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Production of a Monoclonal Antibody by Simultaneous Immunization of Staphylococcal Enterotoxin A and B

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Abstract In this paper, a method of simultaneous immunizing BALB/c mice with staphylococcal enterotoxin (SE) A and B (SEA and SEB) to prepare a monoclonal antibody (3F2) for detecting both of SEA and SEB was developed. The results showed that antibody 3F2 had high titers against both SEA and SEB by enzyme-linked immunosorbent assay (ELISA). The sensitivities of 3F2 to SEA and SEB detected by ELISA were 133.2 and 82.5 ng/mL, respectively, and the detection limits for the two enterotoxins were about 1 ng/mL. The antibody 3F2 had high specificities and affinities to both SEA and SEB, and had no cross-reaction with SEC_1, bovine serum albumin, and ovalbumin. SEs-free skimmed milk samples were spiked with different concentrations of SEA, SEB, or both of them, respectively. Average recoveries of SEA and SEB from the spiked samples were all nearly between 82% and 104%. The result suggested that one cell fusion with simultaneous immunization by multiple antigen to prepare monoclonal antibody against them was possible, simple, and economic. The monoclonal antibody could be used in simultaneous detecting multifarious SEs.

Keywords Simultaneous immunization · Staphylococcal enterotoxin · Monoclonal antibody · Indirect competitive ELISA

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Abbreviations

SEs	Staphylococcal enterotoxins
SEA	Staphylococcal enterotoxin A
SEB	Staphylococcal enterotoxin B
SEC_1	Staphylococcal enterotoxin C_1
BSA	Bovine serum albumin
OVA	Ovalbumin
ELISA	Enzyme-linked immunosorbent assay
icELISA	Indirect competitive ELISA
PBST	Phosphate-buffered saline containing 0.05% Tween 20

Introduction

Staphylococcal enterotoxins (SEs) are a group of related emetic proteins produced by *Staphylococcus aureus* causing various diseases including septicemia, food poisoning, and toxic shock syndrome, as well as bovine mastitis [1–5]. Normally, the SEs are classified into five classical serological types: SEA, SEB, SEC, SED, and SEE. However, recently, the existence of some new serological types of SEs, such as SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, and SEO has been found [6, 7]. According to the difference of amino acid sequence homology, SEs are generally divided into two groups [8, 9]. One group mainly includes SEA, SED, and SEE, the sequence homology between them is from 51% to 81%. The other group mainly includes SEB and SEC, the sequence homology between them is from 42% to 67%. The sequence homology is from 22% to 33% between these two groups. Among them, SEA and SEB are the most common serotypes to cause staphylococcal food poisoning [10, 11].

The wide existence of SEs in foods has driven the development of analytical tools to identify their serotypes and comment of the contaminants [12]. At present, the methods for detecting SEs are mainly based on immunological methods. Among them, enzyme-linked immunosorbent assay (ELISA) is one of the most commonly used method because of its rapid and sensitivity [13]. This technique is now commercially available in kits such as RIDASCREEN, VIDAS, and TECRA [14]. Monoclonal antibodies used in commercial ELISA kits for detecting SEs are mainly prepared by hybridomas technique and immunized with a single antigen of SEs, and it can only detect related enterotoxin [15, 16]. However, staphylococcal food poisoning is not usually caused by single type of SEs [17], and actually in actual testing process, sometimes determining the existence of SEs are more important than identifying SEs serotypes. In this case, it will tremendously cut down detecting workload and time if multifarious SEs can be detected at one testing experiment, and obtaining a kind of monoclonal antibody against multifarious SEs is the first step to solve this problem.

In order to prepare a monoclonal antibody against multifarious SEs, simultaneous immunization was attempted by some researchers during the process of cell fusion [18–20]. Meyer [21] obtained a monoclonal antibody capable of binding to SEA, SEB, SEC_1, SED, and SEE by alternately injecting BABL/c mice with SEs serotypes of A and D. Then Shinagawa [22] reported that two monoclonal antibodies reacting with SEA, SEB, SEC_1, SED, and SEE were obtained by immunizing BABL/c mice with five serotypes of SEs. But they did not extensively analyze the characteristics of antibodies and discuss potential application of such antibodies to detect SEs in samples.

In this paper, a monoclonal antibody against both SEA and SEB prepared by simultaneous immunization with mixture of these two antigens by hybridomas technique

was studied. The characteristics of monoclonal antibody obtained by this method were systemically evaluated by ELISA, and its potential application for detecting SEs in skimmed milk samples was also studied. It would be helpful to understand whether it is possible to obtain a kind of monoclonal antibody against multifarious SEs by the method of simultaneous immunization, and to use it for simultaneous detecting multiple enterotoxins in foods.

Materials and Methods

Materials

Purified SEA, SEB, and SEC_1 were kindly provided by Dr. Jiang from Academy of Military Medical Sciences (Beijing, China). Sp2/0 myeloma cells were obtained from Academy of Animal Science and Technology of Huazhong Agricultural University (Wuhan, China). Freund adjuvant, hypoxanthine, aminopterin, thymidine (HAT), Bovine serum albumin (BSA), and ovalbumin (OVA) were purchased from Sigma (St. Louis, MO, USA). Goat anti-mouse IgG-HRP conjugate was purchased from Sino-American Biotechnology Company (Beijing, China), polyethylene glycol (PEG, mol. wt.4000), RPMI1640, newborn calf serum, and other common reagents were purchased from Sino-pharm Chemical Reagent Company (Shanghai, China).

Animals

Female, 6-week-old BALB/c mice were purchased from the Center for Disease Control and Prevention (Hubei, China) [certificate: SCXK (e) 2008-0005]. Mice were maintained under optimal conditions for hygiene, temperature, and photoperiods (12L:12D), and were allowed food and water ad libitum according to the institutional guidelines for the care and use of laboratory animals. All procedures on animals were approved by the Regulations for the Administration of Affairs Concerning Experimental Animals of Hubei province, China.

Immunization

The mixture of 10 μg SEA and 10 μg SEB were dissolved in 400 μL sterilized saline and emulsified with an equal volume of the Freund's adjuvant. The emulsion solutions were injected intraperitoneally into BALB/c mice (No. 1–3) [23]. The subsequent three immunizations were given by intraperitoneal injections with the same quantity of immunogen emulsified with the incomplete Freund's adjuvant at 2-week intervals. A week later at each booster dose, the mice were bled and the sera were tested by an indirect ELISA. After the fourth bleeding, the mice were given further booster injections of the mixture of 100 ng SEA and 100 ng SEB with intrasplenic injection in the third day before cell fusion.

Determination of Polyclonal Antibody Level in Mice Serum by Indirect ELISA

The process of secreting polyclonal antibody in mice was evaluated by indirect ELISA described in [24]. Briefly, 100 μL of coating antigen (1 μg/mL of SEA or SEB in bicarbonate buffer, pH 9.6) were added to each microwell of the plate respectively and incubated at 4 °C overnight at 90% relative humidity. The plate was washed three times with washing solution (0.1 mol/L phosphate buffered saline, pH 7.5, containing 0.05% Tween 20, PBST) for each well and decanted. The wells were blocked with 5% skimmed

milk in PBST for 2 h at 37 °C. After washing, 100 μL of serum or hybridoma supernatants were added to each well and incubated at 37 °C for 1 h. After another washing procedures, 100 μL of goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate (1:4,000) in PBST was added to each well and incubated at 37 °C for 1 h. Then 100 μL of peroxidase substrate (40 mg of 1, 2-diamino-benzene and 150 μL H_2O_2 in 100 mL of citrate buffer, pH 4.5) were added after washing the plate five times, and incubated at 37 °C for 20 min. The reaction was terminated by the addition of 2 mol/L sulfuric acid. Absorbance at 492 nm was determined in a Multiskan MK3 ELISA reader (Thermo, China). The positive well was defined as titer of the diluted antibody when the OD_{492} nm of positive was 2.1 times than that of negative control (P/N≥2.1).

Production of Monoclonal Antibodies

Monoclonal antibodies were prepared as described in [25]. Mice splenocytes immunized by the mixture of SEA and SEB were mixed with the myeloma cells at the ratio of 5–10:1 and centrifuged. The pellet was mixed by tapping the tube and then 1 mL of 50% (*v*/*v*) PEG 4000 in RPMI-1640 medium was added to it over 1 min with gentle shaking. The fusion reaction was stopped after an addition of 40 mL of RPMI-1640 medium over 5 min. At last, the fused cells were centrifuged for 10 min at 800 rpm. The supernatant was discarded and the fused cell pellet was resuspended in HAT medium and distributed into the 96-well tissue culture plates. The positive hybridoma cells were screened out by the indirect ELISA described above in seventh day after fusion. The hybridoma cells of positive wells were subcloned four times by the limiting dilution. Ascites were produced in paraffin oil-primed BALB/c mice.

Determination of the Sensitivity of Monoclonal Antibody

The optimum working concentrations of coating antigen and antibody were determined by the checkerboard titration. Sensitivity of antibody for SEA and SEB was assayed by indirect competitive ELISA (icELISA), respectively. The procedure of icELISA was similar to that mentioned in "Determination of Polyclonal Antibody Level in Mice Serum by Indirect ELISA". with some modifications. Briefly, after the microplates were coated with the optimized coating antigen concentration and blocked, 50 μL of properly diluted ascites were added to each well and then 50 μL of a serial of concentrations (10^{-1}, 1, 10^1, 10^2, 10^3, 10^4 ng/mL) of competitive antigen (SEA or SEB) in saline were added and incubated at 37 °C for 1 h, respectively. The subsequent steps were the same as in "Determination of Polyclonal Antibody Level in Mice Serum by Indirect ELISA". Fifty percent inhibitory concentration (IC_{50}) was defined as the sensitivity of antibody [23].

Determination of the Specificity of Monoclonal Antibody

The cross-reactivities of antibody in ascites to various SEs (SEA, SEB, and SEC_1) and some proteins (BSA and OVA) were evaluated by their IC_{50} values obtained from icELISA. Cross-reactivity (CR_{50}%) was as follows: $CR_{50}\% = 100 \times IC_{50}$ value of SEA or SEB/IC_{50} value of competitor [26].

Analysis of SEs in Spiked Samples by Antibody 3F2

Potential application for antibody 3F2 to detect SEs was evaluated by spiked samples experiment [27]. Skimmed milk samples were purchased from local supermarket in Wuhan,

free of SEs, and spiked with different concentrations of SEA, SEB, or mixture of equal amount of these SEs, respectively. Four final concentrations (0.5, 5, 25, 50 ng/mL) of each SE for the above samples were prepared, and every concentration were repeated in triple. Milk samples were first gently stirred, and then centrifuged for 15 min at 10^4 rpm. Samples supernatant were analyzed by the optimized icELISA protocol as described in "Determination of the Sensitivity of Monoclonal Antibody". The difference was that 50 μL sample supernatant instead of standard antigen. The samples were diluted with PBS buffer by ten times. The recovery was as follows: Recovery=found concentration/theoretical concentration×100%.

Results and Discussion

Production of Polyclonal Antibodies

Immunized mice were bled at the seventh day after each immunization and the sera were tested at 1:3,200 dilution by indirect ELISA to evaluate the titer. Mice (No. 1–3) immunized by multi-antigen (SEA and SEB) gave a fast immune response to both SEA and SEB (Figs. 1 and 2). They had higher titer in sera against both SEA and SEB after the fourth immunization. They had similar immune response as mice immunized with single enterotoxin (data not given here).

Production of Monoclonal Antibody and Detection of Antibody Titer

Mouse (no. 2) was chose for cell fusion in this study. The 90 hybrid cell lines were obtained by using hybridoma technique. Among these hybrid cell lines, 11 positive cell lines were screened out. Among them, six hybrid cell lines were found to produce antibodies only reacting with SEA, three hybrid cell lines only reacted with SEB, and two hybrid cell lines reacted both with SEA and SEB. After the two hybrid cell lines were subcloned four times, one stable hybrid cell line was obtained, designated 3F2. Antibody 3F2 had well immune reaction to both SEA and SEB, and had a titer of 4×10^5 against SEA and 1×10^5 against SEB, respectively (Fig. 3). The titer of antibody was determined by indirect ELISA (P/N≥2.1).

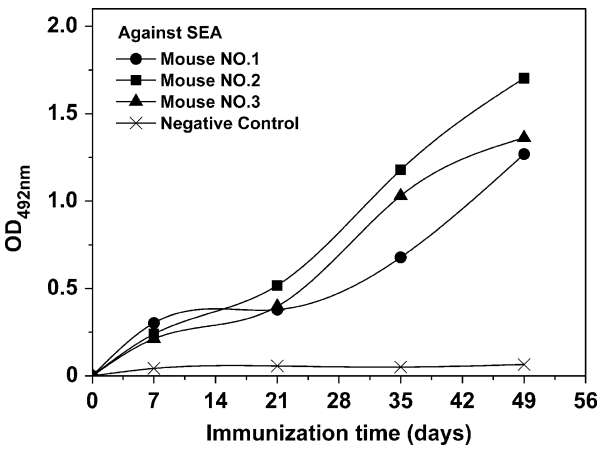

Fig. 1 The process curves of polyclonal antibodies production against SEA. The microwell plates were coated with 1 μg/mL SEA per well

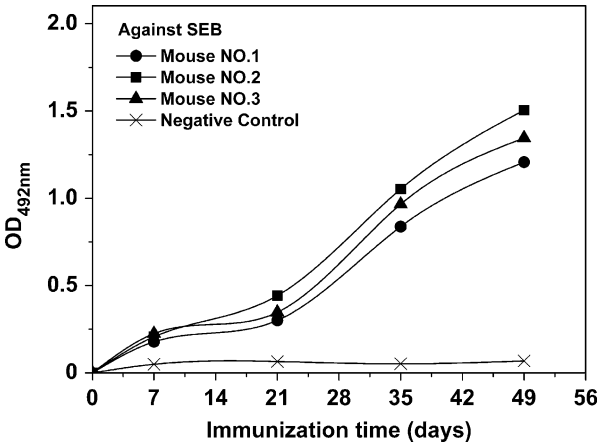

Fig. 2 The process curves of polyclonal antibodies production SEB. The microwell plates were coated with 1 μg/mL SEB per well

Each point represented the mean ± SD of three replicates. Results implied that it was possible to screen a monoclonal antibody against common antigenic determinants of multifarious antigens from a single cell fusion experiment by immunizing mice with a mixture of various antigens. This would overcome the problems of high cost, low efficiency, long period, and complicated process in monoclonal antibody production as usual method. Monoclonal antibody produced against common antigenic determinants of SEA and SEB was able to react with them [21]. This would be elaborately analyzed in following "The Specificity of Monoclonal Antibody".

The Sensitivity of Monoclonal Antibody

The competitive inhibition curves of antibody 3F2 against SEA and SEB were obtained by icELISA described above. Concentrations of SEA and SEB to cause 50% inhibition of binding (IC_{50}) were found to be 133.2 and 82.5 ng/mL, respectively (Figs. 4 and 5). Each point represented the mean ± SD of three replicates. According to the linear range of the curve, the working range to detect both SEA and SEB was assigned to a concentration

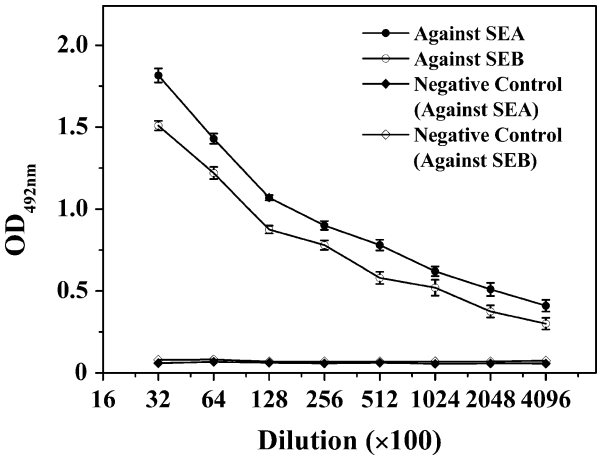

Fig. 3 Titration curve of antibody 3F2 from ascites against SEA and SEB. *Black circle* and *black diamond*: the microwell plates were coated with 1 μg/mL SEA per well; *white circle* and *white diamond*: the microwell plates were coated with 1 μg/mL SEB per well. PBST was used to replace ascites in the negative controls

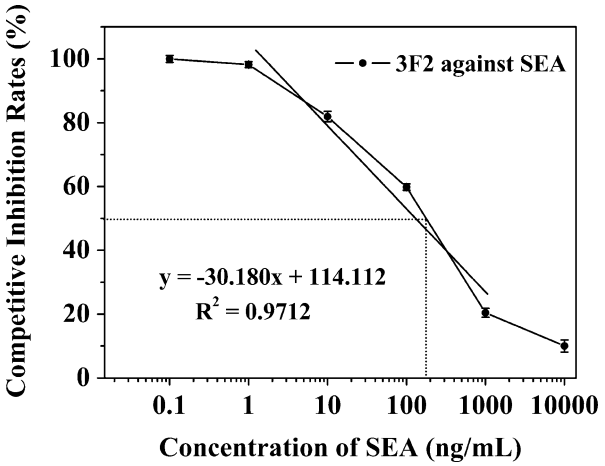

Fig. 4 The competitive inhibition curve of antibody 3F2 against SEA. The microwell plates were coated with SEA, and the competitive antigen was SEA

between 10 to 10^3 ng/mL which gave 20% to 85% inhibition against them. The detection limits for these two enterotoxins are all about 1 ng/mL.

The Specificity of Monoclonal Antibody

To determine the specificity of antibody 3F2, several SEs (SEA, SEB, SEC_1) and other proteins (BSA and OVA) were tested cross-reactivity with it by icELISA. The results demonstrated that monoclonal antibody 3F2 was highly specific for SEA and SEB, with the cross-reactivity rate at 161.45% and 61.94%, respectively. Meanwhile, the antibody showed little affinity with SEC1, BSA and OVA, and the cross-reactivity were all lower than 0.01%. This revealed that 3F2 could specifically recognize common antigenic determinant belonging to both SEA and SEB. This is probably due to amino acid sequence homology between them. Six antigenic determinants are predicted from the hydrophilicity and secondary structure information of SEA and SEE. There are 84% sequence homology between them, and at least four determinants of them are identical [28–30]. Similarly, seven antigenic determinants exist between SEB and SEC1. There are 65% sequence homology

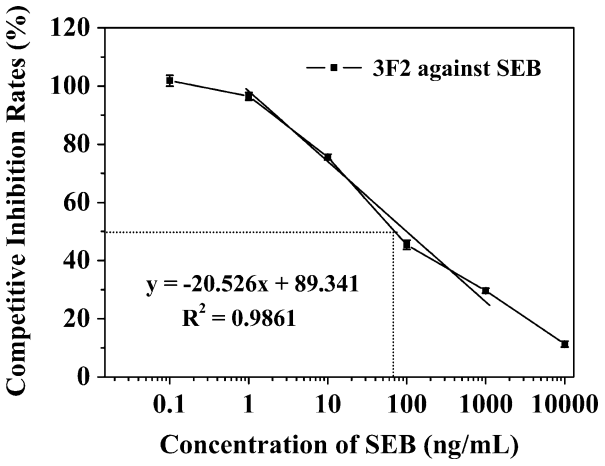

Fig. 5 The competitive inhibition curve of antibody 3F2 against SEB. The microwell plates were coated with SEB, and the competitive antigen was SEB

between them, ant three of these antigenic determinants appear similar [31]. Furthermore, the percentage of amino acid sequence similarity between the two groups of SEs varies from 22% to 33% [9]. Thus, there may have several similarities of secondary structures and amino acid sequence between SEA and SEB, and there may be common antigenic determinants between SEA and SEB. Monoclonal antibody could have specific binding with both SEA and SEB when it was produced with immune response to their common antigenic determinants.

Analysis of SEs in Spiked Samples by Antibody 3F2

The spiked recoveries were used to evaluate the potential application for antibody 3F2 to detect SEs in samples by icELISA (Table 1). The contents of SEA and SEB in all samples were calculated by the linear equation presented in Figs. 4 and 5, respectively. For samples spiked with SEA or SEB, the average recoveries of SEA and SEB were from 88.3% to 103.5% and from 86.2% to 101.6%, respectively. For samples spiked with mixture of SEA and SEB, the average recoveries of the two SEs were from 82.3% to 102.4%. From them, it could be seen that antibody 3F2 had well-spiked recoveries for both SEA and SEB, which indicated that it could accurately determine SEA and SEB in artificially contaminated skimmed milk samples. Thus, it might be applicable for routine screening of multifarious SEs in skimmed milk and other foods.

Conclusion

In summary, monoclonal antibodies are potentially useful for the development of rapid, sensitive, and specific methods for detection of enterotoxins [25]. The results of our study have suggested that it is possible to prepare monoclonal antibodies against both SEA and SEB by simultaneous immunization with mixture of two antigens during the process of cell fusion, and then detect multifarious SEs in one reaction. Developing an immunoassay

Table 1 Spiked recoveries of SEA and SEB from artificially contaminated skimmed milk samples using 3F2 antibody by icELISA

SEs	Spiked (ng/mL)	Theoretical (ng/mL)	Found[a] (ng/mL)	Average recovery ± SD (%)
SEA	5	0.5	0.458±0.034	91.6±7.6
	50	5	4.413±0.015	88.3±4.5
	250	25	25.878±0.031	103.5±8.3
	500	50	46.614±0.053	93.2±5.5
SEB	5	0.5	0.431±0.047	86.2±9.1
	50	5	4.796±0.092	95.9±12.5
	250	25	22.673±0.025	90.7±5.8
	500	50	50.815±0.058	101.6±5.2
SEA+SEB	5+5	1	0.823±0.077	82.3±6.7
	50+50	10	9.061±0.132	90.6±10.7
	250+250	50	51.214±0.066	102.4±10.3
	500+500	100	91.952±0.049	91.95±6.2

[a] Data represent the mean of three replications ± SD

method to simultaneously detect multiple enterotoxins in food will have considerable advantage in saving detection time and workload.

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