Interaction of Fragments B and C of Tetanus Toxin with Neural and Thyroid Membranes and with Gangliosides*

Nicholas P. Morris, Eduardo Consiglio, and Leonard D. Kohn
From the Section on Biochemistry of Cell Regulation, Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20005

William H. Habig† and M. Carolyn Hardegree
From the Bacterial Toxins Branch, Division of Bacterial Products, Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland 20205

Torsten B. Helting§
From the Behringwerke AG, D-3550 Marburg/Lahn, Federal Republic of Germany

Fragment C of tetanus toxin appears to retain most of the determinants of the tetanus toxin molecule insofar as interactions with neural or thyroid membranes and with gangliosides are concerned. Thus, unlabeled fragment C is nearly as effective as unlabeled tetanus toxin in inhibiting the binding of either 125I-labeled toxin or 125I-labeled thyrotropin to neural or thyroid membranes. 125I-labeled fragment C interactions with neural membranes are effectively the same as 125I-labeled tetanus toxin when studied as a function of membrane protein, salt concentration, or pH. Both bind reversibly and have the same specificity for unlabeled ligands. Both interact similarly with gangliosides whether the gangliosides are in aqueous suspensions or imbedded in liposomes, or whether examined as a function of ganglioside concentration or individual ganglioside specificity. Modulation of the lipid composition of liposomes containing gangliosides does not affect the binding of either tetanus toxin or fragment C, although it does alter thyrotropin binding. Fragment C undergoes retrograde axonal transport like tetanus toxin.

In contrast to fragment C, fragment B of tetanus toxin appears to have none of the determinants of the toxin molecule important for receptor interactions in membrane binding assays. In experiments using ganglioside-containing liposomes, fragment B was also unable to inhibit the binding of tetanus toxin but was noted to slightly inhibit thyrotropin binding. Although fragment B does not undergo retrograde axonal transport, some diffuse neural uptake was noted in these assays.

Unlike tetanus toxin, neither fragment C nor fragment B has any effect on the membrane potential of synaptosomal preparations.

The extracellular form of tetanus toxin is believed to contain two nonidentical polypeptide chains (1–4). The heavy chain (Mr · 100,000) contains determinants important for the interaction of the toxin with gangliosides (4, 5) and specific cell surface receptors presumed to include gangliosides as a functional or structural component (6–11). No specific functional role has been associated with the light chain (Mr · 55,000) (4, 5). Both chains are atoxic (1–6).

To define further the structure-function relationships of these subunits, the toxin molecule has been subjected to partial proteolytic cleavage in order to obtain additional fragments. In one study, controlled papain digestion of the toxin molecule resulted in the formation of two fragments defined as B and C (4). Fragment C, with a molecular weight of 47,000 (±5%), is a portion of the heavy chain (4). Although it is capable of eliciting antibodies which block neurotoxicity, it is itself atoxic (4, 6). Fragment B, with a molecular weight of 95,000 (±5%), is composed of Mr · 48,000 (±5%) and 45,000 (±5%) peptides joined by one or more disulfide links (4). One of the component peptides of fragment B is derived from the light chain and the other is derived from the heavy chain (4). Fragment B does not cause the typical signs of tetanus, but in high doses (200 μg or more) can induce a neurological disorder in mice (7).

Using a procedure wherein a radiolabeled ganglioside competes for a soluble protein or for tetanus toxin adsorbed to a Sephadex matrix (6), fragment C was shown to interact with gangliosides only minimally by comparison to the native toxin or its heavy chain. Fragment B did not react at all. The poor interaction between gangliosides and fragment C measured by this means (6) was, however, in contrast both to the ability of antibodies to fragment C to inhibit toxin-ganglioside interactions and to the effectiveness of antibodies to fragment C in preventing neurotoxicity (4, 6). These same activities by tetanus antitoxin have been ascribed to an ability to block toxin-receptor interactions (8, 9). The observation in these studies (4, 6) that tetanus toxin interactions with the gangliosides GM1 and GD1b were effectively the same was also confusing, since other studies (10–15), measuring neurotoxicity or tetanus

* The abbreviations used are: GM1, galactosyl-N-acetylgalactosaminyl-[N-acetyleneuraminyl]-galactosylglycosylceramide; GM2, N-acetylgalactosaminyl-[N-acetyleneuraminyl]-galactosylglycosylceramide; GM3, N-acetylneuraminylgalactosylglycosylceramide; GD1a, N-acetylneuraminylgalactosyl-N-acetylgalactosaminyl-[N-acetyleneuraminyl]-galactosylglycosylceramide; GD1b, galactosyl-N-acetylgalactosaminyl-[N-acetyleneuraminyl]-galactosylglycosylceramide; GD2, N-acetyleneuraminylgalactosyl-N-acetylgalactosaminyl-[N-acetyleneuraminyl]-galactosylglycosylceramide; TSH, thyrotropin; TPP+, tetraphenylphosphonium+.

† To whom correspondence should be addressed.
§ Present address, Sandoz Forschungsinstitut Gesellschaft M.B.H., Brunner Strasse 59, A1235 Wien, Austria.
toxin binding to membranes, showed that \( G_{\text{mV}} \) was significantly more (10-fold) effective in its interactive ability than was \( G_{\text{M1}} \). In sum, although studies of its antisera suggested that fragment C could interact with gangliosides and neural receptors, these interactions could not be detected using a Sephadex matrix adsorption procedure.

The present study uses different assay procedures to measure the abilities of fragments B and C to interact with gangliosides or with tetanus toxin receptors on neural and thyroid membranes. It provides data indicating that fragment C retains all or most of the determinants of the tetanus toxin molecule insofar as receptor or ganglioside interactions are concerned and thus clarifies the above discrepancies. The current studies remain consistent with the hypothesis that the tetanus toxin receptor on neural tissues might have structural and functional relationships to the thyrotropin receptor on thyroid membranes (15-19).

We discuss the relevance of these data to the structure and function of the tetanus toxin molecule.

**MATERIALS AND METHODS**

Tetanus toxin, fragments B and C of tetanus toxin, and bovine TSH were purified preparations obtained as described previously (4, 15-21). The TSH had a thyroid-stimulating activity of 25 ± 3 IU/mg of protein in a mouse bioassay (22); the tetanus toxin had approximately \( 10^6 \) minimum lethal doses/mg of protein as determined by subcutaneous injection into mice (15, 23).

Synthetic dipeptidyl-DL-\( \alpha \)-phosphatidylcholine, synthetic dipeptidyl-\( \alpha \)-phosphatidylcholine, and cholesterol were obtained from Sigma Chemical Co. (St. Louis, Mo.).

The bovine brain ganglioside mixture was obtained from the Nutritional Biochemicals Division of ICN Life Sciences Group (Cleveland, Ohio.). Its approximate composition was 45% \( G_{\text{mV}} \), 10% \( G_{\text{M1}} \), 20% \( G_{\text{mI}} \), and 25% \( G_{\text{T}} \). The purified gangliosides \( G_{\text{mV}} \), \( G_{\text{M1}} \), \( G_{\text{mI}} \), \( G_{\text{T}} \), and \( G_{\text{T}} \) were either obtained as described previously (24) or were isolated from commercial preparations (Supelco, Inc., Bellefonte, Pa.) by preparative thin layer chromatography (25). Each ganglioside used in these experiments was at least 99% pure as judged by densitometric analysis of resorcinol-stained chromatograms (24, 25). Gangliosides were quantitated from their sialic acid content using a micromodification of the resorcinol method of Svensson (26).

Tetanus toxoid (1,900 flocculation units/mg of nitrogen) was purchased from Connaught Laboratories (Toronto, Canada). The actual quantity of tetanus toxoid was calculated using a value of 3,000 flocculation units/mg of nitrogen for pure toxoid (27). Diphtheria toxin contained \( 10^6 \) minimum skin reactive doses/mg of protein that was prepared by Sephadex G-150 chromatography of a concentrated (by ultrafiltration) culture filtrate obtained from Lederle Laboratories. The toxoid content was calculated assuming \( 3 \times 10^6 \) minimum skin reactive doses/mg of pure diphtheria toxoid (29, 28). Bovine glucagon and bovine insulin were obtained from Calbiochem (San Diego, Calif.). Bovine prolactin and bovine growth hormone were the highest purity preparations available from the National Institutes of Health Endocrinology Study Section (Bethesda, Md.). Human chorionic gonadotropin was obtained from Dr. Robert E. Canfield, Columbia University College of Physicians and Surgeons (New York).

Plasma membranes were prepared from rat thyroid and rat brains by using published procedures for the isolation of bovine thyroid plasma membranes (21, 30). To prepare control liposomes and liposomes containing mixed brain gangliosides, the procedure described by Kinsky et al. (31) has been used with some modifications (16, 19, 32, 33).

\( ^{125} \text{I} \)-labeled TSH was prepared and had the properties described previously (21). \( ^{125} \text{I} \)-labeled tetanus toxin and \( ^{125} \text{I} \)-labeled fragments B and C were prepared using the Bolton-Hunter procedure, also as described previously (34). The neurotoxic activity of the iodinated toxoid was about 80% of that of the native toxoid. The radiiodinated toxoid and preparations of fragments B and C co-migrated with their native counterparts in disc gel analysis (35) and had specific radioactivities of 50 to 150 mCi/nmol. The specific radioactivity of TSH was 200 to 400 mCi/nmol.

The binding of \( ^{125} \text{I} \)-labeled toxoid toxin, TSH, fragment B, or fragment C to membranes or to liposomes was assayed by using procedures similar to those described previously (15, 21, 30, 31, 33), i.e. membranes or liposomes were incubated with the iodinated hormone or toxoid in a 100-μl volume containing 0.025 M Tris/acetate, pH 6.0, and 0.08 bovine albumin (5 times recrystallized). Control incubations contained all components except membranes or all components plus a 6,000-fold excess of the unlabeled hormone or toxoid. Unless otherwise noted, standard incubations were for 1 h at 2-4°C, after which the membrane-hormone or membrane-toxoid complex was isolated by filtration through Cellulose filters (EHWP-02500, Millipore Corp., Bedford, Mass.). The filters were washed, dried, and counted as described previously (21, 30). All assays were performed in duplicate or triplicate, and the data are presented as specific counts bound, i.e. control values have been subtracted from experimental values to obtain net counts. Nonspecific binding rarely exceeded 5% of the counts bound in the presence of membranes.

**Fig. 1. Effect of unlabeled fragment B, fragment C, and tetanus toxoid on \( ^{125} \text{I} \)-tetanus toxoid binding to brain membranes (A); \( ^{125} \text{I} \)-tetanus toxoid binding to thyroid membranes (B); and \( ^{125} \text{I} \)-TSH binding to thyroid membranes (C).** In A, membrane protein added in the assay was 0.5 μg; in B and C, membrane protein was 30 μg/assay. \( ^{125} \text{I} \)-Tetanus toxoid added to each assay in A and B was approximately 50,000 cpm; 100% binding was 42,000 cpm. \( ^{125} \text{I} \)-TSH added to each assay in C was 50,000 cpm; 100% binding was 46,000. At concentrations between \( 1 \times 10^{-9} \) and \( 1 \times 10^{-7} \) M, insulin, glucagon, human chorionic gonadotropin (hCG), prolactin, diphtheria toxoid, tetanus toxoid, and growth hormone had no effect on the binding of either \( ^{125} \text{I} \)-labeled tetanus toxoid or \( ^{125} \text{I} \)-TSH to the brain and thyroid membranes. A depicts the average effect of these ligands in this experiment (dashed line without data points); similar results were seen in the experiments detailed in B and C (data not shown).
liquid scintillation spectrometry after the filters were dissolved in Instabray (Yorktown Research, S. Hackensack, N. J.). Corrections and washing were complete within 2 h. Additional washes of unspecific binding were made by diluting the assay buffer 10-fold with assay buffer or with assay buffer containing unlabeled fragment C (1×10⁻⁴ M) or unlabeled toxin (1×10⁻¹ M). The data presented with the unlabeled ligands measure only their effect on ¹²⁵I-labeled tetanus toxin binding. Conditions in all experiments were standard with the exceptions noted; in B through D, assays contained 0.5 μg of membrane protein.

Followed by addition of synaptosomes, filtration, and washing. Values obtained in this manner were subtracted from the data presented. Studies of retrograde axonal transport were performed by an adaptation (16) of the procedure of Price et al. (39).

Protein measurements were made using a colorimetric assay (40); membranes and synaptosome preparations were first digested with NaOH.

RESULTS

The unlabeled fragment C of tetanus toxin inhibits ¹²⁵I-labeled tetanus toxin binding to brain (Fig. 1A) and thyroid (Fig. 1B) membranes nearly as effectively as does unlabeled toxin. It is also nearly as effective as is unlabeled tetanus toxin in inhibiting ¹²³I-TSH binding to thyroid membranes (Fig. 1C). Unlabeled fragment B has, in contrast, no effect on ¹²⁵I-labeled toxin or ¹²³I-TSH binding to brain or thyroid membranes in these experiments (Fig. 1).

As noted in Fig. 1, the effect of fragment C on ¹²⁵I-tetanus toxin binding to neural membranes is specific. Thus, at concentrations 10⁻⁴ to 100-fold higher than unlabeled fragment C or toxin, insulin, glucagon, human chorionic gonadotropin, diphtheria toxin, prolactin, tetanus toxoid, and growth hormone had no effect on the binding of radiolabeled tetanus toxin.

Mouse synaptosomes were prepared using a procedure (36) modified previously in a study of guinea pig synaptosomes (37). Accumulation of [³H]TPP⁺ in these synaptosomes was determined by filtration through Millipore Cellulose filters (0.5 μm) (37, 38). Incubations were carried out at 37°C in reaction mixtures containing Krebs-Ringer phosphate buffer (180 mM NaCl, 3 mM KCl, 2 mM MgSO₄, 10 mM NaH₂PO₄, pH 7.0, 0.01 mM CaCl₂, and 10 mM glucose) and 5 μM [³H]TPP⁺ (final volume, 100 μl). Additions were made as indicated in volumes no greater than 1% of the total assay volume. Reactions were initiated by the addition of 5 μl of the synaptosome suspension (50 to 75 μg of protein) and terminated at given times by the rapid addition of 2.0 ml of ice-cold 0.8 M NaCl, followed by immediate filtration and washing with an additional 2.0 ml of the same solution. Dilution, filtration, and washing were complete within 10 s. Radioactivity was assayed by liquid scintillation spectrometry after the filters were dissolved in Instabray (Yorktown Research, S. Hackensack, N. J.). Corrections for nonspecific adsorption to the filters were made by diluting the reaction mixtures with 0.8 M NaCl prior to addition of synaptosomes.

#### Table I

<table>
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Fig. 4. Binding of 125I-labeled toxin, 125I-labeled fragment C, and 125I-TSH to synthetic liposomes. A, binding of 125I-labeled tetanus toxin and 125I-labeled fragment C to liposomes containing mixed brain gangliosides in a dipalmitoyl phosphatidylcholine matrix as a function of liposome concentration. Stock liposome preparations were diluted 5-fold before being added to these assays, i.e., there were 1 x 10^6 particles/50 μl of liposome suspension. The 125I-labeled toxin and fragment C were added at the same concentrations used in Fig. 2. Unlabeled fragment B or C was present at 5 x 10^{-7} M in a duplicate set of assays. Standard binding conditions were used (see “Materials and Methods”); the moalr ganglioside to phospholipid ratio was 0.08.

125I-labeled fragment C binds to brain membranes as well as 125I-labeled tetanus toxin when studied as a function of membrane protein in the assay (Fig. 2A). In addition, the binding of 125I-tetanus toxin or radiolabeled fragment C to brain membranes is equally salt-sensitive at low membrane concentrations (0.5 μg of membrane protein/assay) (Fig. 2B) and salt-insensitive at high membrane concentrations (>20 μg of membrane protein/assay) (data not shown but similar to that for toxin presented in Ref. 16). As a function of pH, 125I-labeled fragment C binding at low membrane concentrations has a broader pH optimum than does 125I-labeled toxin (Fig. 2C); a broad optimum for the radiolabeled toxin has, however, been observed in assays with higher membrane concentration (16).

The 125I-labeled fragment C bound to membranes is more susceptible to reversal by dilution in buffer than is 125I-labeled toxin (Fig. 2D); unlabeled fragment C is, however, effectively equipotent with unlabeled toxin in reversing 125I-labeled toxin binding in dilution experiments using buffers containing the cold ligand (Fig. 2D). Unlabeled TSH enhanced 125I-labeled fragment C binding to neural membranes in the same manner detailed previously (13) for 125I-labeled tetanus toxin (data not shown). 125I-labeled fragment C binding was otherwise as specific as was 125I-labeled tetanus toxin binding (see Fig. 1A).

Gangliosides inhibit 125I-labeled toxin and 125I-labeled fragment C binding to neural membranes. The inhibition is quantitatively similar for the two labeled ligands (Fig. 3) and the relative potency of individual gangliosides is the same (Table I).

Both radiiodinated ligands can also bind to gangliosides which are imbedded in liposomes. The binding of the toxin and fragment C showed a similar dependency on liposome concentration (Fig. 4A). Fragment B (2.5 x 10^{-7} M) had no effect on the binding of either the 125I-labeled toxin or the 125I-labeled fragment C (Fig. 4A). Unlabeled fragment C (2.5 x 10^{-7} M), in contrast, was able to completely inhibit the binding of either its radiolabeled counterpart or radiolabeled toxin (Fig. 4A).

In a previous report (19), alterations in the lipid matrix of the liposome, i.e., a change from dipalmitoyl phosphatidylcholine to dioleoyl phosphatidylcholine, altered the relative binding properties of TSH and tetanus toxin to the glycoprotein component of the TSH receptor isolated from bovine thyroid membranes and imbedded in liposomes. Thus, TSH binding increased 2- to 4-fold, whereas tetanus toxin binding stayed the same or decreased slightly. The same data were obtained using liposomes containing gangliosides as the “receptor” component (Fig. 4, B and C). 125I-labeled fragment C binding, like that of the 125I-labeled toxin, was unperturbed by the change in matrix phospholipids whether liposomes containing ganglioside or glycoprotein as the receptor component were tested (data not shown).

Fragment C and tetanus toxin inhibited the binding of 125I-TSH to both ganglioside-containing liposomes (Fig. 4D) and to biological membrane preparations (Fig. 1C). Fragment B was able to inhibit slightly 125I-TSH binding to these liposomes (Fig. 4D) but not to membrane preparations (Fig. 1C).

In separate reports (17, 37), we showed that tetanus toxin could perturb the membrane potential of guinea pig synapomome preparations. In these experiments, the permeant lipophilic cation TPP+ was used as the potential probe. These studies showed that tetanus toxoid could not perturb the
potential and that tetanus antitoxin could block the toxin effect. Tetanus toxin, but not fragments B and C, was also able to alter the membrane potential of mouse synaptosome preparations (Table II).

Tetanus toxin is believed to enter the central nervous system by a process known as retrograde axonal transport (39, 41–43). As noted in Fig. 5, fragment C undergoes retrograde transport in a manner similar to that of intact tetanus toxin. Fragment B does not similarly accumulate in the first distal segment to the ligation but does appear to be taken up or internalized in a diffuse pattern (Fig. 5).

**DISCUSSION**

In the present study, we show that the fragment C portion of the heavy chain of tetanus toxin contains many or even most of the determinants on the intact toxin molecule insofar as interactions with membrane receptors and gangliosides are concerned. Thus, fragment C inhibits toxin or TSH binding to neural and thyroid receptors nearly as well as toxin itself (Fig. 1). Fragment C and tetanus toxin have effectively identical properties of binding to neural membranes (Fig. 2) and are similar in their abilities to undergo retrograde axonal transport (Fig. 5). Fragment C interacts with gangliosides in a manner effectively the same as the holotoxin whether the gangliosides are in micellar suspensions or imbedded in liposomes (Figs. 3 and 4; Table I).

The current results can provide an explanation for previous observations (4, 6) that antibodies to fragment C will block both the neurotoxicity of tetanus and ganglioside-toxin interactions. Fragment C, by retaining most or many of the determinants on the holotoxin molecule important for receptor interactions, can elicit antibodies to these determinants. These antibodies, like those present in tetanus antitoxin preparations, can block the initial toxin interaction with neural tissue and thereby prevent the consequent neurological disease.

Despite the fact that the current data are in accord with the antitoxin data (4, 6) and despite the fact that they agree with numerous previous studies of toxin-ganglioside interactions (5, 10–15), it is recognized that the current results, in particular those pertaining to the interaction of fragment C with gangliosides, are in sharp contrast to previous results using a technique wherein the soluble fragment C competes for a radiolabeled ganglioside with tetanus toxin adsorbed to a Sephadex matrix (6). That study showed that equivalent competition for a ganglioside would require well over a 150-fold higher concentration of fragment C than tetanus toxin, by comparison to the less than 2-fold higher concentrations required in radioreceptor studies in this report. That study (6) also showed that tetanus toxin interactions with Gαq and Gβγδ were equivalent, whereas in this and other reports (5, 10–15), 10-fold greater specificity was shown by Gαb.

This discrepancy may have its basis in the results of a recent study of TSH-ganglioside interactions (44). In that study, the conclusion was drawn that the initial TSH-ganglioside association reaction was dominated by charge-charge interactions, whereas the dissociation reaction was dominated by short range forces, most likely hydrophobic. Attention was drawn to the fact that formation of the TSH-ganglioside adduct most likely placed the glycerol-like moiety on the sialic acid residues of the ganglioside (Cα, Cβ, Cα) in an apolar environment and that hydrogen bonding involving the glycerol moiety could be an important component of these short range forces. The inference to the tetanus data is that a Sephadex matrix might provide additional interaction sites for the ganglioside once it has associated with tetanus toxin and that these sites involve hydrogen bonding. Support for such an inference already exists in the observation that the ganglioside-toxin interaction measured by the Sephadex adsorption procedure is sensitive to urea and methanol but not to high concentrations of salts (4, 6). The influence of the Sephadex matrix on ganglioside-ligand interactions is not without precedent. Sephadex-ganglioside interactions with interferon have thus far precluded the use of gangliosides as an affinity tool for interferon purification. In sum, study of the influence of the Sephadex matrix on the ganglioside-toxin interaction may provide insight concerning the influence of other membrane components on receptor-ligand interactions.

Several other points should be noted in conclusion. First, in a previous report (19), we showed that fatty acid changes in the phospholipid matrix could modulate receptor expression by the glycoprotein component of the TSH receptor and that this effect was ligand-specific, i.e. this effect was not seen with tetanus toxin. This report shows that the same phenomena hold for gangliosides used as receptor analogs. Second, the absence of an effect of fragment C on synaptosome membrane potential, despite good binding under these conditions, emphasizes once again the dichotomy between binding and function. Third, the ability of fragments B and C to similarly perturb TSH and tetanus binding to thyroid membranes and ganglioside liposomes again emphasizes the potential relationship in the structure and function of TSH and tetanus receptors (15–19, 45, 46). Finally, we believe that the ability of fragment C to undergo retrograde axonal transport confirms previous results by Dumas et al. (47), who used a fragment derived from tetanus toxin by different procedures.

**REFERENCES**


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*Personal communication by Dr. Christian B. Anfinsen, Laboratory of Chemical Biology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Md.*

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