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Immunochemical Studies on β_1-Bungarotoxin

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CONTENTS

ABSTRACT

Chen-Chung Yang and Hong-Lin Chan (2000) Immunochemical studies on β_1-bungarotoxin. *Zoological Studies* **39**(2): 79-90. β_1-Bungarotoxin (β_1-Bgt) is the main and the most toxic isoform in the β-Bgt family. The toxin consists of 2 dissimilar polypeptide chains: the A chain with 120 amino acid residues and the B chain with 60 amino acid residues, cross-linked by an interchain disulfide bond and possessing weak phospholipase A_2 (PLA_2) activity. The amino acid sequence of the A chain is homologous with those of PLA_2 enzymes from snake venom and the mammalian pancreas, while the B chain serves as a recognition subunit of the toxin towards a specific target presynaptic membrane and blocks the voltage-gated potassium channel.

The numbers of antigenic determinants on β_1-Bgt, A chain, and B chain were determined to be 7, 5, and 2, respectively, by the quantitative precipitin reactions and analysis of the molecular weight of the soluble complex formed from β_1-Bgt and Fab fragments of the purified antibody. Twenty-three stable monoclonal antibodies (mAbs) were prepared against β_1-Bgt, seven of which could inhibit more than 70% of PLA_2 activity of β_1-Bgt and could neutralize the toxicity of the toxin. Continuous epitopes of β_1-Bgt were mapped, and the result indicates that the A chain sequences 31-37, 46-51, 91-98, and 100-106 are the neutralizing epitopes of β_1-Bgt. A hybrid hybridoma that produces bispecific mAb, which recognizes 2 different epitopes on the A chain of β_1-Bgt at peptide sequences 46-51 and 100-106, has been obtained by fusing 2 hybridoma cell lines. The combination of bispecific mAb, with its 2 corresponding epitopes of β_1-Bgt, not only facilitates immuno-complex formation and enhances avidity, but also highly neutralizes the biological activity of β_1-Bgt. 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.

Key words: Bungarotoxin, Presynaptic neurotoxin, Monoclonal antibody, Hybrid hybridoma, Bispecific monoclonal antibody, Peptide vaccine.

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INTRODUCTION

β-Bungarotoxin (β-Bgt), isolated from the venom of *Bungarus multicinctus* (the Taiwan banded krait, Fig. 1) (Lee et al. 1972, Abe et al. 1977, Kondo et al. 1978 1982), is one of the most investigated presynaptic neurotoxins from snake venom. It contains covalently linked A and B chains. There are 3 A chain isoforms (A-1, A-2, and A-3) and 2 B chain isoforms (B-1 and B-2) which combine to form 5 isotoxins (β_1-β_5) (Kondo et al. 1978), and a 6th isotoxin has been purified (Chu et al. 1994). Another novel A chain cDNA has also been cloned and its mutagenesis was studied by Chang et al. (1996a, b). In addition, 2 B chain cDNAs were also cloned, and the recombinant B chains were functionally expressed in *Escherichia coli* (Wu et al. 1998).

β_1-Bgt is the main and most toxic isoform (with a mouse LD_{50} of 19 μg/kg body weight) in the β-Bgt family (Lee et al. 1972, Kondo et al. 1978). The toxin consists of 2 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), cross-linked by an interchain disulfide bond (Kondo et al. 1978) and possessing weak phospholipase A_2 (PLA_2) activity (Strong et al. 1976). The amino acid sequence of the A chain is homologous with those of the PLA_2 enzymes from snake venom and the mammalian pancreas, while the B chain serves as a recognition subunit of the toxin to a specific target cell membrane and blocks the voltage-gated potassium channel (Benishin 1990).

Kwong et al. (1995) studied the crystal structure of β_2-Bgt and proposed a hypothetical mechanism of β-Bgt action. The toxin diffuses away from the site of venom injection and reaches the presynaptic membrane. The B chain binds to the potassium ion channel and this binding event orient the A chain phospholipase subunit toward the membrane. Once hydrolysis occurs, the substrate binding displaces Trp-19, flipping it into the membrane where it provides a hydrophobic anchor. At the same time, fatty acids are released upon phospholipid hydrolysis, which increases the anionic composition of the membrane, thus enhancing the β-Bgt non-specific electrostatic affinity. Eventually, extensive phospholipid hydrolysis interferes with the release of neurotransmitter resulting in the blockage of neural transmission.

Although the enzymatic and the pharmacological properties of β_1-Bgt have been reported and its structure-function relationship has also been studied (Kondo et al. 1978, Yang and Chang 1984, Yang and Lee 1986, Rosenberg et al. 1989), only a few immunological studies of β_1-Bgt were found. These immunological studies on β_1-Bgt include those of Caratsch et al. (1982) who used polyclonal antibody to study β-Bgt. Strong et al. (1984) who utilized PBP-β-Bgt as an antigen to immunize Balb/c mice, and Danse and Kempf (1989) who immunized Balb/c mice with native β-Bgt. However, all these mAbs or polyclonal antibodies could not potentially inhibit the biological activity of the toxin, and no other structural information was offered.

In the present study, the number of antigenic determinants on β_1-Bgt was determined, and the neutralizing epitopes were mapped on β_1-Bgt. The epitope mapping results were utilized to develop a β_1-Bgt bispecific mAb and design a β_1-Bgt peptide vaccine for protecting animals from β_1-Bgt challenge.

NUMBER OF ANTIGENIC DETERMINANTS ON β_1-Bgt

β_1-Bgt polyclonal antibody was prepared according to the method described by Yang and Chan (1999a), and the immunized mice were bled on the 7th day after final immunization. The antisera were precipitated with 50% saturated ammonium sulfate, and the precipitates were purified through a protein G column. As seen from the typical quantitative precipitin curves (Fig. 2) obtained from anti-β_1-Bgt-purified IgG (immunoglobulin) with different amount of antigens, the molar ratios of IgG to β_1-Bgt, A chain, and B chain in the equivalence zone were 3.40, 2.44, and 0.96, respectively. The valence or the number of antibody-combinding sites on 1 antigen molecule was obtained by doubling the molar ratio of antibody to antigen present in the precipitates formed at

Fig. 1. Taiwan banded krait (*Bungarus multicinctus*).

equivalence (Chang and Yang 1969), therefore, the antigenic determinants per molecule of β_1-Bgt, A chain, and B chain would be 7, 5, and 2, respectively. The gel filtration patterns of soluble complexes formed from Fab fragments of precipitating antibodies with β_1-Bgt, A chain, and B chain also confirmed the evidence that β_1-Bgt contains 7, A chain contains 5 and B chain contains 2 antibody-combining sites per molecule (Fig. 3). The antigenicity profile (Fig. 4) (Welling et al. 1985) indicated that 5 regions (44-52, 55-64, 66-73, 94-98, and 108-114) on the A chain and 2 regions (5-13 and 23-33) on the B chain of β_1-Bgt have high antigenicity. Since the numbers of dominant epitopes on β_1-Bgt, A chain, and B chain from the quantitative precipitin reactions (Fig. 2) and the chromatographic analysis (Fig. 3) are the same as those in the antigenicity profile, it is further concluded that 7, 5, and 2 dominant epitopes are located on β_1-Bgt, A chain, and B chain, respectively.

β_1-Bgt-NEUTRALIZING EPITOPE MAPPING

Determination of the binding site of seven β_1-Bgt-neutralizing mAbs

β_1-Bgt mAbs were prepared according to the method described by Yang and Chan (1998). After 73 days of immunization, all Balb/c mice reached a highly immunized state, and the titer of β_1-Bgt antibody in the blood was found to be high. After hybridoma production and selection, 7 mAbs (mAbs 2, 6, 8, 11, 17, 21, and 22) could inhibit more than 70% of the PLA_2 activity of β_1-Bgt (Table 1). The potential toxin-neutralizing activity of these 7 mAbs was tested

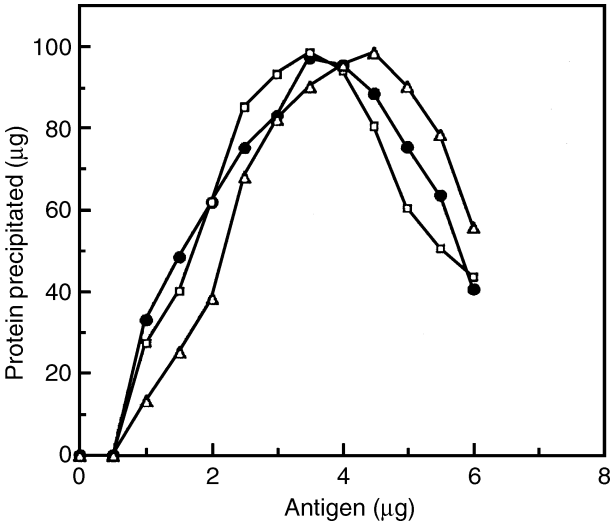


Fig. 2. Quantitative precipitin reactions of β_1-Bgt, A chain, and B chain with β_1-Bgt antibody. Increasing amounts of β_1-Bgt (●), A chain (□), and B chain (Δ) were added to 1 ml of 0.02 M Tris-0.15 M NaCl (pH 7.5) containing 1 mg of antibody (purified from β_1-Bgt antisera by protein G column).

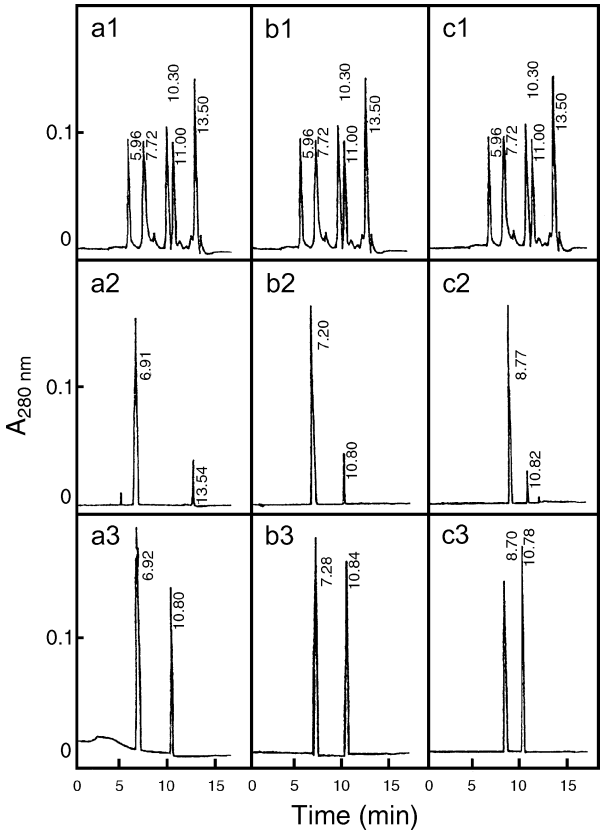


Fig. 3. Gel filtration patterns of soluble complexes formed from Fab fragments of precipitating antibodies with β_1-Bgt, A chain, and B chain on a size-exclusion column. A high-performance size-exclusion chromatographic column was equilibrated and eluted with 0.1% TFA at a flow rate of 1 ml/min, and the effluent was monitored at 280 nm. (a1), (b1) and (c1) show the elution times of the standard proteins: IgM (900 kDa), 5.96 min; IgG (150 kDa), 7.72 min; BSA (67 kDa), 10.30 min; HRP (44 kDa), 11.00 min; and β_1-Bgt (20.5 kDa), 13.50 min. (a2) and (a3) show the soluble complexes formed from Fab fragments and β_1-Bgt at molar ratios 7:1 and 10:1, respectively. The peak emerging at 6.91 min represents the complexes formed from 1 β_1-Bgt and 7 Fab with a molecular weight of 370 kDa, while the peaks eluting at 10.80 and 13.54 min are the Fab fragment (50 kDa) and free β_1-Bgt (20.5 kDa), respectively. (b2) and (b3) show the soluble complexes formed from Fab and A chain at molar ratios of 5:1 and 10:1, respectively. The peak eluting at 7.20 min represents the complexes formed from 1 A chain and 5 Fab at 264 kDa, while the peak at 10.8 min corresponds to a free Fab fragment (50 kDa). (c2) and (c3) show the soluble complexes formed from Fab and B chain at molar ratios of 2:1 and 5:1, respectively. The peak at 8.77 min represents the complexes formed from 1 B chain and 2 Fab at 107 kDa, while the peak at 10.82 min is free Fab (50 kDa).

on mice with various amounts of antibodies which had been incubated with 3 times the LD_{50} dose of β_1-Bgt (the LD_{50} in mice is 19 μg/kg body weight). As shown in table 1, mAbs 2, 6, 8, 11, 17, 21, and 22 could neutralize the toxicity of β_1-Bgt in vivo, with the neutralizing capacities of mAb 2 and mAb 8 found to be particularly high.

β_1-Bgt was denatured by guanidinium hydrochloride and immobilized on a PVDF membrane and then hybridized with the 7 neutralizing mAbs for dot blotting. Six of these mAbs (mAbs 2, 6, 8, 17, 21, and 22) revealed a positive signal on dot blotting indicating that they recognize the continuous epitopes. However, mAb 11 recognized only the non-reduced antigen, implying that mAb 11 recognized the conformational epitope (Table 1). The immunoblotting analysis of the neutralizing mAbs (Fig. 5) revealed that all 6 neutralizing mAbs are bound to the A chain (13.5 kDa) as well as to the intact β_1-Bgt (20.5 kDa), with no evidence indicating that any blot corresponded to the B chain (7 kDa).

Neutralizing epitope mapping of β_1-Bgt with synthetic peptide and proteolytic method

Continuous epitope mapping of the 6 neutralizing mAbs bound to β_1-Bgt was first studied by using the synthetic peptide method. Selecting the possible antigenic peptide epitopes containing the A chain of β_1-Bgt was based on the hydrophilicity, antigenic index, and flexibility profiles (Fig. 4). Synthetic peptide sequences corresponding to A(44-64), A(61-

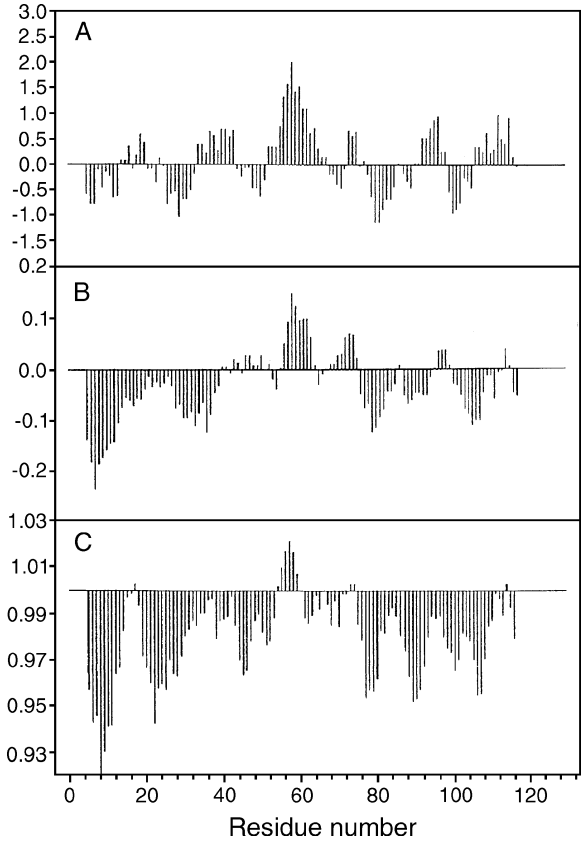


Fig. 4. Hydropathy (A), antigenicity (B), and flexibility (C) profiles calculated from the amino acid sequence of the A chain of β_1-Bgt. Units of measurement: (A) hydrophilicity value (kcal/mol); (B) antigenicity value (log_{10} relative occurrence) as derived from percentages of amino acids; and (C) B value (temperature factor).

Table 1. Characteristics of seven β_1-Bgt-neutralizing mAbs

Clone number	Subclass (heavy chain)	K_A (l/nM)	Inhibition of PLA_2 activity[a] (%)	Neutralizing capacity[b] (LD_{50}/mg mAb)	Dot blotting[c]
2	IgG_1	0.345 ± 0.016	79 ± 9	729 ± 90	+
6	IgG_1	1.010 ± 0.036	78 ± 0	292 ± 42	+
8	IgG_{2b}	8.309 ± 0.542	87 ± 1	987 ± 65	+
11	IgG_{2b}	0.160 ± 0.037	76 ± 9	244 ± 8	–
17	IgG_{2b}	0.073 ± 0.010	73 ± 9	183 ± 27	+
21	IgG_{2b}	0.150 ± 0.025	79 ± 12	186 ± 25	+
22	IgG_{2b}	0.455 ± 0.054	76 ± 10	208 ± 41	+

[a]Inhibition of enzymatic 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 anti-human IgG_1 was used as a control.

[b]The LD_{50} and the neutralizing capacity were calculated according to the method described by Yang and King (1980).

[c]"+" and "–" signs indicate positive and negative results in dot blotting, respectively. A positive signal indicates that the epitope of the corresponding mAb is the continuous form. In contrast, the negative signal indicates that the mAb corresponding to the epitope is conformation dependent.

73), A(91-98), and A(106-118) were prepared and conjugated with BSA. The binding ability indicated that mAb 2 and mAb 8 reacted with peptide containing amino acid sequence 44-64. mAb 21 and mAb 22 reacted with peptide A(91-98). The other 2 neutralizing mAbs, 6 and 17, did not react with any of the 4 synthetic peptides. For the sake of fine-mapping the epitopes of mAb 2 and mAb 8, another 4 peptides A (46-51), A(46-53), A(52-61), and A(54-61) were synthesized. By direct ELISA test, both mAb 2 and mAb 8 reacted with A(44-64), A(46-51), and A(46-53) peptides, indicating that mAbs 2 and 8 recognized the amino acid sequence 46-51 of the A chain. Direct ELISA also revealed that the binding ability of these β_1-Bgt mAbs to BSA-peptide conjugates was in a dose-dependent manner.

In order to determine the continuous epitopes of neutralizing mAb 6 and mAb 17, a sequence of thermolysin, acid protease A (Nakamura and Takahashi 1977), and pepsin were used to hydrolyze the A chain of β_1-Bgt, and the peptide fragments were separated by reverse-phase HPLC on a column of SynChropak RP-P (Fig. 6). The peptide fragments were conjugated with BSA by glutaraldehyde, and the immunoreactivity of mAbs against these fragments was determined by direct ELISA. According to figure 6, fragments T-1, A-1, and A-2 reacted with mAb 17, while fragments P-1, P-2, and P-3 reacted with mAb 6. After analysis of the amino acid compositions of these proteolytic fragments and their location on the amino acid sequence of the A chain of β_1-Bgt, the epitopes of mAbs 6 and 17 were deduced within the sequences 100-106 and 31-37, respectively.

All continuous epitopes of the neutralizing mAbs have been determined by the synthetic peptides and the proteolytic methods. The competitive-antibody-binding inhibition experiments showed that the affinity of these neutralizing mAbs to native β_1-Bgt is compatible with the synthetic peptides (Fig. 7), suggesting that synthetic peptides can form structures comparable with the neutralizing epitopes on β_1-Bgt. Figure 8 summarizes the mapped neutralizing B-cell epitopes and their locations on the 3-dimensional structure of the A chain of β_1-Bgt.

Characterization of the neutralizing epitopes of β_1-Bgt

By means of proteolytic hydrolysis and synthetic peptide method, 6 continuous neutralizing epitopes have been mapped on 4 distinct regions in a sequence of the A chain of β_1-Bgt (Fig. 8). The shortest epitope appear to be 6 amino acid residues and the

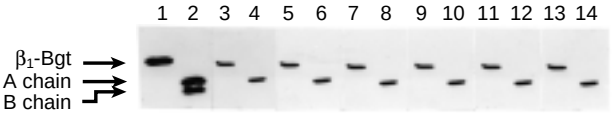


Fig. 5. Immunoblotting analysis of the A and B chains of β_1-Bgt recognized by β_1-Bgt mAbs. Lanes 1 and 2 are SDS-PAGE of intact β_1-Bgt and β_1-Bgt treated with β-mercaptoethanol, respectively. Six odd lanes (3-13), and 6 even lanes (4-14), are intact β_1-Bgt and β_1-Bgt treated with β-mercaptoethanol, respectively. They were resolved by SDS-PAGE, transferred to a PVDF membrane and probed with mAbs 2, 6, 8, 17, 21, and 22, respectively.

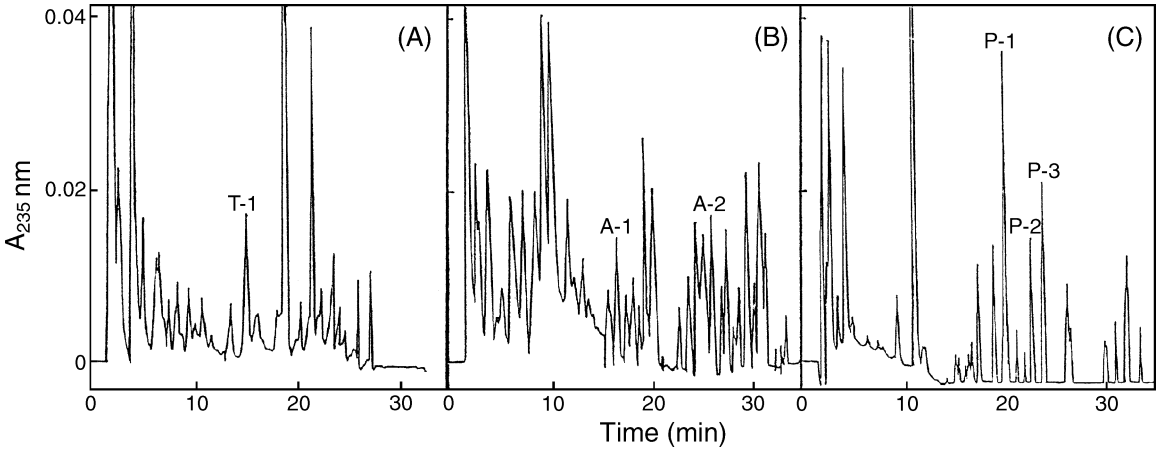


Fig. 6. Separation of the peptide fragments of the A chain of β_1-Bgt after hydrolysis with thermolysin (A), acid protease A (B), and pepsin (C). Proteolytic hydrolysate at 0.1 mg was applied to a SynChropak RP-P column (4.6 mm × 25 cm) equilibrated with 0.1% trifluoroacetic acid and eluted with a linear gradient of 0%-40% acetonitrile for 40 min. The flow rate was 0.8 ml/min, and the effluent was monitored at 235 nm.

longest 8. The hydropathy plot analysis revealed that the epitopes for mAbs 17, 21, and 22 are located in hydrophilic regions, and epitopes for mAb 2 and mAb 8 are in an intermediate region; however, the epitope of mAb 6 is located in a hydrophobic region. On the other hand, the antigenic index profile revealed that the epitopes of mAb 21 and mAb 22 show high antigenicity, epitopes of mAb 2 and mAb 8 are intermediate, while epitopes of mAb 6 and mAb 17 have low antigenicity. Besides, the chain flexibility profile 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, it is implied that the epitope of mAb 6 is located in a region that is hydrophobic, rigid, and inaccessible, indicating that the region is unfavorable for antigenicity. But the experimental result (from ELISA) demonstrated that native β_1-Bgt could be bound by mAb 6, and the corresponding epitope on β_1-Bgt could not be masked by the toxin molecule.

The epitope of mAb 2 and mAb 8 was located at the sequence 46-51 of the A chain of β_1-Bgt, which contains 2 essential catalytic residues, His-48 and Asp-49 (Kwong et al. 1995). 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 enzymatic activity and toxicity. From the standpoint of affinity constants, the affinity of mAb 8 is higher than that of mAb 2; which may possibly account for the inhibition of enzymatic activity and the toxicity of mAb 8 being stronger than that of mAb 2. The experimental results suggest that these 2 mAbs inhibit the biological activity of β_1-Bgt, which appears to be consistent with previous reports (Brunie et al. 1985, Kwong et al. 1995). Asp-92 is also an essential catalytic residue located within the epitope of mAbs 21 and 22; therefore, the biological activities of

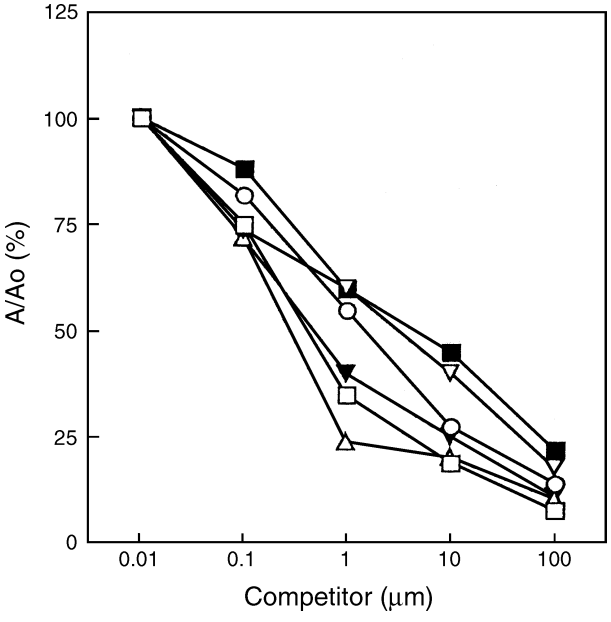
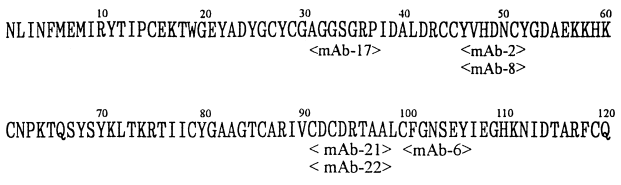


Fig. 7. Inhibition of β_1-Bgt binding to mAbs 2, 6, 8, 17, 21, and 22 by peptide competitors. A/A_o is the determined ratio (%) of the absorbance at 450 nm in the presence (A) or absence (A_o) of a competitor. mAbs at 10 μg/ml were used, and the synthetic peptide competitors were 0.01~100 μm. The binding 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) (□), A(100-106) (▽), A (46-51) (○), A(31-37) (△), A(91-98) (■), and A(91-98) (▼), respectively.

(A)



(B)

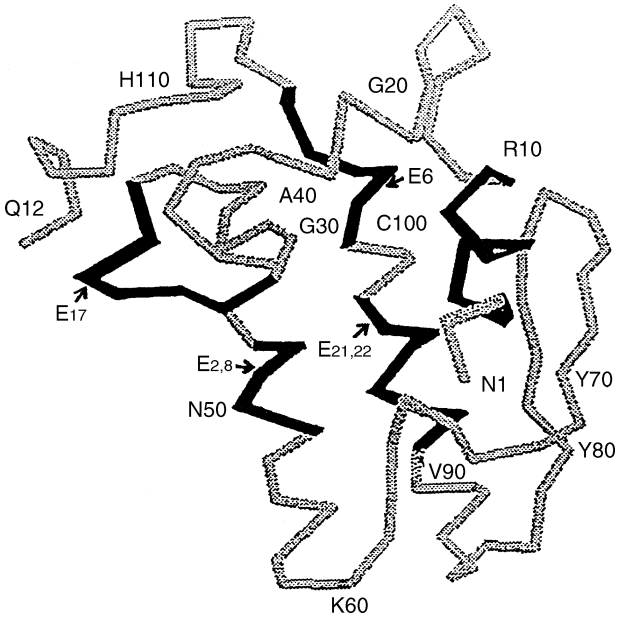


Fig. 8. Possible epitopes of 6 continuous neutralizing mAbs on the amino acid sequence (A) and on the 3-dimensional structure of the A chain of β_1-Bgt (B). E_2, E_6, E_8, E_{17}, E_{21}, and E_{22} indicate the mapped neutralizing epitopes of mAb 2, 6, 8, 17, 21, and 22, respectively. The backbone of the toxin is indicated with gray lines, and neutralizing epitopes with black lines. On the other hand, the A chain amino acid sequence from Ile 3 to Ile 9, which was detected as a minor epitope of the toxin, possesses weak antigenicity.

β_1-Bgt might be blocked by these 2 mAbs. According to bovine pancreatic-PLA_2 crystal structure, the carbonyl oxygens of Tyr-28, Gly-30, and Gly-32, the carboxylate oxygens of Asp-49, and 2 water molecules form an oxygen cage that sequesters a Ca^{2+} ion (Dijkstra et al. 1981). From the above observation, we can infer that mAb 17 can block Gly-32, and inhibit the PLA_2 activity of β_1-Bgt.

By means of these neutralizing epitope mapping results, it is possible to conclude that A chain sequences 31-37, 46-51, 91-98, and 100-106 of β_1-Bgt are all important for PLA_2 enzymatic activity and lethal toxicity. By contrast, ammodytoxin C or crotoxin, the presynaptically neurotoxic PLA_2 or β-neurotoxin, contains its toxicity site near the C-terminal part of the PLA_2 subunit of the toxin (Curin-Serbec et al. 1991 1994, Krizaj et al. 1994).

PREPARATION AND CHARACTERIZATION OF β_1-Bgt BISPECIFIC mAb

Production of hybrid hybridoma and preparation of bispecific mAb

The binding avidity of the mAb (2+6) mixture to β_1-Bgt was noted to be 9.4 fold higher than that of mAb 6 and 31 fold higher than that of mAb 2 (Table 2). Moreover, the neutralizing capacity of the mAb (2+6) mixture towards β_1-Bgt was about 2 to 4.9 times higher than that of either mAb 2 or mAb 6, yet the inhibition of PLA_2 activity did not increase as much. However, for other neutralizing mAb combinations, mAb (2+17), mAb (2+21), and mAb (6+17), no obvious cooperative effects were observed. The biological activity of β_1-Bgt was also not observed to be further inhibited by any of these antibody combinations.

According to the method described by Milstein and Cuello (1983 1984), the hybridoma lines capable of producing mAb 2 (against epitope A[46-51]) and mAb 6 (against epitope A[100-106]) were labeled with FITC and TRITC, respectively. After PEG fusion and culturing, cells that emitted a double fluorescent signal were collected and tested for secretion of bispecific mAb against epitopes A(46-51) and A(100-106). Subsequent purification of bispecific mAb was performed by ammonium sulfate precipitation and protein G purification, followed by separation on a hydroxylapatite column (Yang and Chan 1999b). As seen from figure 9c, 6 protein peaks were obtained from the hybrid antibody mixture. In contrast, 4 and 2 peaks were obtained from clone 2 and clone 6 parental lines, respectively (Fig. 9a, b). As expected, the first 2 peaks in figure 9c showed no antibody activity, while hybrid antibody activity was observed in peaks 4 and 5. The equal valence of the bispecific mAb to 2 epitopes was observed only in the peak 5 fraction, but not in the peak 4 fraction. These 2 peaks are located between the peaks that contain the anti-epitope A(46-51) activity (peak 3 in figure 9c or peak 4 in figure 9a) and the anti-epitope A(100-106) activity (peak 6 in figure 9c or peak 2 in figure 9b).

Characterization of anti-β_1-Bgt bispecific mAb

Bispecific mAb is a hybrid molecule comprised of 2 different heavy chains, γ_1 and γ_{2b}; the former is donated by mAb 2 and the latter by mAb 6 (Yang and Chan 1999b). The specificity of bispecific mAb was determined by ELISA against epitope A(46-51) and epitope A(100-106). The results indicate that the bifunctional reactivity of bispecific mAb is comparable to the activities of the 2 parental mAbs, i.e., the ability of parental mAb 2 towards epitope A(46-51) as well as the ability of parental mAb 6 towards epitope A (100-106). While only bispecific mAb, either as supernatant, ascites, or affinity-purified product, was observed to react in the dual assay, however, the mixture of mAb 2 and mAb 6 yielded a negative result on the dual assay, thereby emphasizing the fact that the dual reactivity of bispecific mAb resides on a single molecule of antibody alone.

As shown in table 2, bispecific antibodies reveal as much as 45- to 150-fold higher avidity than does individual monospecific mAb. The mixture of mAb 2

Table 2. Analysis of affinity constants of monospecific and bispecific mAbs and their Fab and $F(ab')_2$ fragments

	K_A (l/nM)	Enhancement factor[a]
mAb 2	0.30 ± 0.05	0.3
mAb 6	0.98 ± 0.04	1.0
mAb(2+6)	9.40 ± 0.30	9.4
Bispecific mAb	45.00 ± 0.13	45.0
Fab of mAb 2	0.19 ± 0.01	0.2
Fab of mAb 6	0.70 ± 0.08	0.7
Fab of mAb(2+6)	0.40 ± 0.04	0.4
Fab of bispecific mAb	0.45 ± 0.05	0.5
$F(ab')_2$ of mAb 2	0.21 ± 0.07	0.2
$F(ab')_2$ of mAb 6	0.78 ± 0.06	0.8
$F(ab')_2$ of mAb(2+6)	8.60 ± 0.14	8.7
$F(ab')_2$ of bispecific mAb	7.80 ± 0.20	8.0

[a]Enhancement factor was calculated by (K_A of 2 mAb mixtures or bispecific mAb/K_A of higher-affinity single mAb).

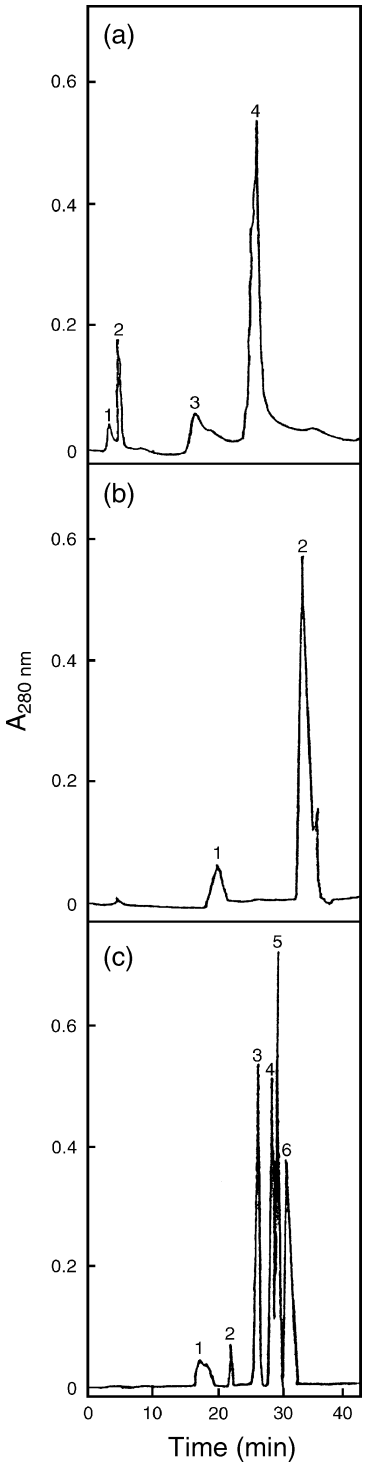


Fig. 9. Hydroxylapatite column chromatographic profiles of antibodies of hybridoma clone 2 (a), clone 6 (b), and hybrid hybridoma (c). Protein G-purified immunoglobulin fractions were applied to a hydroxylapatite column (100 × 7.8 mm, Bio-Gel HPHT, Bio-Rad Laboratories, Richmond, CA) equilibrated with 0.01 M phosphate buffer, pH 6.8, and eluted with a linear gradient from 0.01 to 0.3 M sodium phosphate buffer, pH 6.8, for 30 min. In (a), only peak 4 belongs to immunoglobulin, while peak 2 in (b) and peaks 3, 4, 5, and 6 in (c) are all immunoglobulins.

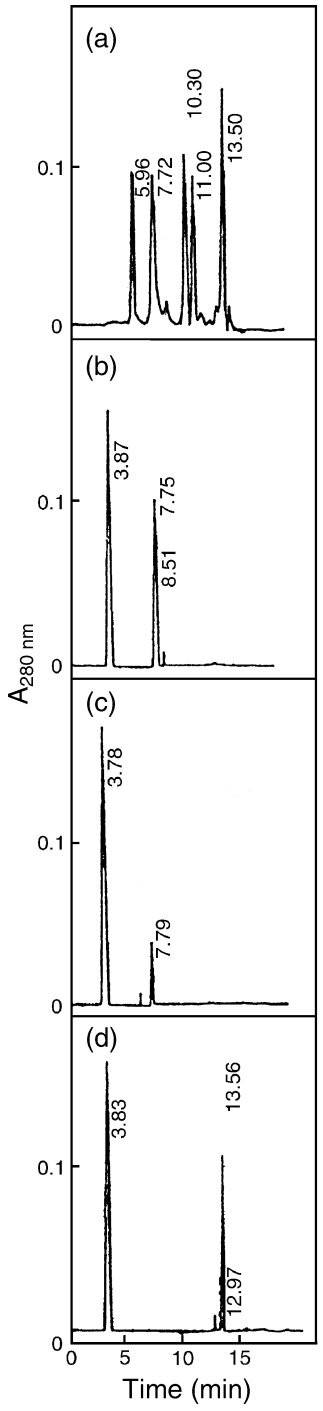


Fig. 10. Gel filtration patterns of soluble complexes formed from bispecific mAb and β_1-Bgt on a size-exclusion column. (a) indicates the elution time of protein standards: 5.96 min, IgM (900 kDa); 7.72 min, IgG (150 kDa); 10.30 min, BSA (67 kDa); 11.00 min, HRP (44 kDa); and 13.50 min, β_1-Bgt (20.5 kDa). (b), (c), and (d) show the elution patterns of the bispecific mAb after incubation with β_1-Bgt at molar ratios of 2:1, 1:1 and 1:2, respectively. The peak at 3.8 min indicates the β_1-Bgt-bispecific mAb complexes, and the peak at 7.7 min indicates free bispecific mAb (150 kDa), while that at 13.56 min indicates free β_1-Bgt (20.5 kDa).

and mAb 6, and bispecific mAb could, respectively, inhibit 86% and 98% of the PLA_2 enzymatic activity of β_1-Bgt, which is potentially more than that of mAb 2 (79%) or mAb 6 (78%) alone. Besides, the results of neutralizing toxicity experiments also revealed that the neutralizing capacity of bispecific mAb to β_1-Bgt (1818 LD_{50}/mg mAb) is much higher than that of either mAb 2 (729 LD_{50}/mg mAb) or mAb 6 (292 LD_{50}/mg mAb).

Mechanism of avidity enhancement by bispecific mAb

As shown in table 2, bispecific mAb could enhance the avidity towards β_1-Bgt by 45-150 fold. However, the affinity constants of bispecific mAb to epitope A(46-51) (0.34 l/nM) and also to epitope A (100-106) (1.10 l/nM) were found to be similar to those of mAb 2 (0.30 l/nM) and mAb 6 (0.98 l/nM), respectively, which imply that the recombination of heterologous heavy chains in a single bispecific mAb does not significantly alter the avidity of any one of the 2 paratopes towards the corresponding epitope or β_1-Bgt. Also shown in table 2, the avidity enhancement of Fab fragments of the mAb (2+6) mixture as well as bispecific mAb did not differ significantly from those of mAb 2 and mAb 6. In addition, the pepsin cleavage of bispecific mAbs into bivalent $F(ab')_2$ fragments exhibited a similar-magnitude avidity to that of the mAb (2+6) mixture; moreover, the avidity of bispecific mAb was 5 times higher than that of the mAb (2+6) mixture. All these results prove that the bivalency of the toxin-antibody interaction and the interaction between 2 different heavy chains induce avidity enhancement and increase the ability of reducing the biological activity of β_1-Bgt.

As shown in figure 10, the gel filtration patterns indicate that all the soluble complexes formed by bispecific mAb and β_1-Bgt, at different molar ratios, emerged in the void volume, which means that the molecular weight of the soluble complex is around 6000 kDa or greater. According to this result, we may propose that the 1st binding site of the bispecific mAb interacts with its corresponding epitope, which in turn changes the overall conformation of β_1-Bgt and then facilitates the interaction between the other paratope and its corresponding epitope in the same toxin molecule. After a subsequent chain reaction, multiple complexes of β_1-Bgt and bispecific mAb were formed.

From the results of avidity enhancement, enzymatic activity inhibition, and toxicity neutralizing, it is found that these biological properties are almost all positively correlated. That is, the higher the avidity, the lower the biological activity of the β_1-Bgt-antibody complex observed.

NEUTRALIZING EPITOPE MAPPING AS A BASIS FOR β_1-Bgt PEPTIDE VACCINE DESIGN

Protective capacity of peptide-immunized mice

Synthetic peptides A(31-37), A(46-51), A(91-

Table 3. Survival ratio of peptide-immunized mice

Peptide immunized[a]	Survival ratio with challenge by β_1-Bgt[b]		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
Scrambled peptide[c]	0%	0%	1.17	0.70	1 ± 1	0 ± 0	ND[d]	ND[d]
BSA control	0%	0%	1.33	–	0 ± 0	–	ND[d]	–

[a]Peptides were conjugated with BSA using glutaraldehyde.
[b]For each survival ratio experiment, 25 mice were used.
[c]The sequence of scrambled peptide was MVRVFPNVYVYSNIYIYPNIGIAF.
[d]"ND" means not detectable (neutralizing capacity was lower than 10).

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. Subsequently, they were injected intramuscularly into Balb/c mice with Freund's complete adjuvant and then with Freund's incomplete adjuvant. Table 3 compares the survival ratios of peptide-immunized mice that were challenged with 10 x LD_{50} or 5 x LD_{50} of β_1-Bgt. These results indicate that all synthetic peptides, A(31-37), A(46-51), A(91-98), and A(100-106), had the capacity to protect the mice from β_1-Bgt challenge, yet mice immunized with synthetic peptides A(46-51) and A(100-106) were better 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 both PLA_2 activity and β_1-Bgt toxicity (Table 3). In contrast, the scrambled peptide-immunized (MVRVFPNVYVYSNIYIYPNIGIAF) or BSA-control-immunized mice had undetectable protective activity.

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

A passive transfer experiment was performed to test whether the anti-peptide-immunized sera could produce protective immunity against β_1-Bgt in mice. Donor Balb/c mice were immunized with BSA-conjugated peptides A(31-37), A(46-51), A(91-98), A(100-106), and scrambled peptide. The sera were collected 3 d after the last booster injection and pooled, while control serum was obtained from mice immunized with BSA alone. Undiluted pooled sera (0.5 ml), peptide-specific antibodies (0.25, 0.5, or 1 mg), or immunoglobulin-free serum were injected intravenously into male Balb/c mice. After 12 h, the recipient mice were challenged with 5 x LD_{50} of β_1-Bgt. The results show that all peptide-immunized sera had the ability to protect passively transferred mice from toxin challenge to varying degrees (Table 4). Furthermore, injection of mice with peptide-specific antibody purified from anti-peptide sera showed a distinct protective effect against toxin challenge in a dose-dependent manner. On the other hand, all immunoglobulin-free sera fractions had no protective effect. The results indicate that immunoglobulin purified from immune sera proved to be a major component for protecting mice from toxin challenge, and the protection induced by the peptide is antibody mediated.

CONCLUSIONS

The numbers of antigenic determinants in β_1-Bgt, A chain, and B chain were determined to be 7, 5, and 2, respectively, by quantitative precipitin reactions and analysis of the soluble complexes formed from β_1-Bgt and Fab fragments of the antibody. Twenty-three stable mAbs were prepared against β_1-Bgt, of which 7 could inhibit more than 70% of PLA_2 activity of β_1-Bgt and neutralize the toxin. Six of these neutralizing mAbs (mAbs 2, 6, 8, 17, 21, and 22) recognized the continuous epitopes on the A chain of β_1-Bgt while one (mAb 11) recognized a conformational epitope on the toxin. The continuous epitopes of the 6 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

Table 4. Survival ratios of native mice passively transferred with anti-peptide sera or peptide-specific antibody[a]

	Injection of 0.5 ml of antisera	Injection of immunoglobulin-free serum	Injection of various amounts of peptide-specific antibodies		
			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
Scrambled peptide	0	0	0	0	0
Control (BSA)	0	0	–	–	–

[a]Challenge with 5 LD_{50} of β_1-Bgt after passive transfer of antisera, peptide-specific antibody, or immunoglobulin-free serum fractions. For each survival ratio experiment, 25 mice were used.

46-51; mAbs 21 and 22 recognized residues 91-98; and mAb 6 recognized residues 100-106. The competitive antibody-binding inhibition experiments showed that the affinity of these neutralizing mAbs to native β_1-Bgt is compatible with synthetic peptides.

A hybrid hybridoma (tetradoma) that produces bispecific mAbs, which recognizes 2 different epitopes on the A chain of β_1-Bgt at peptide sequences 46-51 and 100-106, has been obtained by fusing 2 hybridoma cell lines. Bispecific mAbs were observed to inhibit 98% of the enzymatic activity of β_1-Bgt and completely neutralize its lethal toxicity. Besides, the avidity between bispecific mAb and β_1-Bgt was noted to be 4.5×10^{10} (l/nM), which is about 45-150 fold higher than the avidity of its 2 parental mAbs, indicating that the binding of bispecific mAb with its 2 epitopes on β_1-Bgt, which facilitates immuno-complex formation and enhances avidity, also highly neutralizes the biological activity of β_1-Bgt.

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, peptide-immunized sera were passively injected into Balb/c mice and a significant protective effect was also observed. In conclusion, the present study demonstrates 4 fine-mapped linear neutralizing epitopes of β_1-Bgt, and these epitopes occupy the catalytic and toxic essential residues. According to this result, a β_1-Bgt synthetic-peptide vaccine was designed, and a protective effect was observed; moreover, β_1-Bgt-bispecific mAb was prepared for neutralizing the toxicity and enzymatic activity of the toxin.

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ABBREVIATIONS

β_1-Bgt	β_1-Bungarotoxin
mAb	Monoclonal antibody
PLA_2	Phospholipase A_2
LD_{50}	50% lethal dose
FITC	Fluorescein isothiocyanate
TRITC	Tetramethyl rhodamine isothiocyanate
PEG	Polyethylene glycol
Fab	Fragment of antigen binding

臺灣雨傘節腱前神經毒 (β-Bungarotoxin) 之免疫化學研究

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β_1-Bungarotoxin (β_1-Bgt) 是雨傘節蛇毒中最主要且最具毒性的腱前神經毒。這個帶有磷脂酶活性的毒蛋白由兩個不相同的多胜肽經由雙硫鍵的連結所組成。其一稱為 A 鏈，它由 120 個胺基酸所組成；另一稱為 B 鏈，由 60 個胺基酸所構成。A 鏈的氨基酸序列類似於蛇毒或哺乳類胰臟所產生的磷脂酶序列；而 B 鏈可與特定目標細胞膜做專一性之結合並且有阻絕電位敏感性鉀通道的能力。利用定量沉澱反應以及抗體 Fab 片段與毒蛋白所形成之可溶性複合物分析得知每分子 β_1-Bgt 含有七個主要的抗原決定基，其中五個位於 A 鏈上，二個位於 B 鏈上。此外，23 個 β_1-Bgt 的單株抗體被製備出來，其中有 7 個可抑制毒蛋白酵素活性達百分之七十以上，並能中和毒蛋白的毒性。藉著表位分析，得知這些中和抗體的表位是位於 A 鏈蛋白序列 31-37，46-51，91-98 以及 100-106。將能認知毒蛋白表位 46-51 及 100-106 的融合瘤二度融合，所產生的融合融合瘤可分泌雙專一性單株抗體，此種抗體可引起免疫複合物之形成以及增進親合力效價，並可高度中和 β_1-Bgt 的生物活性。更進一步，將上述的這四段抗原決定基合成胜肽來免疫老鼠，再施以高劑量的 β_1-Bgt，發現免疫後的老鼠對毒蛋白有相當高的耐受性。

關鍵詞：臺灣雨傘節蛇毒，腱前神經毒，單株抗體，融合融合瘤，雙專一性單株抗體，胜肽疫苗。

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