Production and characterization of anti-peptide monoclonal antibodies with specificity for staphylococcal enterotoxins A and B ☆

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Abstract

A synthetic peptide containing selected epitopes from staphylococcal enterotoxin A (SEA) and enterotoxin B (SEB) was used to produce monoclonal antibodies (Mabs) to respective enterotoxins in a single fusion procedure. The peptide inhibited the reaction of polyclonal anti-SEA or anti-SEB antisera with their homologous enterotoxin, thus showing that the chosen epitopes are part of the antibody-inducing enterotoxin sequences. Two Mabs, Mab-A and Mab-B, reacted with both the peptide and with either SEA or SEB. Used in a double antibody sandwich ELISA, the Mabs were able to quantitate the native SEA or SEB toxins at nanogram levels. © 1999 Elsevier Science B.V. All rights reserved.

1. Introduction

Strains of Staphylococcus aureus produce a large variety of toxic molecules that may induce pathological conditions in man and animals (Freer and Arbuthnott, 1983). Of these, the staphylococcal enterotoxins (SE) have been extensively investigated because of their involvement in staphylococcal food poisoning (Bergdoll, 1989) and other staphylococcal related diseases (Bergdoll and Chesney, 1991). The SE are a group of eight single-chain simple proteins (25 to 28 kD) which contain a disulfide loop in their molecule.

The nucleotide and amino acid sequences of SE have been determined (for review see (Marrack and Kappler, 1990 and Micusan and Thibodeau, 1993). The SE although similar in biological activity, are classified serologically on the basis of antigenic diversity as SEA, SEB, SEC 1-3, SED, SEE (Bergdoll, 1989) and SEH (Ren et al., 1994).

There are no chemical methods which can be employed routinely for SE detection. Immunological methods are the only ones which can detect and identify a given SE. However, these procedures necessitate the availability of specific high affinity antisera. Limitations associated with the use of polyclonal antisera include (i) the requirement for a constant supply of highly purified SE as immunizing antigen, (ii) batch to batch variation in antisera quality, (iii) the possibility of occurrence of toxic shock during immunization and (iv) inability to identify antigenic determinants or biologically active

sites. Some of these limitations may be overcome by the production and use of uniform and specific monoclonal antibodies (Mabs) (Thompson et al., 1984, 1986; Lapeyre et al., 1987; Shinagawa et al., 1991).

This report describes the production of anti-SEA and anti-SEB Mabs. The immunogen used was not native SE but rather a synthetic peptide containing SEA and SEB amino acid sequences corresponding to predicted antigenic epitopes (Singh and Betley, 1989; Singh et al., 1988).

2. Materials and methods

2.1. Animals and myeloma cell line

Female Balb/c mice, age 6 to 7 weeks were purchased from Charles River Ltd. (St-Constant, QC). The non-secretor myeloma cell line NS-1 (ATCC TIB 18) was obtained from ATCC (Rockville, MD).

2.2. Reagents and equipment

Staphylococcal enterotoxins, S. aureus exfoliative toxin, α-hemolysin, toxic shock syndrome toxin 1 (TSST-1), and antisera to staphylococcal enterotoxins were purchased from Toxin Technology Inc. (Sarasota, FL). Adjuvant RAS R-700 was obtained from Cedarlane Laboratories (Hornby, ON). Biotinylated sheep anti-mouse IgG [F(ab')₂], biotinylated donkey anti-rabbit IgG [F(ab')₂], streptavidin-biotinylated horseradish peroxidase complex and mouse monoclonal antibody isotyping kit were obtained from Amersham Canada Ltd. (Oakville, ON). Micro BCA* protein assay reagent and Inject Activated Immunogen Kit were purchased from Pierce Chemical Co. (Rockford, IL). Cell culture media, Protein Biotinylation System and 3, 3', 5, 5'-tetramethylbenzidine (TMB) were obtained from GIBCO-BRL (Burlington, ON) and Falcon tissue culture labware from Fisher Scientific (Montreal, QC). Immulon-3 and-4 flat-bottomed 96 well microplates were purchased from Dynatech Laboratories Inc. (Alexandria, VA). Hi Trap Protein A and G (5 ml columns) were purchased from Pharmacia-Biotech (Ste-Anne de Bellevue, QC) The microplate ELISA

reader, model LE-309 was purchased from Bio-Tek Instruments Inc. (Winooski, VT). Supelcosil LC-318 and Supelcosil LC-DABS columns were obtained from Supelco (Oakville, ON) and HPLC equipment from Waters Ltd. (Mississauga, ON).

2.3. Peptide synthesis

The primary structure of the peptide contains the first eight N-terminal amino acids from SEA, a spacer of four glycine residues, and a sequence of amino acids 67 to 74 from SEB i.e. SEKSEEING-GGGKNKDLADK and is thereafter abbreviated A-G4-B. For conjugation with carrier proteins, two identical A-G4-B peptides were synthesized with a cysteine added to either the N-terminal or C-terminal part of the molecule. A glycine molecule acted as a spacer between the added cystein and the rest of the molecule. For inhibition studies the peptide was synthesized without the cysteine and glycine residues. The peptides were manufactured by Vetrogen Corp. (London, ON). All the peptides were purified to more than 95% homogeneity by reverse phase HPLC on a 5 × 4.6 cm Supelcosil LC-318 column. Elution was achieved by a 0–100% gradient of acetonitrile in water containing 0.1% (v/v) trifluoroacetic acid. Amino acid analysis was performed after hydrolysis of peptides in 6N HCl (containing 0.05% phenol) at 110°C for 48 h, followed by HPLC chromatography on a 15 cm × 4.6 mm Supelcosil LC-DABS column with 25 mM KH_2PO_4, pH 7.0 and acetonitril/methanol (70:30) as mobile phase. The results confirmed the expected amino acid composition.

2.4. Peptide conjugation to carrier proteins

For immunization purpose, the cysteine N-terminal and cysteine C-terminal A-G4-B peptides, in equal quantities, were conjugated with a carrier protein, keyhole limpet hemocyanin (KLH). For selection of Mabs reacting with the peptide mixture, the carrier protein KLH was replaced with bovine serum albumin (BSA). Conjugation was performed using the Inject Activated Immunogen kit as indicated by manufacturer. The degree of conjugation was determined by using Ellman's Reagent (5,5'-dithio-bis-2-nitrobenzoic acid) and calculated based

on the difference in absorbance at 412 nm between the peptide solution before and after conjugation. An average of 18 peptide molecules per molecule of KLH and 12 peptide molecules per molecule of BSA were conjugated by this method.

2.5. Monoclonal antibody production, purification and characterization

Female Balb/c mice were injected intraperitoneally with 200 μg of peptide-conjugate emulsified in RAS R-700 adjuvant. Booster injections prepared as above were made every 2 weeks for 2 months. After an additional month of "rest", the mice were given, intravenously, an equal mixture of unconjugated peptides (50 μg/mouse) in 0.2 ml of sterile PBS. Three days later, the mice were killed and the spleens were aseptically removed. Hybridization with mouse myeloma cell line NS-1 was performed as previously described (Bhatti et al., 1994). Ten days after fusion, culture wells were screened visually for cell growth and supernatants from positive wells were tested by ELISA for the presence of anti-peptide, anti-SEA and anti-SEB antibodies. Cells from positive wells were further cloned in soft agarose (Coffino et al., 1972). Larger volumes of Mabs were obtained using the dialysis tubing technique (Jwo and LoVerde, 1988). Mabs were purified by affinity chromatography on a 5 ml Hi Trap Protein G column according to manufacturer's instructions. Protein concentration of purified antibodies were determined using Micro BCA* protein assay reagent. Purified Mabs were characterized for isotype and light chain content by use of a mouse monoclonal antibody isotyping kit.

2.6. Purification and biotinylation of rabbit anti-SEA and anti-SEB antibodies

IgG fractions of rabbit anti-SEA or anti-SEB antisera were obtained by $(NH_4)_2 SO_4$ precipitation (35%) followed by affinity chromatography over Hi Trap Protein A-Sepharose under conditions described by the manufacturer. The IgG containing fractions were pooled, dialyzed against 1M NH_4HCO_3, pH 8.0 and lyophilized. For use in quantitative ELISA, the IgG fractions were suspended in PBS and absorbed with purified mouse IgG to eliminate potential cross-

reactivity with mouse immunoglobulin. After centrifugation at 10 000 rpm, the IgG fractions were biotinylated using a Protein Biotinylation System and procedures as provided by the manufacturer.

2.7. Immunoassays

The reactivity and specificity of Mabs with purified SEA, SEE, SED, SEB and SEC1 were evaluated by an indirect ELISA. Briefly, microtiter plates Immulon-3 or -4 (depending on the antigen) were coated, by overnight incubation at 4°C, with 100 μl/well of antigens (A-G4-B-BSA, SE or antigenically unrelated S. aureus toxins at 0.25-1 μg/well or A-G4-B peptide at 0.25–0.75 μg/well) diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.4. Plates were then washed with PBS-0.5% Tween-20 (PBS-T) and blocked for 1 h at 37°C with 200 μl/well of PBS containing 0.2% gelatin. Primary antibodies (anti-SEA, anti-SEB or Mabs) were then added (100 μl/well) and incubated for a further 2 h at 37°C. The optimal concentration of primary antibodies was established as the antibody dilution which gave the highest absorbance in preliminary tests determined by a checker board titration. The plates were washed extensively with PBS-T and antigen–antibody complex was then developed by incubation for 1 h at 37°C with corresponding biotinylated second antibody. Wells were washed again with PBS-T, then streptavidin-biotinylated peroxidase complex was added followed by incubation at room temperature for 15–30 min. Plates were washed again with PBS-T followed by addition of TMB substrate. After incubation for 15 min at room temperature 50 μl of 2M H_2SO_4 was added to each well to stop the reaction and optical densities were read at 450 nm.

For competitive inhibition studies, rabbit anti-SEA or anti-SEB antiserum was mixed with increasing concentrations of SEA, SEB or A-G4-B peptide (total volume 1 ml) in 1.5 ml conical-bottom microcentrifuge tubes pre-coated with BSA. After incubation for 1 h at 37°C, the tubes were centrifuged and the supernatant used as the primary antibody on SEA or SEB pre-coated Immulon-4 microtiter plates. The ELISA reaction was then developed as described above.

For quantitative determination of SEA or SEB, a

double antibody sandwich ELISA was performed. Briefly, Immulon-4 plates were coated (100 μl/well) with either Mab-A or Mab-B antibody diluted at 5 μg/ml in carbonate–bicarbonate buffer, pH 9.4 and incubated overnight at 4°C. The wells were washed with PBS-T then were blocked with 0.2% gelatin (200 μl/well) for 1 h at 37°C and washed again with PBS-T. Dilutions of purified SEA or SEB (0–10 ng/ml in PBS) were added to triplicate wells. The plates were incubated for 2 h at 37°C and then washed with PBS-T. Biotinylated rabbit anti-SEA or anti-SEB (IgG fraction) at a concentration of 10 μg/ml (100 μl/well) was added to the wells and plates were incubated for 2 h at 37°C. The plates were washed with PBS-T, then 100 μl of strep-tavidin-biotinylated peroxidase complex (diluted 1:1000) was added to each of the wells and the plates again incubated for 30 min at 37°C. The plates were thoroughly washed with PBS-T followed by the addition of TMB substrate solution (100 μl/well) The plates were incubated at room temperature until the desired colour intensity was achieved, at which point the reaction was stopped with 50 μl/well of 2N H_2SO_4. The absorbance was measured at 450 nm as described above.

Negative antibody controls were run simultaneously by substitution of Mab-A and Mab-B with an equivalent concentration of a Mab of the same isotype but having no reactivity to either SEA or SEB. In other controls, SEA and SEB were replaced by antigenically non-related *S. aureus* toxins *i.e.* exfoliative toxin, α-hemolysin and TSST-1. Readings were considered positive when OD_{450} was greater than the mean OD_{450} plus two standard deviations (S.D.) of the negative controls. The OD values of controls were subtracted from each data point. Standard curves were generated by linear regression analysis (Ritchie et al., 1981).

3. Results and discussion

3.1. Reactivity of A-G4-B peptide with polyclonal antibodies to SEA and SEB

Synthetic peptides as well as cleavage peptides obtained by enzymatic or chemical degradation are recognized as having immunogenic potential and used to obtain specific antibodies to native proteins (Briand et al., 1985; Mayer and Walker, 1987), to locate active epitopes (Talbot et al., 1988; Hugues et al., 1992) or to identify biologically active sites (Pontzer et al., 1989; Komisar et al., 1994). To produce Mabs reacting with SEA or SEB we used a synthetic peptide containing two putative immunogenic regions from SEA and SEB sequences which were spaced by four glycine residues. Reactivity of A-G4-B peptide with anti-SEA or anti-SEB antisera was tested in competitive inhibition experiments. The reactivity of polyclonal rabbit anti-SEA and anti-SEB antisera with the native SE was reduced following pre-treatment with the peptide as evidenced by reduction in ELISA titer. This fact demonstrates that each SEA-derived and SEB-derived sequence from the A-G4-B peptide was recognized and reacted with antibodies present in the respective polyclonal antisera. As expected, inhibition of anti-SEA or anti-SEB antisera with homologous native SE was more evident than in the case of A-G4-B peptide (Fig. 1).

Although SEA has a high degree of sequence homology with SED and SEE (Micusan and Thibodeau, 1993; Bayles and Iandolo, 1989; Couch et al., 1988), the A-G4-B peptide, at reasonable concentrations, did not inhibit significantly the reaction of respective antisera with their homologous SE (data not shown). This fact can be explained by a difference in the *N*-terminal amino acid sequences between SED or SEE and the SEA sequence included in the A-G4-B peptide. SEB and SEC 1-3 also show important sequence homology (Micusan and Thibodeau, 1993; Bohach and Schlievert, 1987). However, the SEB sequence of A-G4-B peptide is quite different from that corresponding to SEC 1-3 and the peptide did not inhibit the reaction between anti-SEC1 antiserum and SEC1 (data not shown). The above findings strongly suggest that the A-G4-B peptide contains epitopes similar to those in native SEA or SEB which are recognized by antibodies present in the polyclonal antisera. However, the relatively reduced inhibition of the reaction between the antisera and their homologous enterotoxins by the A-G4-B peptide indicates that the sequences chosen might not be major antigenic determinants.
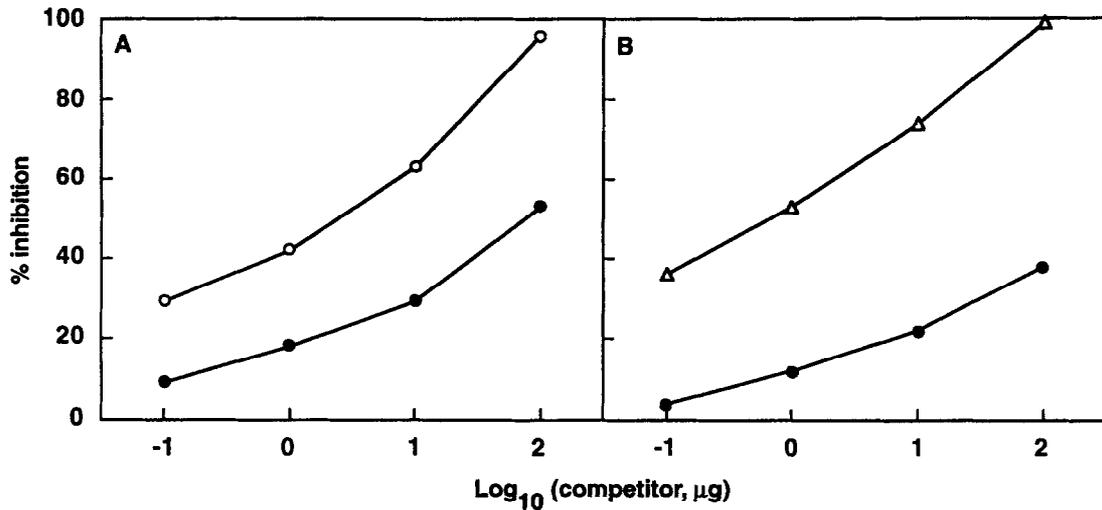
Fig. 1. Competitive inhibition experiments of: (A) rabbit anti-SEA antiserum with SEA (O-O) and A-G4-B peptide (●-●); (B) rabbit anti-SEB antiserum with SEB (△-△) and A-G4-B peptide (●-●). The rabbit antisera were diluted to give an ELISA OD_{450} value of 1.0 ± 0.2 when reacted with 1 μg/well coated SE. Each point represents the mean of three separate replicates.

3.2. Selection of hybridoma clones producing antibodies with SEA or SEB specificity

Based on the above results, we proceeded to immunize mice with A-G4-B-KLH conjugate, with the aim to obtain specific Mabs for SEA and SEB within a single fusion experiment. A relatively high percentage of viable hybridomas i.e. 42% was obtained from two 96 well plates. However, only 8% of these (6 wells) secreted antibodies reacting in ELISA with A-G4-B-BSA conjugate coated on Immulon-3 microplates. These positive clones were further selected for specific reactivity with native molecules of either SEA or SEB. Two wells contained hybridoma cells secreting Mabs reacting with SEA and the remaining four wells showed SEB specificity. After cloning of cells in soft agarose, we selected two Mabs designated Mab-A and Mab-B having specificity for SEA and SEB, respectively.

3.3. Reactivity of Mab-A and Mab-B

Larger quantities of Mab-A and Mab-B were produced using the dialysis tubing method (Jwo and

LoVerde, 1988). Besides the low cost, the main advantage of this method is the high purity of Mabs obtained compared to the ascites fluid method which often contains natural anti-SE antibodies, thus reducing the specificity characteristic of Mabs (personal observation). Both Mabs were of IgG_1, κ isotype. Titration curves of Mab-A and Mab-B were performed by ELISA on Immulon-4 microplates coated with either A-G4-B peptide or SEA and SEB. Both Mabs reacted more strongly with the peptide than with native homologous SE (Fig. 2). This finding is often observed in producing antibodies to peptides. Because of conformational changes imposed by the folding of the polypeptide chain, antibodies generated by peptides may react less or fail to react with native protein (Jemmerson and Blankenfield, 1989).

For the same concentrations of SEA or SEB coated on microtiter plates, Mab-A gave always a higher OD_{450} signal than Mab-B even though tested in equivalent concentrations. In this context, previous studies have shown that accessible and flexible protein regions, such as N- or C-terminal regions, are the sites most likely to react with anti-peptide antibodies (Hopp, 1986; Dyson et al., 1988). Since the N-terminal portion of the A-G4-B peptide is
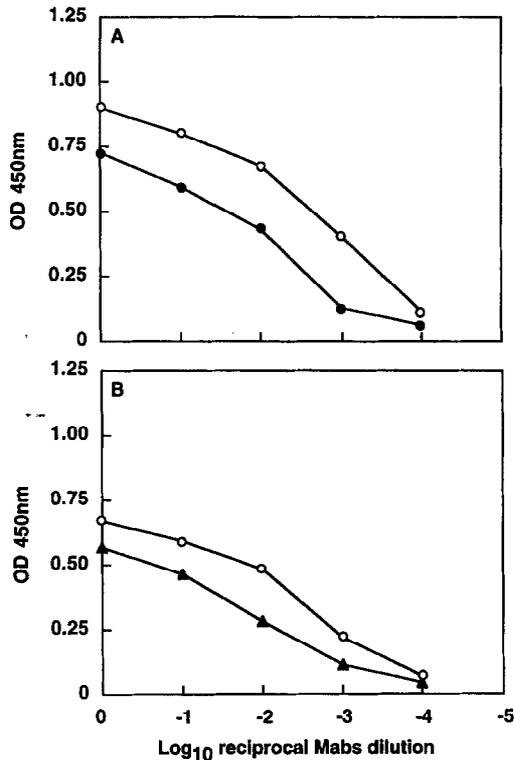
Fig. 2. ELISA titration curves of anti-peptide Mabs: (A) Mab-A with A-G4-B peptide (O-O) and SEA (●-●); (B) Mab-B with the A-G4-B peptide (O-O) and SEB (▲-▲). Peptide concentration for wells coating was 50 ng/well and that of SEA or SEB was 25 ng/well. Both Mabs had an initial concentration of 75 μg/ml. Each point represents the mean of duplicate wells.

similar in sequence to the N-terminal portion of SEA, the Mab-A may more easily recognize this region than Mab-B which is directed to an inside sequence of SEB.

The specificity of Mab-A was tested in ELISA for reactivity with other *S. aureus* toxins such as α-hemolysin, exfoliative toxin and TSST-1 as well as SED and SEE. The reaction of Mab-A with these toxins was negative since the OD_{450} readings obtained were identical of those of the controls. There was however, a weak reaction with SEE, but only at higher antibody concentrations which are improper for maximum OD_{450} signal in reaction with SEA. Mab-B antibody was shown to be specific for SEB only.

3.4. Quantitative measurements of SEA and SEB with Mab-A and Mab-B

Several ELISA methods for detection and quantitation of SE using Mabs have been described and many of them have a high sensitivity (Thompson et al., 1984, 1986; Lapeyre et al., 1987; Shinagawa et al., 1991). However, because of the high sequence similarities between SEA, SED and SEE and between SEB and SEC1-3, some Mabs have exhibited cross-reactivity with several SE (Lapeyre et al., 1987; Edwin et al., 1986; Goyache et al., 1992). This is of no consequence when the detection of SE is desired rather than a specific identification. The latter may be obtained only after screening many hybridoma clones or with polyclonal antisera after intensive absorption with undesired SE. The Mabs obtained using A-G4-B peptide as immunogen are highly specific for SEA and SEB and may therefore prove useful for specific identification and quantitation of these enterotoxins. When employed in a double antibody sandwich ELISA, Mab-A and Mab-B were used to quantitate SEA or SEB at nanogram levels (Fig. 3). Applied to food extracts spiked with SEA or SEB, Mab-A and Mab-B detected these enterotoxins at a concentration close to the input SE concentration added to the sample (data not shown). In
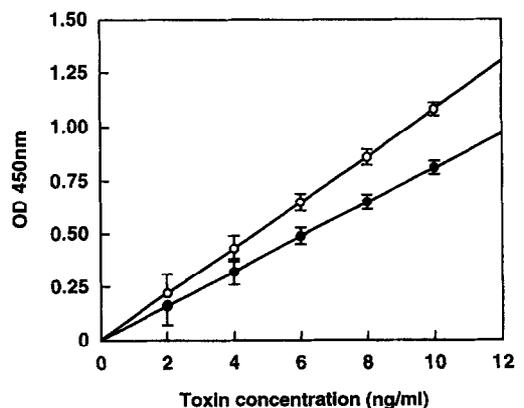
Fig. 3. Quantitative ELISA standard curves for detection of SEA (●-●) or SEB (O-O). Anti-peptide Mab-A and Mab-B were used as capture antibodies at a concentration of 5 μg/ml. Each data point is the mean of five separate experiments and vertical bars indicate±S.D. from the mean.

summary, our results show that synthetic peptides, based on carefully selected epitopes spaced by neutral or weakly hydrophobic amino acid residues, are useful for generating Mabs with different specificities in a single immunization and fusion procedure.

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