrying the HGPRT gene of spleen cells and showing the characteristics of constant growth and proliferation of tumor cells can grow and reproduce in HAT medium; thus, a fused hybridoma can be obtained after screening in HAT culture medium.

In this study, the method of *in vivo* induction in animals was applied. Whether a hybridoma readily generates ascites in mice depends on the growth characteristics of the hybridoma, which are related to both the antigen for the antibody and the amount of inoculated cells. Prior to immunization of the mice with the cells, the injection of Freund's incomplete adjuvant effectively suppressed the immune function of the peritoneal macrophages and lymphocytes of the mice, and the mice could therefore better adapt to the hybridoma with the characteristic of malignant growth transplanted in their abdominal cavity. In this environment, the hybridoma cells were able to gradually proliferate and secrete antibody in the ascites. This study successfully screened hybridoma cell lines that secrete an anti-fumonisin monoclonal antibody and prepared a monoclonal antibody with high affinity and high specificity against fumonisin, which provides a reliable technique for the rapid detection of fumonisin B_1. Based on this cell line as well as the monoclonal antibody it secrets, the next step of developing the immunological detection methods could be performed, for example, the method based on ELISA (Enzyme-Linked Immuno Sorbent Assay) or TRFIA (Time-resolved fluorescence immunoassay) technology. And further, after appraising the credibility, sensitivity and specificity of the method, it will be supply a fast and sensitive time-resolved immunofluorescence analysis assay to detect FB_1 in food or other products.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

FINANCIAL DISCLOSURE

This work was supported by grants from: The Project for Key Medicine Discipline Construction of Guangzhou Municipality (2013-2015-07).

The funders of the study had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Received on 06-08-2015 Accepted on 19-08-2015 Published on 10-09-2015

http://dx.doi.org/10.6000/1927-5129.2015.11.68