Neutralizing epitope mapping of six β_1 -bungarotoxin monoclonal antibodies and its application in β_1 -bungarotoxin peptide vaccine design

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Twenty three stable monoclonal antibodies (mAbs) against β_1 -bungarotoxin (β_1 -bgt) were prepared by the hybridoma technique. Seven of the 23 mAbs (mAbs 2, 6, 8, 11, 17, 21 and 22) could inhibit more than 70 % of phospholipase A₂ activity of β_1 -bgt and neutralize the toxin. Six of these neutralizing mAbs (mAbs 2, 6, 8, 17, 21 and 22) recognized continuous epitopes on the A chain of β_1 -bgt and the other one (mAb 11) recognized a conformational epitope on the toxin. The continuous epitopes of these six mAbs were mapped using synthetic peptide and proteolytic enzymes. Experimental results indicate that mAb 17 recognized the A-chain residues 31–37; mAbs 2 and 8 recognized residues 46–51; mAbs 21 and 22 recognized residues 91–98; and

mAb 6 recognized residue 100–106. The competitive-antibody-binding inhibition experiments showed that the affinity of these neutralizing mAbs to the native β_1 -bgt is compatible with synthetic peptides. Furthermore, mice immunized with BSA-conjugated A-chain-peptide sequences A(31–37), A(46–51), A(91–98) or A(100–106) were protected from a high-dose β_1 -bgt challenge. Subsequently, the peptide-immunized sera were passively injected into Balb/c mice and a significantly protective effect was also observed. To our knowledge, this study is the first systematic demonstration of multiple neutralizing B-cell epitopes of β_1 -bgt, and this study is also the first report of the protective synthetic-peptide vaccine against β_1 -bgt challenge.

INTRODUCTION

β -Bungarotoxin (β -bgt), isolated from the venom of *Bungarus multicinctus* (the Taiwan banded krait) [1–4], is one of the most investigated presynaptic neurotoxins from snake venom. β -Bgt contains covalently linked A and B chains. There are three A-chain isoforms (A-1, A-2 and A-3) and two B-chain isoforms (B-1 and B-2) which combine to form five isotoxins (β_1 – β_5) [3]; a sixth isotoxin has also been purified [5]. Another novel A-chain cDNA has also been cloned by Chang et al. [6]. β_1 -Bgt is the main and the most toxic isoform (with a mouse LD₅₀ of 19 μ g/kg of body weight) in the β -bgt family [2,3]. The toxin consists of two dissimilar polypeptide chains: the A-1 chain with 120 amino acid residues (13.5 kDa) and the B-1 chain with 60 amino acid residues (7 kDa), crosslinked by an interchain disulphide bond [3] and possesses weak phospholipase A₂ (PLA₂) activity [7]. The amino acid sequence of the A chain is homologous with those of PLA₂ enzymes from snake venoms and the mammalian pancreas, whereas the B chain serves as a recognition subunit of the toxin to a specific target cell membrane and blocks the potassium channel [8].

Recently, Kwong et al. [9] proposed the detailed mechanism of β -bgt action from their studies on the crystal structure of β_2 -bgt. The enzymic and pharmacological properties of β_1 -bgt have been reported and its structure–function relationship has also been studied [3,10]. However, only two studies on the immunological properties of β_1 -bgt have been described. Strong et al. [11] and Danse and Kempf [12] used modified β -bgt to immunize animals, however, these modifications could not reduce the toxin's toxicity and enzymatic activity.

In this study, seven β_1 -bgt-neutralizing monoclonal antibodies (mAbs) were prepared and the protective effect on the toxin *in vivo* was investigated. By using synthetic peptides and proteolysis of β_1 -bgt, we were able to locate the neutralizing mAbs contact sites on the β_1 -bgt molecule. Furthermore, according to the

epitope-mapping results, four peptide fragments that could potentially protect mice from a high dose of toxin challenge have proved to be candidates for a synthetic peptide vaccine.

EXPERIMENTAL

Materials

β -Bgt and its isotoxins from *B. multicinctus* venom were isolated as previously described [13,14], and further purified by reversed-phase HPLC on a SynChropak RP-P column (4.6 mm $\times$ 25 cm; SynChrom, Lafayette, IN, U.S.A.). The purity was verified by PAGE.

Dithiothreitol, SDS, BSA, 2,6,10,14-tetramethylpentadecane (pristane), Dulbecco's modified Eagle's medium and acid protease A were purchased from the Sigma Chemical Co., St Louis, MO, U.S.A. Nitro Blue Tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate and anti-mouse IgG (heavy and light) alkaline phosphatase conjugated were obtained from Promega, Madison, WI, U.S.A. Hypoxanthine/aminopterin/thymidine (HAT) and hypoxanthine/thymidine (HT) selection media, polyethyleneglycol (PEG) 4000 and peroxidase-conjugated goat anti-mouse IgG (H + L) were purchased from Boehringer Mannheim GmbH, Mannheim, Germany. Keyhole-limpet haemocyanin (KLH), *m*-maleimidobenzoyl-*N*-hydroxysulphosuccinimide ester (sulfo-MBS), glutaraldehyde and micro BCA protein assay kit were purchased from Pierce, Rockford, IL, U.S.A. Immuno-precipitin (formalin-fixed *Staphylococcus aureus* cells) was purchased from Gibco BRL, Gaithersburg, MD, U.S.A. Thermolysin and pepsin were purchased from Worthington Biochemical Corporation, NJ, U.S.A. All other reagents were of analytical grade.

Preparation of β_1 -bgt mAbs

mAbs were produced essentially according to the method described by Köhler and Milstein [15] with some modification.

Abbreviations used: β_1 -bgt, β_1 -bungarotoxin; KLH, keyhole-limpet haemocyanin; mAb, monoclonal antibody; PLA₂, phospholipase A₂; HAT, hypoxanthine/aminopterin/thymidine; HT, hypoxanthine/thymidine; PEG, polyethyleneglycol; sulfo-MBS, *m*-maleimidobenzoyl-*N*-hydroxysulphosuccinimide ester.

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Balb/c mice were immunized with increasing doses of β_1 -bgt (0.1–15 $\mu\text{g}/\text{mouse}$) over a period of 10 weeks. An additional boost (20 $\mu\text{g}/\text{mouse}$) was injected 3 days before fusion. Spleen cells (3×10^7) were fused with myeloma cells NS-1 (1×10^7) using PEG 4000. After removal of PEG 4000, cells were suspended in a HAT selection medium and cultured with HAT and HT selection media until myeloma cells disappeared. Hybrid-containing wells were tested by an enzyme immunoassay and positive clones were subcloned by limiting dilution. Hybridoma cells (2.5×10^6) producing mAbs were injected intraperitoneally into pristane-primed Balb/c mice. Ascites fluids were withdrawn and the mAbs were precipitated by 50 % saturated $(\text{NH}_4)_2\text{SO}_4$ and purified through a column of high-performance Protein G (10 mm $\times$ 10 cm). The purity of the mAbs was verified by SDS/PAGE and their subclasses were determined by enzyme immunoassay using the Mouse MonoAb-ID Kit (Zymed Laboratories Inc., CA, U.S.A.).

Dot blotting of denatured β_1 -bgt

Dot blotting of denatured β_1 -bgt was performed, essentially according to the method described by Hawkes [16] with some modifications. Denatured β_1 -bgt (100 $\mu\text{g}/\text{ml}$ in 0.1 M phosphate buffer (pH 7.6) containing 6 M guanidine hydrochloride) on a 1 cm² Immobilon-PPVDF membrane (Millipore Corp., Bedford, MA, U.S.A.) were incubated with a primary antibody solution ($\times 1000$) containing 5 % non-fat milk at 37 °C for 2 h. The filter was washed three times each in PBS/Tween 20 and PBS, and incubated with a secondary antibody solution, alkaline phosphatase-conjugated goat anti-mouse IgG ($\times 5000$) containing 5 % non-fat milk, at 37 °C for 2 h. After rinsing the filter in a substrate buffer (100 mM Tris/100 mM NaCl/5 mM MgCl_2 , pH 9.5), one drop of substrate solution containing 6.6 μl of Nitro Blue Tetrazolium and 3.3 μl of 5-bromo-4-chloro-3-indolyl phosphate were added. The blot was developed at room temperature with agitation until the band became purple, and was then rinsed with PBS containing 20 mM EDTA to stop the reaction.

Hydrolysis of the A chain of β_1 -bgt with proteolytic enzymes

Reduced and S-carboxymethylated A chain of β_1 -bgt (1 mg) was dissolved in: (i) 300 μl of 50 mM ammonium bicarbonate buffer at pH 7.8, and thermolysin was added to give a protein:enzyme ratio of 50:1 (w/w); (ii) 250 μl of 5 % acetic acid (pH 2.0), and acid protease was added (in a ratio of 30:1, w/w); and (iii) 250 μl of 10 mM HCl (pH 2.0) and pepsin was added (in a ratio of 50:1, w/w). Digestion was performed at 37 °C for 3 h and the hydrolysate was filtered through a 0.22 μm filter and lyophilized. The lyophilized hydrolysates were separated on a SynChropak RP-18 column (4.6 mm $\times$ 25 cm) equilibrated with 0.1 % trifluoroacetic acid and eluted by a linear gradient of 0–40 % acetonitrile for 40 min. Peptide fragments were collected and lyophilized for ELISA [17] and amino acid analysis.

Peptide conjugation to carrier protein

All of the synthetic peptides were synthesized from Chiron, Victoria, Australia. The molecular mass of synthetic peptides was confirmed by electrospray-ionization MS; all of them were of purity exceeding 85 %. Glutaraldehyde coupling [18] and sulfo-MBS coupling [19] were used for conjugating synthetic peptide to carrier protein. For synthetic peptides bound covalently via amine groups to the carrier protein, 0.75 mg of peptide in 100 mM sodium phosphate buffer (pH 8.0) was mixed with 0.75 mg of carrier protein, and made up to 100 μl with buffer. Subsequently, 2 μl of 25 % glutaraldehyde was added to the

reaction mixture and left for 20 min at room temperature. Then, 2 μl of 15 % glutaraldehyde was added and left for another 15 min. Finally, 20 μl of 1 M glycine buffer (pH 6.0) was added to quench the glutaraldehyde. The conjugate for immunization was dialysed against PBS to remove free peptide. For synthetic peptides bound covalently via sulphhydryl groups to the carrier protein, 0.1 mg of sulfo-MBS was mixed with 1 mg of BSA in 150 μl of conjugation buffer (0.083 M sodium phosphate/0.9 M NaCl/0.1 M EDTA, pH 7.2) and incubated at room temperature for 1 h. After dialysing the mixture with conjugation buffer, 1 mg of synthetic peptide was added to the mixture. The mixture was incubated at room temperature for another 2 h and the conjugate was dialysed against PBS to remove free peptide.

Immunization of mice with BSA-peptide conjugate

Each BSA-peptide conjugate (100 μg) was emulsified in Freund's complete adjuvant and injected intramuscularly into a Balb/c mouse. Subsequently, five doses of booster injection with 1 mg of BSA-peptide conjugate emulsified in Freund's incomplete adjuvant were administered at weekly intervals. The titre of anti-peptide antibody in serum was determined by ELISA that was coated with 100 μl of KLH-peptide conjugate (1 $\mu\text{g}/\text{ml}$).

Affinity chromatography of peptide-specific antibody preparation on KLH-peptide-Sepharose

Covalent coupling of KLH-peptide to CNBr-activated Sepharose 4B was carried out according to the method described by Yang et al. [20]. An aliquot of the KLH-peptide-Sepharose was poured into a column (10 mm $\times$ 10 cm) and equilibrated with PBS. The $(\text{NH}_4)_2\text{SO}_4$ -purified antibody solutions against BSA-peptide were applied by passage through the column of KLH-peptide-Sepharose. The column was washed with 100 ml of PBS and 80 ml of 0.1 M acetate buffer/0.5 M NaCl (pH 4.0). The peptide-specific antibodies were then eluted with 0.53 M formic acid/0.15 M NaCl (pH 2.05); the effluent was neutralized immediately with 1.0 M glycine/NaOH buffer (pH 12.0). The antibody preparations were dialysed against PBS and concentrated in a visking tube under a vacuum.

Other tests

Determinations of PLA_2 activity, lethal toxicity, PAGE, amino acid analysis and sequence determination, and immunoblot analysis were performed in essentially the same manner as previously described [21,22].

RESULTS

Characterization of β_1 -bgt mAbs

Twenty three stable hybridoma lines were generated in an experiment in which 960 wells were plated, of which 455 contained colonies and 51 showed significant β_1 -bgt-binding activity. mAbs produced by 23 stable hybridoma cell lines were incubated with subclass-specific goat anti-mouse Ab in an ELISA plate for isotyping. Nineteen out of the 23 mAbs were sub-isotyped as IgG2b, while the others were IgG1. The specificity of these hybridoma lines was also tested by ELISA and immunoprecipitation against β_1 -bgt; the results indicated that these mAbs could specifically bind to β_1 -bgt. Affinity constants of these mAbs were measured by the solid-phase ELISA titration method and ranged from 10^6 – 10^{10} l/mol (results not shown).

Inhibition of enzymic activity was performed *in vitro* with the same molar ratio of antibody that was preincubated with 3 μg of β_1 -bgt in 100 μl of PBS at 37 °C for 3 h. A non-specific mouse

Table 1 Characteristics of seven β_1 -bgt-neutralizing mAbs

Clone number	Subclasses (heavy chain)	KA* (l/nmol)	% Inhibition of PLA ₂ activity†	Neutralizing capacity‡ (LD ₅₀ /mg mAb)	Dot blotting§
2	IgG1	0.345 ± 0.016	79 ± 9	729 ± 90	+
6	IgG1	1.010 ± 0.036	78 ± 0	292 ± 42	+
8	IgG2b	8.309 ± 0.542	87 ± 1	987 ± 65	+
11	IgG2b	0.160 ± 0.037	76 ± 9	244 ± 8	—
17	IgG2b	0.073 ± 0.010	73 ± 9	183 ± 27	+
21	IgG2b	0.150 ± 0.025	79 ± 12	186 ± 25	+
22	IgG2b	0.455 ± 0.054	76 ± 10	208 ± 41	+

* Affinity was determined according to the method described by Friguet et al. [28].

† Inhibition of enzymic activity was performed *in vitro* with the same molar ratio of antibody that was preincubated with 3 μ g of β_1 -bgt in 100 μ l of PBS at 37 °C for 3 h. A non-specific mouse anti-human IgG1 was used as a control.

‡ The LD₅₀ and the neutralizing capacity were calculated according to the method described by Yang and King [21].

§ The + and — signs mean the positive and negative results in dot blotting, respectively. The positive signal indicates that the epitope of corresponding mAb is the continuous form. In contrast, the negative signal indicates the mAb corresponding epitope is conformation-dependent.

Figure 1 Immunoblotting analysis of the A and B chains of β_1 -bgt recognized by β_1 -bgt mAbs

Lanes 1 and 2, SDS-PAGE of intact β_1 -bgt and β_1 -bgt treated with β -mercaptoethanol, respectively. Six odd lanes (3–13), and six even lanes (4–14), are intact β_1 -bgt and β_1 -bgt treated with β -mercaptoethanol, respectively. They were resolved by SDS/PAGE, transferred to a PVDF membrane and probed with mAbs 2, 6, 8, 17, 21 and 22, respectively.

anti-human IgG1 was used as a control. Seven out of 23 mAbs (mAbs 2, 6, 8, 11, 17, 21 and 22) could inhibit more than 70 % of the PLA₂ activity of β_1 -bgt (Table 1). The potential toxic neutralizing activity of these 23 mAbs was tested on mice with various amounts of antibodies which had been preincubated with three times the LD₅₀ dose of β_1 -bgt (the LD₅₀ is 19 μ g/kg of body weight). As shown on Table 1, mAbs 2, 6, 8, 11, 17, 21 and 22 could neutralize toxicity of β_1 -bgt *in vivo*, the neutralizing capacities of mAb 2 and mAb 8 were particularly high.

β_1 -Bgt was denatured by guanidium hydrochloride and immobilized on PVDF membrane, then hybridized with the seven neutralizing mAbs for dot blotting. Six of these mAbs (mAbs 2, 6, 8, 17, 21 and 22) revealing a positive signal on dot blotting implies that they recognize continuous epitopes, whereas mAb 11 recognizing only the non-reduced antigen implies that the mAb 11 recognized the conformational epitope (Table 1).

Immunoblotting analysis of the neutralizing mAbs revealed that all the six mAbs bound to the A chain (13.5 kDa) as well as to the intact β_1 -bgt (20.5 kDa) (Figure 1). No evidence suggested that any blot corresponded to the B chain (7 kDa). The control experiments using Amido-Schwarz staining demonstrated that both A and B chains were equally transferred to the PVDF membrane under the blotting conditions used.

Epitope mapping of β_1 -bgt with synthetic peptides and proteolysis methods

Continuous epitopes for six neutralizing mAbs binding to β_1 -bgt were studied by using synthetic peptides and proteolysis methods. Selecting the possible antigenic peptide epitopes contained in the A

Figure 2 Hydropathy (A), antigenicity (B) and flexibility (C) profiles calculated from the amino acid sequence of A chain of β_1 -bgt

Units of measurement: (A) hydrophilicity value (kcal/mol); (B) antigenicity value ($\log_{10}$ relative occurrence), derived from percentages of amino acids; and (C) B value (temperature factor), calculated from the 31 proteins of known three-dimensional structure and normalized by dividing by the mean B value of the α -carbon atoms of the proteins [25].

chain of β_1 -bgt was based on the hydrophilicity, antigenic index and flexibility profiles (Figure 2). Synthetic peptide sequences corresponding to A(44–64), A(61–73), A(91–98) and A(106–118)

(Table 2) showed average hydrophilicity values of 0.7, 0.5, 0.5 and 0.5, respectively, average antigenic values of 0.05, 0.04, -0.01 and -0.02, respectively, and average flexibility values of 0.99, 1.00, 0.98 and 0.99, respectively.

The toxicity-neutralizing mAbs were incubated with these four BSA-conjugated synthetic peptides and their binding abilities were detected. Experimental results indicated that mAbs 2 and 8 reacted with peptide containing amino acid sequence 44–64. mAbs 21 and 22 reacted with peptide A(91–98). The other two neutralizing mAbs 6 and 17 did not react with any of the four synthetic peptides. For the sake of fine-mapping the epitopes of mAbs 2 and 8, another four peptides (Table 2; A(46–51), A(46–53), A(52–61) and A(54–61) were synthesized. By direct ELISA test, mAbs 2 and 8 reacted with A(44–64), A(46–51) and A(46–53) peptides, indicating that both mAbs 2 and 8 recognized the amino acid sequence 46–51 of the A chain. The direct ELISA also revealed that the binding ability of these β_1 -bgt mAbs to BSA–peptide conjugates was in a dose-dependent manner (Figure 3).

To determine the continuous epitopes of neutralizing mAbs 6 and 17, thermolysin, acid protease A and pepsin were sequentially used to hydrolyse the A chain of β_1 -bgt and the peptide fragments were separated by reversed-phase HPLC on a column of SynChropak RP-P (Figure 4). The peptide fragments were conjugated with BSA by glutaraldehyde and the immuno-reactivity of mAbs against these fragments was determined by direct ELISA. According to Figure 4, fragments T-1, A-1 and A-2 reacted with mAb 17. On the other hand, fragments P-1, P-2 and P-3 reacted with mAb 6. Table 3 lists the amino acid compositions of these proteolytic fragments and their location on the amino acid sequence of the A chain of β_1 -bgt is shown in Figure 5(a). According to these results, the epitopes of mAbs 6 and 17 were within the sequences 100–106 and 31–37, respectively.

All of the continuous epitopes of neutralizing mAbs have been determined by synthetic peptides or by the proteolysis method. The competitive antibody-binding inhibition experiments showed that the binding activity of these neutralizing mAbs to native β_1 -bgt is compatible with the corresponding synthetic peptides (Figure 6), suggesting that synthetic peptides can form structures comparable with the neutralizing epitopes on β_1 -bgt. Figure 5(b) summarizes the mapped neutralizing B-cell epitopes.

Protective capacity of peptide-immunized mice

Peptides A(31–37), A(46–51), A(91–98) and A(100–106) containing the neutralizing epitopes of the toxin identified with β_1 -bgt mAbs were synthesized and coupled to the carrier protein (BSA). They were injected intramuscularly into Balb/c mice with Freund's complete adjuvant and then with Freund's incomplete adjuvant. The peptide-specific antibodies in the sera of mice were detected by ELISA at weekly intervals. As Figure 7 reveals, the titre of specific antibodies went up to a maximum about 4 weeks after the first immunization.

Table 4 compares the survival ratios of peptide-immunized mice that were challenged with $10 \times LD_{50}$ or $5 \times LD_{50}$ of β_1 -bgt. These results indicate that all of these synthetic peptides, A(31–37), A(46–51), A(91–98) and A(100–106), had the capacity to protect mice from β_1 -bgt challenge, yet mice immunized with synthetic peptides A(46–51) and A(100–106) were more protected than those with A(31–37) and A(91–98). IgG and peptide-specific antibody purified from immunized sera also preserved the capacity to inhibit PLA₂ activity and toxicity of β_1 -bgt (Table 4). In contrast, the scramble peptide (MVRVFPNVYVYSNIYIY-PNIGIAF) or BSA-control-immunized mice had undetectable protective activity.

Table 2 Synthetic peptides for neutralizing epitope mapping*

Peptide	Sequence
A(44–64)	CCYVHDNICYGDAEKKHKCNPK
A(61–73)	CNPKTQSYSYKLT
A(91–98)	CDCDRTA
A(106–118)	Cys†-YIEGHKNIDTARF
A(46–51)	YVHDNC
A(46–53)	YVHDNICYG
A(52–61)	YGDAEKKHKC
A(54–61)	DAEKKHKC

* Synthesis of peptides was based on hydrophilicity, antigenic index and flexibility profiles.
† To conjugate the peptide to the carrier protein (BSA), the cysteine residue was added to the N-terminal end of the synthetic peptide to offer a free thiol group to the conjugating linker (sulfo-MBS).

Figure 3 Direct ELISA displaying the binding ability of β_1 -bgt mAbs to BSA–peptide conjugates

ELISA was performed essentially according to the method described by Tanaka et al. [17]. 100 μ l of BSA or BSA–peptide conjugates (1 μ g/ml) were coated on to the ELISA plate and different concentrations of β_1 -bgt mAb were added. mAb-2 was incubated with BSA–A(46–51) ($\square$) or BSA ($\blacksquare$); mAb-8 with BSA–A(46–51) (∇) or BSA ($\blacktriangledown$); mAb-21 with BSA–A(91–98) ($\circ$) or BSA ($\bullet$); and mAb-22 with BSA–A(91–98) ($\triangle$) or BSA ($\blacktriangle$).

Reduction of mortality by passively transferred anti-peptide sera or peptide-specific antibodies

To test whether anti-peptide-immunized sera could produce protective immunity against β_1 -bgt in mice, a passive transfer experiment was performed. Donor mice were immunized with BSA-conjugated peptides A(31–37), A(46–51), A(91–98), A(100–106) and scramble peptide. Sera were collected 3 days after the last booster injection and pooled. Control serum was obtained from mice immunized with BSA alone. The undiluted pooled sera (0.5 ml), peptide-specific antibodies (0.25, 0.5 or

Figure 4 Separation of the peptide fragments of the A chain of β_1 -bgt after hydrolysis with thermolysin (A), acid protease A (B) and pepsin (C)

0.1 mg of proteolytic hydrolysate was applied to a SynChropak RP-P column (4.6 mm $\times$ 25 cm) equilibrated with 0.1% trifluoroacetic acid and eluted with a linear gradient of 0–40% acetonitrile for 40 min. The flow rate was 0.8 ml/min and the effluent was monitored at 235 nm.

Table 3 Amino acid composition of peptides obtained from digestion of A chain of β_1 -bgt with thermolysin, acid protease A and pepsin

Numbers in brackets denote the calculated number of amino acid residues in peptide.

Amino acid	Thermolysin	Acid protease A		Pepsin		
	T-1	A-1	A-2	P-1	P-2	P-3
Lys				1.0(1)		
His				1.3(1)		
Arg	0.8(1)	0.8(1)	1.1(1)	1.3(1)	1.7(2)	2.1(2)
Asp	1.7(2)	2.0(2)	3.1(3)	2.6(3)	2.9(3)	
Thr				1.0(1)	2.0(2)	1.8(2)
Ser	1.1(1)	1.1(1)	0.9(1)	1.1(1)	1.1(1)	1.0(1)
Glu				1.6(2)	1.8(2)	1.1(1)
Pro	1.1(1)	1.1(1)	0.7(1)			
Gly	5.1(5)	3.8(4)	3.2(3)	2.2(2)	3.4(3)	3.5(3)
Ala	1.8(2)	0.8(1)	2.1(2)	0.9(1)	4.7(5)	5.0(5)
CM-Cys*	1.7(2)	0.7(1)		1.0(1)	4.1(4)	4.2(4)
Val				0.9(1)	1.4(1)	
Ile	0.8(1)		1.0(1)	2.0(2)	2.0(2)	1.3(1)
Leu			1.1(1)	1.0(1)	1.1(1)	
Tyr	1.8(2)			1.1(1)	1.1(1)	1.0(1)
Phe				2.1(2)	0.9(1)	1.0(1)
Total	15.9(17)	8.3(9)	12.1(12)	19.8(19)	27.3(28)	27.4(26)
Corresponding peptide on the sequence	23–39	29–37	31–42	100–118	81–108	81–106

* Cysteine was determined as carboxymethylcysteine.

1 mg) or immunoglobulin-free serum were injected intravenously into male Balb/c mice. After 12 h, the recipient mice were challenged with $5 \times LD_{50}$ of β_1 -bgt. According to these results, all of the peptide-immunized sera had the ability to protect passively transferred mice from toxin challenge to varying degrees (Table 5). Furthermore, mice injected with peptide-specific antibody purified from anti-peptide sera had a distinct protective effect against toxin challenge in a dose-dependent manner. In contrast, all of the immunoglobulin-free sera fractions had no protective effect.

Figure 5 Location of the peptide fragments T-1, A-1, A-2, P-1, P-2 and P-3 (a) and the possible epitopes of six linear neutralizing mAbs (b) on the amino acid sequence of the A chain of β_1 -bgt

DISCUSSION

Both polyclonal and monoclonal antibodies are considered highly effective tools in examining protein structure and function. Therefore, in this study, β_1 -bgt mAbs are prepared for the study of the structure and function of β_1 -bgt and related toxins.

Figure 6 Inhibition of β_1 -bgt binding to mAbs 2, 6, 8, 17, 21 and 22 by peptide competitors

The wells were coated with 100 μ l of β_1 -bgt (1.2 μ M). A/A_0 is the determined ratio (%) of the absorbance at 450 nm in the presence (A) or absence (A_0) of a competitor. 10 μ g/ml mAbs were used and the synthetic peptide competitors were 0.01–100 mM. The binding of mAbs 2, 6, 8, 17, 21 and 22 to solid-phase β_1 -bgt was efficiently inhibited by synthetic peptide competitors A(46–51) ($\square$), A(100–106) (∇), A(46–51) ($\circ$), A(31–37) ($\triangle$), A(91–98) ($\blacksquare$) and A(91–98) ($\blacktriangledown$).

Figure 7 Titre of the anti-peptide antibodies during immunization period

The ELISA plate was coated with corresponding KLH-peptide conjugates [KLH-A(31–37); KLH-A(46–51); KLH-A(91–98); KLH-A(100–106) and KLH-scramble peptide] and detected by goat anti-mouse IgG (H + L). The titre of anti-peptide A(31–37) antibody in the sera was detected by KLH-A(31–37) ($\square$) or KLH ($\blacksquare$); the titre of anti-peptide A(46–51) antibody by KLH-A(46–51) ($\diamond$) or KLH ($\blacklozenge$); the titre of anti-peptide A(91–98) antibody by KLH-A(91–98) ($\circ$) or KLH ($\bullet$); the titre of anti-peptide A(100–106) antibody by KLH-A(100–106) ($\triangle$) or KLH ($\blacktriangle$) and the titre of anti-scramble peptide antibody by KLH-scramble peptide (∇) or KLH ($\blacktriangledown$).

Immunization of animals is critical in preparing mAbs, particularly for the low-LD₅₀ toxin, β_1 -bgt, because a high dose of toxin antigen may kill the animal and a low dose of antigen may induce a weak immune response. To prevent these problems, animals were immunized by a low dose of toxin with a high immunization number. After 73 days immunization, all Balb/c mice reached a highly immunized state and the titre of β_1 -bgt Ab in blood was high.

By means of proteolytic hydrolysis and synthetic peptide methods, six continuous neutralizing epitopes have been mapped on to four distinct regions in a sequence of the toxin. The shortest epitopes appear to be six amino acid residues and the longest eight. The hydropathy plot analysis [23] (Figure 2A) revealed that the epitopes for mAbs 17, 21 and 22 are located in hydrophilic regions, epitopes for mAbs 2 and 8 are in an intermediate region, but the epitope of mAb 6 is located in a hydrophobic region. On the other hand, the antigenic index profile [24] (Figure 2B) predicted that the epitopes of mAbs 21 and 22 are high in antigenicity, epitopes of mAbs 2 and 8 are intermediate, and epitopes of mAbs 6 and 17 are low in antigenicity. The chain flexibility profiles [25] (Figure 2C) revealed that the epitopes of mAbs 2, 8, 17, 21 and 22 are located on intermediate flexibility regions; however, the epitope of mAb 6 is located on a low flexibility region. From these predictions, the epitope of mAb 6 is located in a region predicated to be hydrophobic, rigid and inaccessible, indicating that the region is unfavourable for antigenicity. The fact that the antibody binding was demonstrated *in vitro* with native protein (in ELISA) clearly indicates that this epitope could not be masked in a protein molecule.

The epitope of mAbs 2 and 8 was located at the sequence 46–51 of the A chain of β_1 -bgt, which contained two essential catalytic residues, His-48 and Asp-49. From a structural perspective, His-48 and Asp-49 are responsible for catalytic activity and constructing the catalytic network; thus, blocking His-48 and Asp-49 may potentially inhibit enzymic activity and toxicity. From the standpoint of affinity constants, the affinity of mAb 8 is higher than that of mAb 2; this may possibly account for the inhibition of enzymic activity and toxicity by mAb 8 being stronger than mAb 2. The experimental results apparently suggest that these two mAbs inhibit the biological activity of β_1 -bgt, which appears to be consistent with the previous reports [9,26]. Asp-92 is also an essential catalytic residue located within the epitope of mAbs 21 and 22; therefore, the biological activities of β_1 -bgt might be blocked by these two mAbs. According to bovine pancreatic-PLA₂ crystal structure, the carbonyl oxygens of Tyr-28, Gly-30 and Gly-32, and the carboxylate oxygens of Asp-49 and two water molecules form an oxygen cage that sequesters a Ca²⁺ ion [27]. From the above observation, we can infer that mAb 17 can block Gly-32, and inhibit the PLA₂ activity of β_1 -bgt.

Most neutralizing B-cell epitopes are conformational rather than continuous, even in the case of continuous neutralizing epitopes, and the conformation of synthetic peptides does not consistently maintain a structure identical to the linear epitopes on native β_1 -bgt protein, thereby limiting the development of an efficient synthetic-peptide vaccine. Therefore, it is important when designing the peptide vaccine to analyze the conformational or continuous neutralizing epitopes and to study the identity of neutralizing epitopes on the synthetic peptide with reference to those on native β_1 -bgt. Our results indicated that the neutralizing mAbs (mAbs 2, 6, 8, 17, 21 and 22) reacted with their corresponding synthetic-epitope sequences, and the binding of these neutralizing mAbs to β_1 -bgt was efficiently inhibited by the corresponding synthetic peptides. This finding suggests that these synthetic peptides can form conformations comparable to

Table 4 Survival ratio of peptide-immunized mice

Peptide immunized*	Survival ratio with challenge of β_1 -bgt†		IgG and peptide-specific Ab					
			Concentration (mg/ml)		% Inhibition of PLA ₂ activity		Neutralizing capacity (LD ₅₀ /mg Ab)	
	10 × LD ₅₀	5 × LD ₅₀	IgG	Specific Ab	IgG	Specific Ab	IgG	Specific Ab
A(31–37)	16%	44%	2.02	0.63	23 ± 4	48 ± 7	41 ± 7	121 ± 16
A(46–51)	48%	96%	2.26	0.69	26 ± 6	57 ± 4	158 ± 9	470 ± 45
A(91–98)	20%	64%	1.98	0.54	21 ± 8	48 ± 7	52 ± 8	123 ± 22
A(100–106)	32%	76%	2.12	0.59	20 ± 3	47 ± 3	88 ± 8	227 ± 11
Scramble peptide‡	0%	0%	1.17	0.70	1 ± 1	0 ± 0	ND§	ND
BSA control	0%	0%	1.33	—	0 ± 0	—	ND	—

* Peptides were conjugated with BSA by glutaraldehyde.

† For each survival ratio experiment, 25 mice were used.

‡ The sequence of the scramble peptide was MVRVFPNVYYSNIYIPNIGIAF.

§ ND, not detectable (neutralizing capacity was lower than 10).

Table 5 Survival ratios of native mice that were passively transferred with anti-peptide sera or peptide-specific Ab*

	Inject 0.5 ml of antisera	Inject immunoglobulin-free serum fractions	Inject various amounts of peptide-specific Abs		
			0.25 mg	0.5 mg	1 mg
A(31–37)	8%	0%	28%	64%	100%
A(46–51)	24%	0%	60%	100%	—
A(91–98)	12%	0%	32%	76%	100%
A(100–106)	20%	0%	40%	92%	100%
Scramble peptide	0%	0%	0%	0%	0%
BSA control	0%	0%	—	—	—

* Challenge with 5 × LD₅₀ of β_1 -bgt after passive transfer of antisera, peptide-specific Ab or immunoglobulin-free serum fractions. For each survival ratio experiment, 25 mice were used.

the neutralizing epitopes on the native β_1 -bgt and become potential candidates for peptide vaccine.

In this study, peptides A(31–37), A(46–51), A(91–98) and A(100–106) induced antisera which not only revealed the protective capacity in experiments *in vivo*, but also displayed the neutralizing of PLA₂ activity in experiments *in vitro* (Table 4). However, passive protection experiments (Table 5) with anti-A(31–37), A(46–51), A(91–98) and A(100–106) antisera indicate that antibody purified from immune sera proved to be a major component for protecting mice from toxin challenge. These combined results demonstrated that the protection induced by the peptide is antibody-mediated. On the other hand, T-cell responses also play an essential role in B-cell proliferation and activation. The T-cell epitopes in this vaccination experiment were not localized in the synthetic peptides but within the carrier protein.

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