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Inhibition of tetanus toxin fragment C binding to ganglioside G_{T1b} by monoclonal antibodies recognizing different epitopes

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Abstract

Anti-tetanus toxoid monoclonal antibodies would be useful in exploring the relationship of tetanus toxin structure to its function. Tetanus toxin fragment C has been shown to be responsible for binding to neurons via gangliosides. Eleven new and two previously derived monoclonal antibodies specific for tetanus toxin fragment C were shown to recognize five different fragment C epitopes, two of which were overlapping. Three of these epitopes participate in the binding to ganglioside G_{T1b}. One epitope was defined by a monoclonal antibody that did not inhibit the interaction between fragment C and ganglioside. This antibody however, was blocked from binding to fragment C by antibodies that were able to inhibit the fragment C-ganglioside interaction. Published by Elsevier Science Ltd.

Keywords: Tetanus toxin fragment C; Monoclonal antibodies; Ganglioside G_{T1b}

1. Introduction

Tetanus toxin (TeNT), one of the most potent toxins known [1–3], is synthesized as a 150 kDa single polypeptide chain [4]. This polypeptide is cleaved into a heavy chain of 100,000 kDa and a light chain of 50,000 kDa which are held together by a disulfide bond [4,5] and form the active toxin. Digestion of the holotoxin with papain results in fragment B which is comprised of the TeNT light chain and the amino terminal half of the TeNT heavy chain (H_N), and fragment C (H_C) which contains the carboxy terminal half of the heavy chain [6].

TeNT toxicity requires several steps: binding to neurons, internalization, retrograde axonal transport, and transynaptic transport prior to its intoxicating action [1,3,7]. These various functions have been assigned to different domains of TeNT. In vitro experiments have suggested that H_C is responsible for binding to neurons through gangliosides while the H_N fragment plays a role in internalization and membrane translocation [1,3,7,8]. The requirement of gangliosides for neuronal binding of tetanus toxin has recently been confirmed in spinal cord neuron cultures under conditions where gangliosides could not be synthesized [9] and in vivo in $\beta_{1,4}$-*N*-acetylgalactosaminyl transferase knock-out mice which cannot synthesize gangliosides [10]. Recombinant H_C has recently been shown to be responsible for both the specific binding and internalization into spinal cord neuron cultures [11]. The TeNT light chain contains the catalytic domain, a Zn^{2+}-dependent metalloprotease which cleaves synaptobrevin, a protein found on synaptic vesicles in nerve terminals [1].

TeNT is specific for intoxication of neurons. The mechanism of TeNT binding to neurons is not completely understood however, as many cell types express gangliosides but are not susceptible to tetanus intoxication. Although the related botulinum toxins (BoNT) also bind gangliosides and cleave synaptobrevin or related substrates [12], the clinical manifestation of

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BoNT is different from TeNT. Both TeNT and BoNT bind to the presynaptic terminal of the neuromuscular junction (NMJ) of motor neurons and are endocytosed. While BoNT blocks the release of neurotransmitters at the NMJ, TeNT undergoes retrograde axonal transport, exits the cell and is taken up by inhibitory interneurons of the spinal cord. Here TeNT blocks neurotransmitter release. To account for the difference in sites of action between TeNT and BoNT, it has been proposed that a toxin-protein interaction occurs subsequent to ganglioside binding. This putative toxin-protein interaction would be responsible for the specificity of these toxins [13]. Thus, H_C would be important for the specificity of toxin activity as well as for ganglioside binding.

Monoclonal antibodies (mAbs) against TeNT have been useful as probes of tetanus toxin structure and function. Anti-tetanus toxoid (TT) mAbs developed by Kenimer et al. [14] contained two which were specific for H_C. One of these mAbs inhibited the binding of H_C to ganglioside while the other enhanced binding [15]. In order to more fully define the relationship between structure and function of H_C, we developed an additional 11 H_C-specific mAbs. Analysis of the variable region diversity (V_H–V_L pairs) of all 13 mAbs revealed four groups which recognize five H_C epitopes based on mAb cross-blocking studies and the inhibition of H_C binding to ganglioside G_{T1b}. Three of these H_C epitopes are blocked from binding to ganglioside G_{T1b} by the mAbs that define them. The remaining two epitopes do not participate in binding to ganglioside G_{T1b}.

2. Materials and methods

2.1. Anti-H_C mAbs

A 6 week-old female BALB/c mouse was immunized IP with 250 μg of tetanus toxoid (TT, a gift from Dr Willie Vann, CBER) suspended in 5% Maalox and boosted IP 90 days later. Three days after the boost, the spleen was harvested, cells were fused with Sp2/0, and hybridomas were propagated as described [16]. Supernatants from fusion wells were screened by ELISA for positive binding to TT and negative binding to BSA as a control protein antigen. Microfluor microtiter plate (Dynex Technologies, Chantilly, VA, USA) wells were coated with 100 μl of 5 μg/ml TT or BSA in bicarbonate buffer overnight at 4°C. Wells were rinsed 3× with 260 μl of PBS/0.5% BSA and blocked for 2 h at room temperature with a final 260 μl of PBS/0.5% BSA. Plates were emptied and 100 μl of supernatant were added to single wells for 1 h at room temperature. Plates were washed 4× with PBS/0.1% Tween (PBS-T) and 100 μl of alkaline phosphatase-labeled goat anti-mouse heavy and light chain antibody (Southern Biotechnology Associates, Birmingham, AL, USA) were added to the wells for 1 h at room temperature. After washing 4× in PBS-T, 100 μl of 5 μg/ml 4-methylumbellyferyl phosphate were added to the wells and the plate was read in a microplate fluorimeter. Cells from positive wells were cloned by limiting dilution and rescreened as described above. Supernatants from each TT-positive clone were screened by ELISA for their ability to bind to two preparations of recombinant H_C (prepared by Dr Willie Vann, CBER and purchased from Roche, Indianapolis, IN, USA). ELISA's were performed as described above except that wells were coated overnight with 1 μg/ml H_C in bicarbonate buffer pH 9.0 at 4°C. Antibodies were isotyped by ELISA with the mAb-based isotyping kit from Pharmingen (San Diego, CA) according to the manufacturer's instructions.

2.2. V_H/V_L cloning and sequencing

Total RNA from H_C-specific clones was isolated using the TRIZOL method (Life Technologies, Gaithersburg, MD, USA), according to the manufacturer's instructions. Tubes containing RNA (15 μg), 2 μl of 0.5 μg/ml oligo (dT) and water to 27 μl were incubated at 70°C for 10 min and then placed on ice. Ten microliter 5× reverse transcriptase buffer (Life Technologies, Gaithersburg, MD, USA), 6 μl 10 mM dNTPs, 5 μl 0.1 M DTT, 2 μl Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD, USA), and water to 50 μl were added to each tube. Reverse transcription was performed as follows: room temperature, 10 min/42°C, 50 min/90°C, 5 min/4°C, 10 min. Finally, 1 μl of RNAse H was added to each tube and incubated for 20 min at 37°C. Two microliters of each cDNA was used to amplify hybridoma immunoglobulin heavy and light chain sequences. All PCR reactions were performed in a DNA thermocycler 480 (PE Biosystems, Foster City, CA, USA) in 100 μl reactions with 1.5 mM $MgCl_2$, 0.05 mM dNTPs, and 50 pmol each primer under the following cycling conditions: 95°C, 5 min; 52°C anneal, 50 s/72°C extension, 1.5 min/95°C denaturation 15 s (30 cycles); and 72°C, 10 min, 4°C hold. All light chains were amplified using the L5 (5′-GCTCGTGATGACCCAGACTCCA-3′) and L9 (5′GCGCCGTCTAGAATTAACACTCATTCCTGTTGAA-3′) primer pair [17]. All IgG_1 heavy chains were amplified using VH1 (5′-GAGGTGAAGCTGGTGGAGWCWGG-3′) or VH2 (5′-GAGGTCCAGTTGCAGCAGWCWGG-3′) 5-prime primers [18] with the H12 [19] (5′AGGCTTACTAGTACAATCCCTGGGCACAAT-3′) 3-prime primer. IgG2 heavy chains were amplified with VH1 or VH2 and H15 (5′ACTGACTCAGGGAAGTAGCC-3′).

PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen, San Diego CA) according to manufacturer's instructions and two to three clones for each reaction were sequenced in both the forward and reverse directions.

2.3. Quantitation of hybridoma supernatants

Supernatants from each of the 13 H_C hybridomas were collected and concentrated approx. 20-fold with Macrosep centrifugal concentrators with 100 K membranes (Filtron Technology Corporation, Northborough, MA, USA). IgG concentrations were determined by ELISA with either IgG_1 or IgG_{2b} standards (Pharmingen, San Diego, CA, USA). ELISAs were performed as above using goat anti-mouse kappa (Southern, Birmingham, AL, USA) at 1 μg/ml as a capture reagent.

2.4. Cross blocking ELISAs

For cross blocking, 5E4, 35F7, 72B9, 81H10, 87C10, 18.1.7, and 18.2.12.6 mAbs were purified and labeled with biotin. A dilution curve (1:1000–1:512,000) was generated for each biotinylated mAb and a dilution that resided in the middle of the linear segment of the curve was used for subsequent ELISAs.

Cross blocking experiments were performed as follows: ELISAs were performed as described above with rH_C at 1 μg/ml as coating antigen. Two-fold serial dilutions of hybridoma supernatants from 10 μg mAb/ml to 4.8 ng mAb/ml in PBS-T were added to wells (100 μl of each dilution) in triplicate for 1 h at room temperature. (The fusion partner for hybridomas 18.1.7 and 18.2.12.6, P3-X63Ag8, expresses endogenous antibody. Therefore, the exact concentration of H_C-specific IgG in these supernatants is not known.) After washing, 100 μl of the appropriate dilution of each biotin-labeled antibody were added to each well for 20 min at room temperature followed by the addition of 100 μl streptavidin-alkaline phosphatase at 1:5000 (Amersham Life Sciences, Piscataway, NJ, USA) for 20 min at room temperature. Wells were washed and developed as described above. Biotinylated antibodies with and without self competitor were used as controls. Monoclonal antibody 12C11 was also included as a negative control on each plate. This mAb was generated from the same BALB/c mouse as the H_C-specific mAbs and binds TT but not H_C.

ELISAs were also performed with polyclonal anti-tetanus serum from guinea pigs which were vaccinated with a commercial tetanus vaccine. This serum was generated at CBER for use in neutralizing assays that serve as a measure of the potency of the vaccine and was a gift from Christine Anderson (CBER/OCBQ). The assay was the same as described above except that guinea pig serum serial dilutions from 1:4 to 1:1024 were used as a competitor. Results are presented as the percent inhibition using the value of the 1:1024 dilution for each biotin-labeled mAb as 0% inhibition (no inhibition compared to controls without serum). Each dilution was added to wells in triplicate.

2.5. G_{T1b} binding ELISAs

A dilution of biotinylated-rH_C that resulted in saturation of ganglioside G_{T1b} binding was chosen for use in the assay described below. For 18.2.12.6 however, a dilution of biotinylated-rH_C from the middle of the binding curve was chosen in order to observe enhancement of rH_C binding to G_{T1b}. Microtiter plate wells were coated with 100 μl of 1 μg/ml G_{T1b} (Matreya, Inc., Pleasant Gap, PA, USA) in methanol and plates were left at room temperature overnight to allow evaporation of methanol. Beginning at 20 μg/ml, rH_C-reactive hybridoma supernatants were diluted in serial two-fold dilutions in PBS-0.5% BSA to 0.625 μg/ml. Diluted antibodies were mixed with an equal volume of biotinylated-rH_C diluted 1:250 in PBS-0.5% BSA and incubated overnight at 4°C. After the ganglioside G_{T1b} coated wells were blocked for 2 h at room temperature in PBS–0.5% BSA, 100 μl of each rH_C/antibody mix was added to wells in quadruplicate and incubated for 2 h. Plates were washed 4× in PBS-T and incubated for 1 h at room temperature with 100 μl/well streptavidin-alkaline phosphatase diluted 1:5000 in PBS–0.5% BSA. Plates were washed and developed as described above. Monoclonal antibody 12C11 was included as a negative control on each plate.

Table 1
Heavy and light chain gene usage of tetanus fragment C hybridomas

Hybridoma	IgG class	V_H family	J_H	Vκ family	Jκ
5E4	IgG_{2b}	V_HQ52N	J_H4	Vκ12/13	Jκ1
19G1	IgG_1	V_HQ52N	J_H4	Vκ12/13	Jκ1
24D3	IgG_1	V_HQ52N	J_H4	Vκ12/13	Jκ1
27H3	IgG_1	V_HQ52N	J_H4	Vκ12/13	Jκ1
42C10	IgG_1	V_HQ52N	J_H4	Vκ12/13	Jκ1
64B9	IgG_1	V_HQ52N	J_H4	Vκ12/13	Jκ1
76A8	IgG_1	V_HQ52N	J_H4	Vκ12/13	Jκ1
87C10	IgG_1	V_HQ52N	J_H4	Vκ12/13	Jκ4
35F7	IgG_1	V_HJ558	J_H2	Vκ9A	Jκ2
72B9	IgG_{2b}	$V_H36–60$	J_H2	Vκ4,5	Jκ5
81H10	IgG_1	$V_H36–60$	J_H2	Vκ19/28	Jκ5
18.1.7	IgG_1	$V_H36–60$	J_H2	Vκ19/28	Jκ1
18.2.12.6	IgG2	V_HJ558	J_H3	Vκ9A	Jκ4

3. Results

3.1. Anti-H_C mAbs

Eleven monoclonal antibodies specific for TeNT H_C were produced from the spleen of a BALB/c mouse immunized with tetanus toxoid. Nine hybridomas expressed IgG_1 and two (5E4 and 72B9) were IgG_{2b} (Table 1). All of the mAb's recognized tetanus toxoid and rH_C by ELISA (data not shown). Clonality of hybridomas was determined by the patterns of heavy and light chain gene rearrangement by Southern analysis (data not shown) and by sequencing V_H and Vκ genes to determine family usage as well as distinctive V(D)J junctions.

The variable region gene usage of the heavy and light chains is shown in Table 1. The D_H regions for all mAbs were too short to assign to any family. Eight of the new monoclonal antibodies utilized V genes from the V_HQ52N heavy chain family recombined with J_H4. All mAbs in this group expressed Vκ12,13 light chains. Analysis of the rearrangement patterns and sequences of the V(D)J junctions of the V_HQ52N/Vκ12,13 hybridomas established five different clones among this group. The Vκ12,13 genes rearranged with Jκ1 in all clones except 87C10 which rearranged with Jκ4 and thus, represents an independent clone. Besides 87C10, hybridomas 24D3 and 64B9 are derived from unique clones. Hybridomas 5E4 and 76A8 likely represent switch variants that share a common origin. Clones 19G1, 27H3 and 42C10 are also derived from the same clone.

Of the remaining hybridomas, 72B9 and 81H10 utilized V_H36-60 heavy chains but had different light chains, using Vκ4/5 and Vκ19/28 respectively. Hybridoma 35F7 utilized V_HJ558 heavy and Vκ9A light chains. Thus of the eleven hybridomas, eight are derived from unique clones.

Two previously derived anti-H_C hybridomas were also sequenced [14]. Hybridoma 18.1.7 utilized the same V_H–Vκ families as 81H10, V_H36–60/Vκ19/28. The individual V_H36–60 genes expressed by these two hybridomas however, are distinct. The Vκ19/28 gene(s) rearranged to different Jκ genes (Jκ5 for 81H10 and Jκ1 for 18.1.7) and may represent different members of this family but there are not enough data to make this determination. Hybridoma 18.2.12.6 expressed the same V_H–Vκ families as 35F7, V_HJ558/Vκ9A, but the V_HJ558 genes represent different members of this family. The Vκ9A light chains rearranged to different Jκ genes (Jκ2 for 35F7 and Jκ4 for 18.2.12.6) and may also utilize different members of the Vκ9A family.

3.2. Cross blocking of mAb binding to rH_C

To determine if the H_C-binding monoclonal antibodies recognized distinct or overlapping epitopes, cross blocking ELISA's were performed. Supernatants from each hybridoma were tested for their ability to block the binding of biotin labeled 87C10 and 5E4 (V_HQ52N, Vκ12/13), 35F7 and 18.2.12.6 (V_HJ558, Vκ9A), 81H10 and 18.1.7 (V_H36–60, Vκ19/28) and 72B9 (V_H36–60, Vκ4/5) to H_C. As a negative control, antibody 12C11, a tetanus toxoid-specific mAb which does not bind to H_C, was included in each assay. Fig. 1 shows representative ELISA data from cross blocking experiments with biotinylated 5E4 [Fig. 1(A)], 35F7 [Fig. 1(B)], 18.2.12.6 [Fig. 1(C)], and 72B9 [Fig. 1(D)]. Results for all antibodies are summarized in Table 2. Four cross blocking patterns distinguish four epitopes. Epitope 1 is identified by mAbs from the V_HQ52N, Vκ12/13 group (87C10 and 5E4) which were blocked only by members of this group. 35F7 (V_HJ558, Vκ9A) characterizes epitope 2. It was not blocked from binding H_C by 18.2.12.6 which utilizes the same V_H–Vκ families, and was partially blocked by 81H10 and 18.1.7 (V_H36–60, Vκ19/28) and 72B9 (V_H36–60, Vκ4/5). 18.2.12.6 binding to H_C was not blocked by any mAb other than itself and defines epitope 3. While 18.2.12.6 and 35F7 express the same V_H–V_L families, different members of the V_HJ558 family are utilized which may account for the different epitope recognition. 72B9 had the reciprocal blocking pattern to 35F7. Its binding to rHc was blocked by itself, 81H10 and 18.1.7 and was partially blocked by 35F7. 81H10 and 18.1.7 share this blocking pattern. This set of mAbs defines epitope 4.

3.3. Blocking of mAb binding to H_c with polyclonal serum

As part of lot release testing of tetanus vaccines, guinea pigs are vaccinated with tetanus vaccine and the serum is used in neutralizing assays to assess potency of the vaccine. A batch of anti-tetanus guinea pig serum produced for this purpose was tested for its ability to block the binding of each biotin-labeled monoclonal antibody to rHc. As seen in Fig. 2, binding of all monoclonal antibodies to rH_c was inhibited by the polyclonal serum. Fifty percent inhibition was reached for all mAbs at dilutions ranging from 1:4 to 1:64.

3.4. Inhibition of binding to ganglioside G_{T1b}

Each mAb was tested for its ability to inhibit biotin-labeled rH_C binding to immobilized ganglioside G_{T1b} (Fig. 3). As shown in Fig. 3(A), all V_HQ52N, Vκ12/13 monoclonal antibodies blocked binding of rH_C to G_{T1b}. Supernatant from 12C11, which binds TeNT, but not H_C, did not block binding to G_{T1b}.

35F7 and 18.2.12.6, which both use V_HJ558 heavy

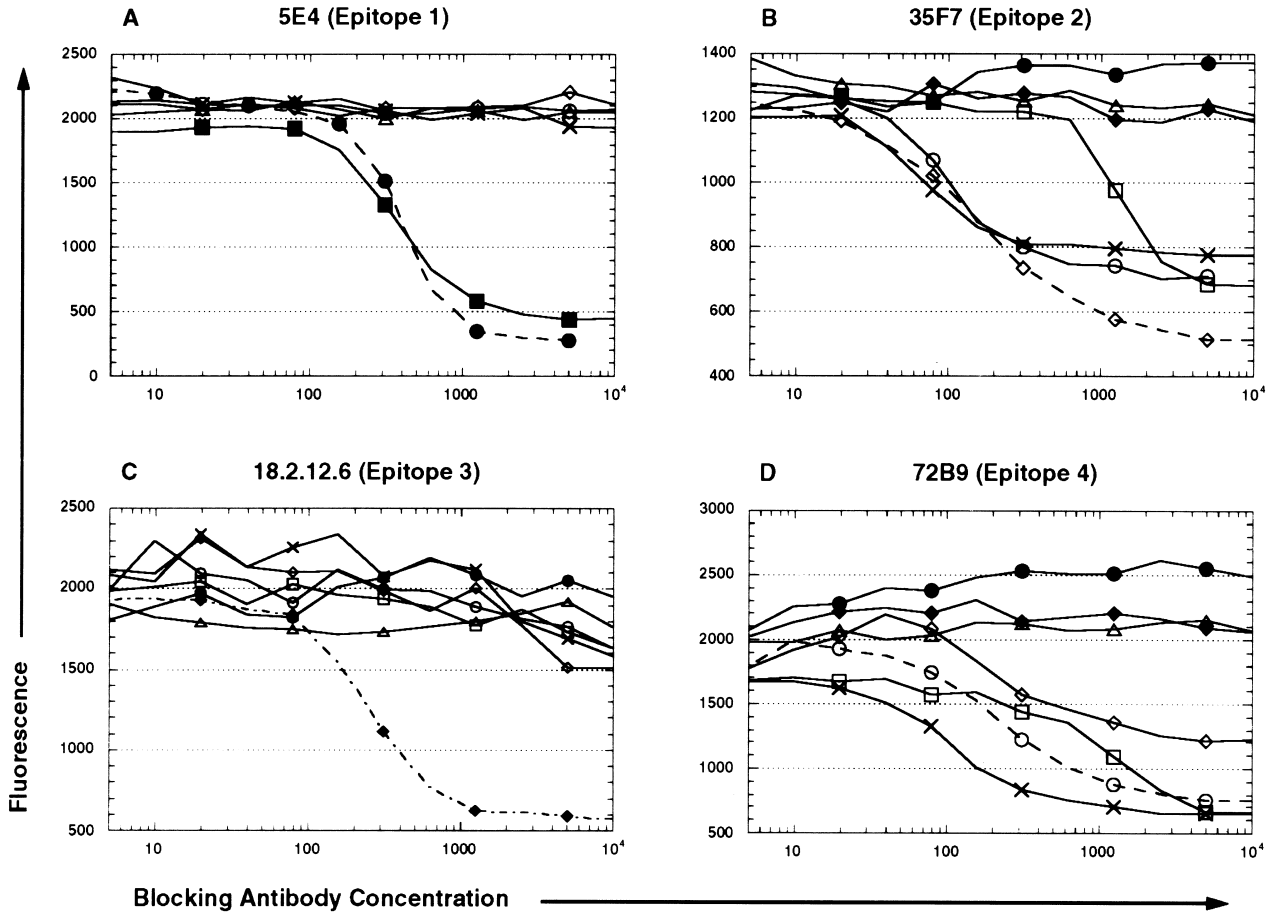

Fig. 1. Representative data from cross blocking of fragment C-specific monoclonal antibodies. Biotinylated antibodies are listed on the top of each panel. (A) 5E4 (V_HQ52N, Vκ12/13), (B) 35F7 (V_HJ558, Vκ9A), (C) 18.2.12.6 (V_H36–60, Vκ19/28), (D) 72B9 (V_H36–60, Vκ4,5). Each point is the average of three wells. The unlabeled self controls for each experiment are shown by a dotted line. Antibody concentrations were determined by ELISA. ■, 19G1; ●, 5E4; ◇ ,35F7; ○, 72B9; ◆, 18.2.12.6; □, 18.1.7; ×, 81H10; △, 12C11.

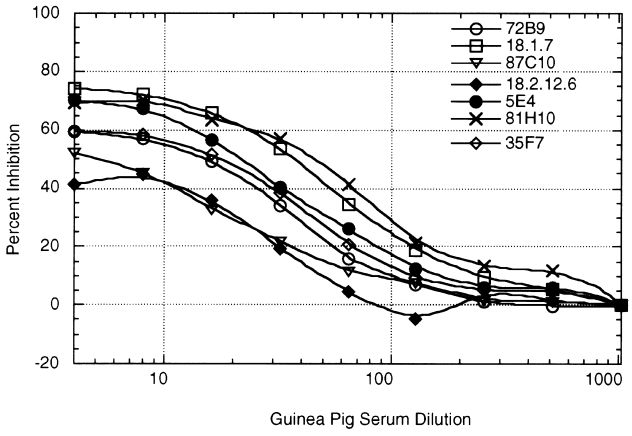

Fig. 2. Cross-blocking of fragment C-specific monoclonal antibodies with serum from guinea pigs immunized with tetanus vaccine. Percent inhibition was calculated by dividing average fluorescence values for each dilution by the average flourescence of the 1:1024 dilution (no inhibition compared to controls without serum). Each point is the average of three wells. ■, 19G1; ●, 5E4; ◇, 35F7; ○, 72B9; ◆, 18.2.12.6; □, 18.1.7; ×, 81H10; ▽, 87C10; △, 12C11.

chains and Vκ9A light chains, but recognize different epitopes of H_C, exhibit opposite profiles [Figs. 3(B) and 3(C)]. As demonstrated previously [15], 18.2.12.6 enhances binding of H_C to ganglioside G_{T1b}. Conversely, 35F7 is able to completely abolish binding at a concentration of 2.5 μg/ml. Both 81H10 and 18.1.7 inhibited ganglioside binding. 81H10 blockage was complete at 2.5 μg/ml while 18.1.7 inhibition approached completeness at 20 μg/ml, which agrees with previous observations [15]. It should be noted that the fusion partner for hybridoma 18.1.7 (as well as 18.2.12.6) was P3-X63Ag8 which expresses endogenous IgG_1,κ antibody. Antibody derived from this hybridoma is therefore, a mixture of endogenous antibody and antibody specific for H_C.

72B9, like 81H10 and 18.1.7, contains a V_H36–60 heavy chain but uses a different light chain family. Unlike 81H10 however, 72B9 failed to block H_C binding to G_{T1b} coated plates and thus identifies a fifth epitope [Fig. 3(B)].

4. Discussion

Tetanus toxin can be separated into two fragments, each with its own function. H_C, the carboxyl-terminal half of the TeNT heavy chain, binds to neurons through an interaction with gangliosides, primarily G_{T1b}, and may also bind to unidentified protein(s) on the neuronal presynaptic membrane [13]. Halpern and Loftus [15], demonstrated that deletion of amino acids 448-458 from the carboxyl terminus of recombinant H_C resulted in its inability to bind to both gangliosides and neuronal cells while deletion of the amino terminal 263 residues had no effect on binding. Deletion of the 5 carboxy-terminal amino acids did not affect binding and a peptide corresponding to the C-terminal 20 amino acids did not block ganglioside binding [15]. These results suggest that the carboxy terminal end of H_C is involved in binding to neurons either as a direct binding site for gangliosides or in providing the necessary conformation for binding. The crystal structure of H_C shows that the C-terminal 10 amino acids as well as those residues spatially surrounding the C-terminus are exposed to solvent, would not participate in intramolecular contacts and, therefore, be available for binding to gangliosides [20]. Taken together, these results suggest that the carboxyl terminus of H_C contains critical residues for binding to cells. In order to further characterize the G_{T1b} binding site of H_C, we have generated a panel of 11 monoclonal antibodies specific for TeNT H_C. These antibodies were used in both cross-blocking experiments and G_{T1b} binding inhibition assays to define different epitopes on H_C. Two previously defined anti-H_C mAbs, 18.1.7 and 18.2.12.6, which block and enhance binding to G_{T1b} respectively, were also included in the analysis.

Our data showed that the 13 mAbs use 4 different V_H–V_L combinations but that mAbs with similar V_H–V_L pairs can bind distinct H_C epitopes and have opposite affects on the binding of to Hc to ganglioside (35F7 and 18.2.12.6). 35F7 blocks H_C binding to ganglioside G_{T1b} while 18.2.12.6 enhances binding. This difference is likely due to the usage of different V region genes from the same V_H and V_L families.

Another set of 3 mAbs (72B9, 81H10, and 18.1.7) block each other from binding H_C and express related V_H genes. One mAb in this set (72B9) utilizes a different Vκ family and is unable to block binding of H_C to ganglioside G_{T1b} while the other mAbs can. In this case, the three mAbs use members of the V_H36–60 family (18.1.7 uses a distinct member of this family) but 72B9 uses a Vκ4/5 L chain while the others utilize the Vκ19/28 family. Thus, while the antibody heavy chain must contribute to overall antigen recognition, the light chain may play a more important role in the specificity of binding. These data suggest that 72B9 recognizes a distinct epitope from 81H10 and 18.1.7 that lies too close, either linearly or spatially, to distinguish the epitope by cross-blocking. All five groups

Table 2
Cross-blocking of fragment C-specific mAbs

	Biotin Labeled Antibody						
	V_HQ52N,Vκ12/13		V_HJ558,Vκ9A		V_H36–60, Vκ19/28		V_H36–60, Vκ4,5
Labeled antibody	5E4	87C10	35F7	18.2.12.6	81H10	18.1.7	72B9
Blocking antibody							
V_HQ52N,Vκ12/13							
5E4	+	+	−	−	−	−	−
19G1	+	+	−	−	−	−	−
24D3	+	+	−	−	−	−	−
27H3	+	+	−	−	−	−	−
42C10	+	+	−	−	−	−	−
64B9	+	+	−	−	−	−	−
76A8	+	+	−	−	−	−	−
87C10	+	+	−	−	−	−	−
V_HJ558,Vκ9A							
35F7	−	−	+	−	±	±	±
18.2.12.6	−	−	−	+	−	−	−
V_H36−60, Vκ19/28							
81H10	−	−	±	−	+	+	+
18.1.7	−	−	±	−	+	+	+
V_H36−60, Vκ4,5							
72B9	−	−	±	−	+	+	+
V_HQ52, Vκ1							
12C11	−	−	−	−	−	−	−

of mAbs likely recognize linear epitopes, however, as all react with recombinant H_C by Western blotting (data not shown).

The requirement for TeNT binding to gangliosides as a first step in intoxication has recently been confirmed [9,10]. Of the five epitopes defined by our set of mAbs, three block binding of H_C to ganglioside G_{T1b}. This blocking activity could be due to mAbs interfering with the direct binding of H_C to ganglioside G_{T1b} or alternatively, the epitopes defined by these mAbs together form one ganglioside binding site or may represent independent ganglioside binding sites. It would be important to determine if all ganglioside binding sites are required for subsequent internalization of the toxin or if one binding site is sufficient. These mAbs will be useful as probes of H_C residues responsible for ganglioside binding.

The remaining two epitopes, defined by 18.2.12.6 and 72B9 are not involved with fragment C binding to ganglioside. As we and others have shown, 18.2.12.6 enhances binding to ganglioside G_{T1b} [15,21]. The nature of this enhancement is not understood but may be due to a conformational change in H_C upon antibody binding. Alternatively, 18.2.12.6 may be able to bind two molecules of H_C and orient them in such a way that one does not stearically hinder the other from binding to gangliosides [21]. Both 18.2.12.6 and 72B9 are of potential interest as they may recognize epitopes involved with other functions of H_C such as protein receptor binding or internalization. Further study of these mAbs could elucidate the specific binding of TeNT to neurons.

Finally, all mAbs were inhibited by a polyclonal guinea pig anti-tetanus serum from binding to H_C. As this serum was raised for lot release testing of a commercial tetanus vaccine, these results suggest that the epitopes defined by this set of mAbs may be important for immunity to tetanus. Work is underway to define these epitopes.

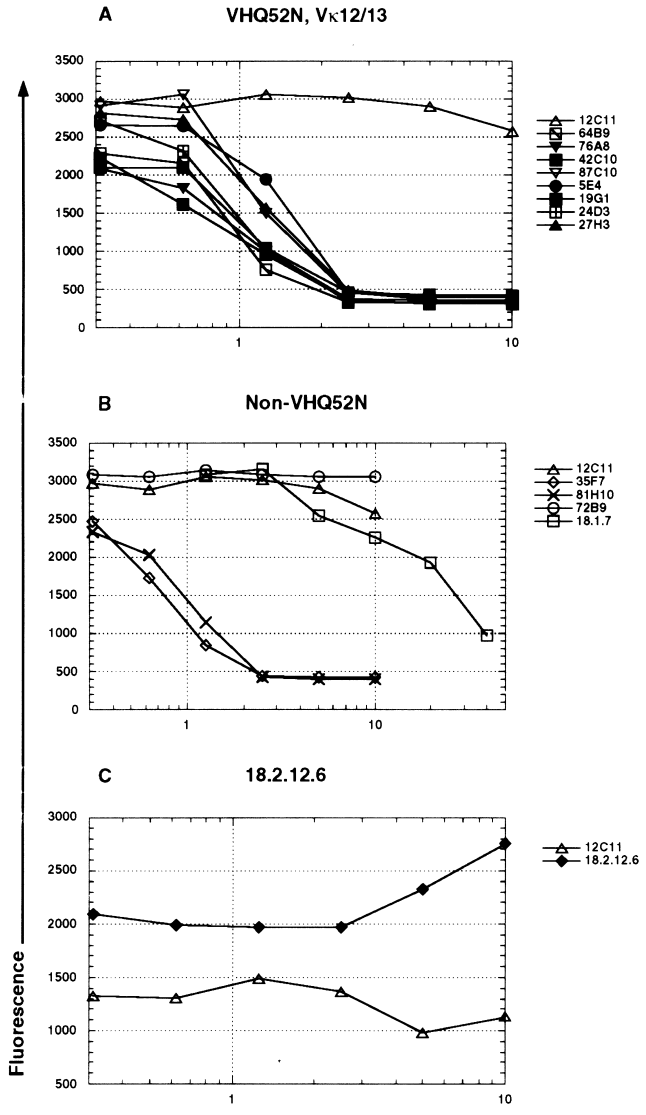

Fig. 3. Inhibition or enhancement of H_C binding to ganglioside G_{T1b}. Biotin labeled H_C was incubated with supernatants from (A) V_HQ52N, Vκ12/13 antibodies, (B) non-V_HQ52N, Vκ12/13 antibodies, (C) 18.2.12.6. Each point represents the average of four wells.

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