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Characterization of Fragment C and Tetanus Toxin Binding to Rat Brain Membranes

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SUMMARY

The binding characteristics of ^{125}I-labeled tetanus toxin and ^{125}I-labeled fragment C to rat brain membranes have been studied. Fragment C is a proteolytically derived portion of the holotoxin. Tetanus toxin is slightly more potent than fragment C in displacing ^{125}I-labeled tetanus toxin bound to rat brain membranes. Conversely, fragment C is more effective than toxin in displacing bound ^{125}I-labeled fragment C. However, the binding curves for the two compounds are in both cases parallel, suggesting that they compete for the same site. The equilibrium affinity constants for the binding of tetanus toxin and fragment C as obtained by both direct and indirect binding experiments were found to be in the nanomolar range (2–12 nM). This relatively high affinity is in contrast with the large number of binding sites (estimated as 2–9 nmoles/mg of protein). This concentration is consistent with the reported content of the gangliosides, G_{T1} and G_{D1b}, in rat brain. The specificity of the toxin binding site has been tested; a number of putative neurotransmitters as well as inhibitors of axonal transport were unable to affect the binding at concentrations several orders of magnitude higher than the toxin or fragment C. The anticonvulsant drug diazepam, reported useful in the treatment of human tetanus, was equally inactive in displacing ^{125}I-labeled tetanus toxin binding. The possible pharmacological relevance of the binding site for tetanus toxin and this fragment is discussed.

INTRODUCTION

Tetanus toxin is one of the most toxic materials known to man. It is a protein composed of two subunits of nonidentical polypeptide chains: a heavy chain of approximately 100,000 daltons and a light chain of 50,000 daltons (1, 2). Neither subunit alone is toxic, although the heavy chain retains the ability to bind to specific gangliosides (3). Papain digestion of the holotoxin can cleave the heavy chain to produce an atoxic fragment (fragment C) of about 47,000 daltons (2) which retains ganglioside-binding properties (4, 5). The remainder of the heavy chain with the attached light chain is referred to as fragment B (2).

Wasserman and Takaki (6) demonstrated in 1898 that tetanus toxin could be adsorbed to brain homogenates. Since then the interaction of tetanus toxin with brain binding sites has been widely studied (reviewed in ref. 7). However, the target organ for tetanus toxin is thought to be the gray matter of the spinal cord; toxin does not ordinarily reach the forebrain and cerebellum, although profound behavioral changes follow microinjection of this protein into the brain (8, 9).

The exact mechanism by which this toxin acts is not known, although there is evidence that the release of many centrally acting neurotransmitter substances such as glycine, GABA,[4] noradrenaline, and acetylcholine is affected (10, 11).

In this paper we characterize the binding of tetanus toxin and fragment C to brain membranes with respect to affinity and receptor population and their interaction with putative transmitters and drugs that could affect neural function.

MATERIALS AND METHODS

Homogeneous tetanus toxin was prepared by the method previously outlined (12). Tetanus toxin had approximately 2×10^7 minimum lethal doses per milligram of protein as determined by subcutaneous injection into 15- to 18-g mice. Fragment C was obtained from Dr. Torsten Helting (Behringwerke AG, Marburg/Lahn, Federal Republic of Germany). Iodination of the holotoxin and fragment C were performed using the Bolton

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[4] The abbreviation used is: GABA, γ-aminobutyric acid.

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and Hunter reagent (13) (New England Nuclear Corporation, Boston, Mass.; 1500 Ci/mmole). This iodination method has yielded a labeled toxin with physicochemical and biological properties very close to those of the native molecule (14), and its use for the study of brain binding sites has already been reported (5). The purity of the labeled toxin and its fragment was assessed by gel electrophoresis: over 95% of the radioactivity comigrated with the native protein. The specific radioactivity of both labeled proteins was 1–2 μCi/μg of protein. The iodinated toxin retained over 75% of its original toxicity. One minimum lethal dose of freshly prepared ^{125}I-labeled tetanus toxin corresponds to about 300 cpm.

Diazepam was obtained from Roche Laboratories (Nutley, N. J.) [*methyl*-^{3}H]Diazepam (60 Ci/mmole) was obtained from New England Nuclear Corporation. Vinblastine and colchicine were obtained from Eli Lilly and Company, (Indianapolis, Ind.). All other compounds were reagent grade or higher.

Membrane preparation and binding assay. Adult rats (Osborne-Mendel, 300–350 g) or adult guinea pigs (Hartley, 300–350 g) were decapitated and the brains were quickly excised and collected in ice-cold buffer (5 mM Tris-acetate, pH 6). Homogenization was carried out in a Teflon-glass apparatus at 4°. The homogenate was centrifuged at 10,000 × *g* for 15 min. The pellet was rehomogenized and centrifuged at 1,000 × *g* for 5 min to remove large debris. The supernatant was carefully aspirated and centrifuged again at 10,000 × *g* for 15 min. The resulting pellet was washed and centrifuged in the same way three more times and, finally, resuspended in a small volume of buffer (about 20 mg of protein per milliliter) and frozen in small aliquots at −70°. Protein was determined according to the method of Lowry *et al.* (15). A similar procedure was employed when membranes from other tissues were prepared. Rat brains were dissected on ice and specific brain regions were identified with the aid of a stereotaxic atlas (16). The tissue was then processed as described for whole brains.

The usual binding assay was performed in triplicate by incubating the labeled protein (20,000–100,000 cpm) and membranes (0.02 μg of membrane protein) together for 1 hr at 0–4° in a total volume of 0.1 ml containing 25 mM Tris-acetate, pH 6.0, and 2.5% bovine serum albumin. These conditions had previously been determined to be nearly optimal for binding with respect to pH, temperature, and ionic strength (5, 17). The membrane-protein complex was isolated by vacuum filtration of the incubation mixture through 0.5-μm Millipore membranes, Type EHWP. After five washes with 1 ml each of cold assay buffer, the filters were counted in a Beckman γ counter at an efficiency of 50%. Filter-bound radioactivity (i.e., with no membranes) was usually 1–2% of the total counts added and was used as nonspecific binding. This was found to be an acceptable control at the low membrane protein concentrations routinely used, especially since similar values were obtained in the presence of a large excess (1000-fold or greater) of unlabeled toxin or fragment C. Specific binding is therefore the total radioactivity bound minus the non-specific filter-bound radioactivity.

Data Analysis. Scatchard analysis was performed on data obtained from the displacement curve using fixed amounts of ^{125}I-labeled ligand and varying concentrations of unlabeled ligand (indirect binding), as well as from experiments in which the ^{125}I-labeled ligand was varied (direct binding). Binding parameters (affinity and binding capacity) were obtained by using the computer program SCATFIT. This program calculates a nonlinear least-squares curve fit for a Scatchard model with from two to five parameters, corresponding to one, two, or three classes of binding sites, the third of which is nonsaturable. A choice of the "best model" is provided according to the residual mean square for the different fits and *F*-tests of the decreases in the sums of squares from model to model (18).

RESULTS

Saturation of the binding of ^{125}I-labeled tetanus toxin and ^{125}I-labeled fragment C. The binding of ^{125}I-labeled tetanus toxin to membranes *in vitro* is a saturable process. The specific binding of ^{125}I-labeled tetanus toxin, shown in Fig. 1, began to reach a plateau level when between 1 and 1.5 picomoles of radioligand were added per assay, i.e., 10–15 nM. The plateau level was stable with up to 0.5 μM added toxin. Half-maximal binding was detected at about 2–3 nM labeled toxin. Figure 2 shows the displacement of bound ^{125}I-labeled tetanus toxin (0.35 nM) with native "cold" toxin. Half-maximal displacement (IC_{50}) was obtained at about 5 nM, whereas 1 μM unlabeled toxin reduced the binding to virtually zero. Conventional Scatchard plots of these data are included in the *insets* of Figs. 1 and 2.

When the direct binding data were subjected to computer analysis, the best fit indicated a single population of binding sites with a dissociation constant (K_D) of 2.5 ± 0.3 nM. The binding capacity was 4.0 ± 1.5 nmoles/mg of protein. Computer analysis of indirect binding data (Fig. 2) yielded values in reasonable agreement with the above parameters (see Table 1). This further confirmed that our preparation of ^{125}I-labeled tetanus toxin was

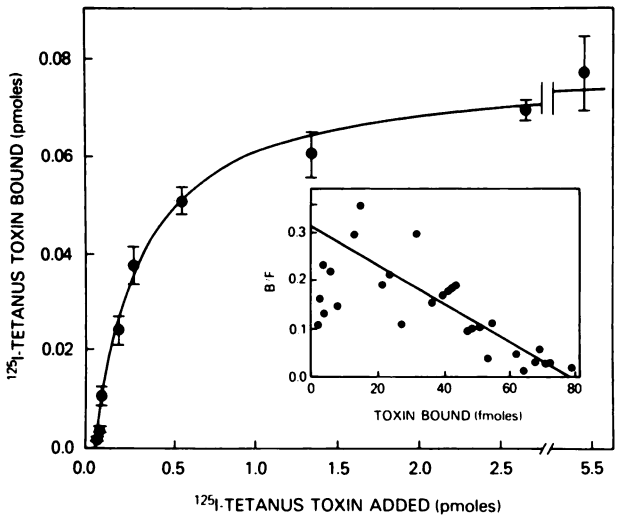

FIG. 1. *Binding of ^{125}I-labeled tetanus toxin to rat brain membranes (0.02 μg in 0.1 ml) after 60 min of incubation at 0–4°*

Each *point* represents the mean ± standard error for three determinations. The lines were computer-drawn with the use of the SCATFIT program.

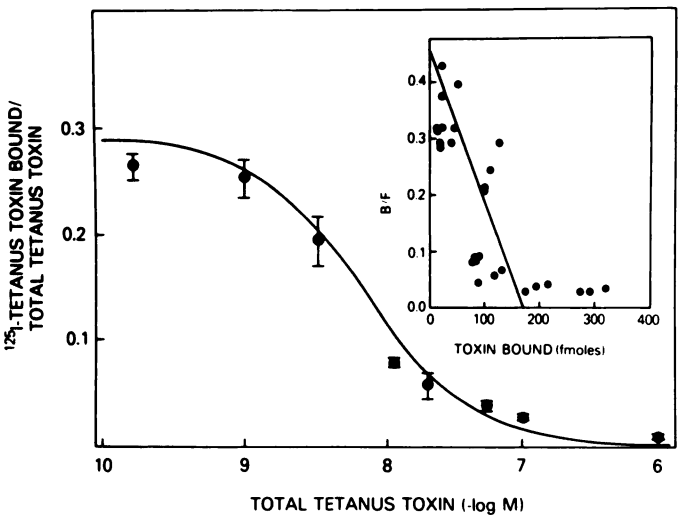

FIG. 2. *Displacement of ^{125}I-labeled tetanus toxin by unlabeled tetanus toxin*

The radiolabeled toxin (0.35 nM) was incubated with 0.02 μg of rat brain membranes in the presence of different amounts of cold toxin. Each *point* represents the mean ± standard error for three determinations. The lines were computer-drawn with the use of the SCATFIT program.

TABLE 1

Constants for the interaction of ^{125}I-labeled tetanus toxin and ^{125}I-labeled fragment C with brain membranes from rats

The K_D and receptor (R) values were determined with the use of SCATFIT program. Best fit was obtained with a two-parameter model assuming one K_D and one receptor population.

Method	^{125}I-labeled tetanus toxin		^{125}I-labeled fragment C	
	K_D	R	K_D	R
	nM	*nmoles/mg protein*	*nM*	*nmoles/mg protein*
Direct binding of ligand	2.5 ± 0.3	4.0 ± 1.5	12.8 ± 2.4	9.0 ± 0.5
Displacement by unlabeled ligand	4.1 ± 0.7	8.5 ± 1.0	2.9 ± 1.2	1.5 ± 0.5

indistinguishable from the native protein, at least insofar as binding to membrane receptors is concerned.

The binding of ^{125}I-labeled fragment C to rat brain membranes showed characteristics of saturability, half-maximal binding, and a specific to nonspecific ratio very similar to those of ^{125}I-labeled toxin (data not shown). Computer analysis of ^{125}I-labeled fragment C binding gave a single population of binding sites. The calculated parameters are shown in Table 1.

Kinetics of ^{125}I-labeled tetanus toxin binding. The rate of association of ^{125}I-labeled tetanus toxin with rat brain membranes is shown in Fig. 3A. Binding approached a plateau at about 15 min and reached equilibrium at 60 min. Half-maximal binding appeared to be attained at about 7 min. When a 10-fold lower tissue concentration was used, the level of binding at equilibrium was substantially reduced, but the times for half-maximal binding remained similar. The association rate constant, (k_1), calculated from the first two points of the binding curve using the integrated rate equation, was 0.733×10^6 M^{-1} sec^{-1}.

The dissociation rate of ^{125}I-labeled tetanus toxin from the rat brain binding site was studied by incubating the membranes at 4° for the equilibrium binding time (60 min) and then adding 1 μM cold toxin; the ^{125}I-labeled tetanus toxin remaining bound was measured at different time intervals (Fig. 3B). The dissociation had a half-time of 6 min and was almost maximal after 30 min.

The equilibrium constant, as calculated from the ratio of the two rate constants (k_{-1}/k_1), where k_{-1} is 1.92×10^{-3} sec^{-1}, had a value (2.6 nM) in reasonable agreement with that estimated from saturation plots.

Mutual competition of tetanus toxin and fragment C binding. The displacement curves of inhibition of ^{125}I-labeled tetanus toxin binding by tetanus toxin and fragment C appeared to be parallel over their full length (Fig. 4A). Tetanus toxin produced half-reduction of binding (IC_{50}) at 4.5 nM, a value close to the calculated equilibrium affinity constant; fragment C was weaker than tetanus toxin in inhibiting the ^{125}I-labeled tetanus toxin binding and had an IC_{50} of 15 nM. Values for maximal

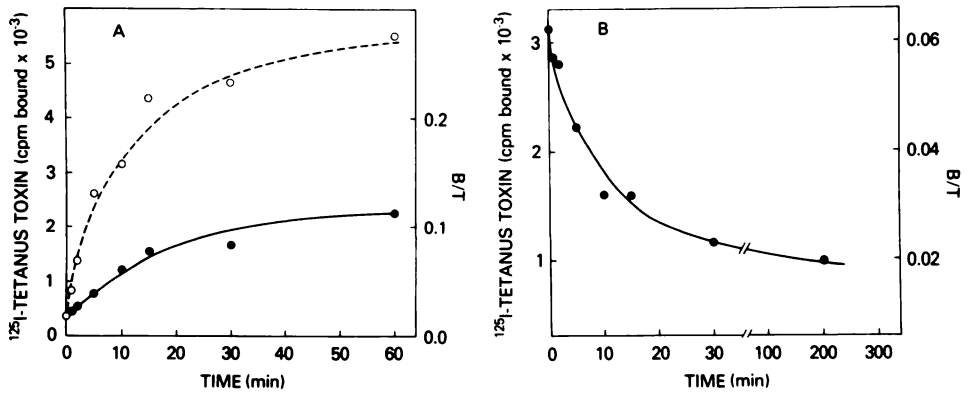

FIG. 3. *Rate of binding and displacement of ^{125}I-labeled tetanus toxin and rat brain membranes*

A 1-ml reaction mixture was prepared for these assays, and at the appropriate time 100 μl were removed for determination of the counts per minute bound.

A. Brain membranes at concentrations of 2 μg/ml (○) and 0.2 μg/ml (●) were mixed with toxin, 220,000 cpm/ml (0.4 nM).

B. After incubation of membranes (0.2 μg/ml) and ^{125}I-labeled tetanus toxin (0.8 nM) after 1 hr, a 200-fold excess of cold toxin was added at time zero. All data are the means of duplicate determinations.

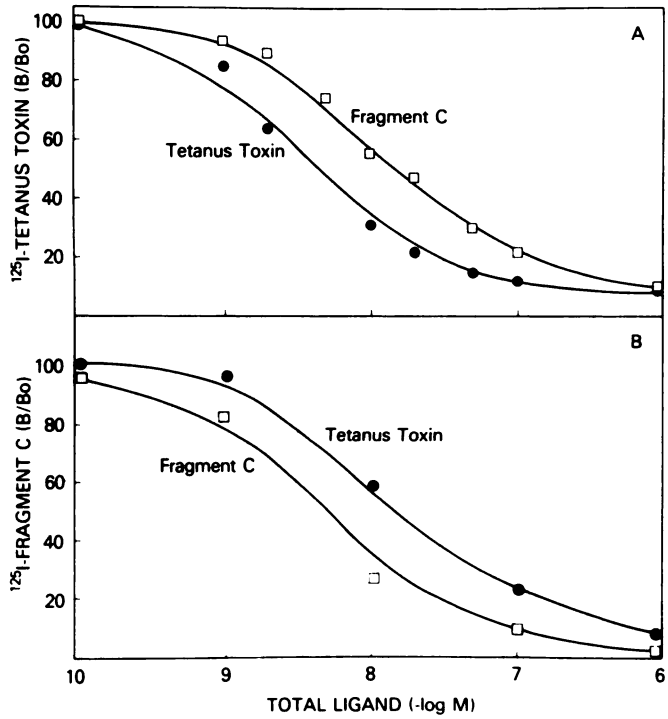

FIG. 4. *Competitive binding curves for ^{125}I-labeled tetanus toxin and ^{125}I-labeled fragment C using the homologous unlabeled proteins*
All data are the means of duplicate determinations.

A. Inhibition of ^{125}I-labeled tetanus toxin binding by tetanus toxin and fragment C.

B. Inhibition of ^{125}I-labeled fragment C binding by tetanus toxin and fragment C.

displacement by tetanus toxin and fragment C were similar and corresponded to the maximal binding capacity of the two ligands.

When the inhibition of ^{125}I-labeled fragment C binding was examined, the displacement dose-response curves for nonradiolabeled fragment C and tetanus toxin were again parallel (Fig. 4B), but here fragment C appeared to be more potent (IC_{50} = 3.5 nM) than toxin (IC_{50} = 16 nM). Again the two dose-response curves reached a plateau at concentrations of unlabeled ligands consistent with their respective binding capacities.

Therefore, although the two ligands appeared to bind to the same membrane site (as indicated by parallelism of the dose-response curves), there were small differences between their affinities for ^{125}I-labeled tetanus toxin and ^{125}I-labeled fragment C binding sites. The different affinities measured by the different techniques do not negate the possibility that the binding sites are on the same macromolecule and perhaps even comprise the same binding region. The ligand in excess could determine the conformation of the receptor and therefore the affinities for different ligands.

Tissue specificity of ^{125}I-labeled tetanus toxin binding. Figure 5 shows the binding of ^{125}I-labeled fragment C to membrane preparations of guinea pig brain, adrenal, and liver. The ligand appears to show tissue specificity in that it binds to brain better than it does to adrenal or liver membranes. However, significant binding can still be detected in the latter two tissues, especially if large amounts of membrane are used.

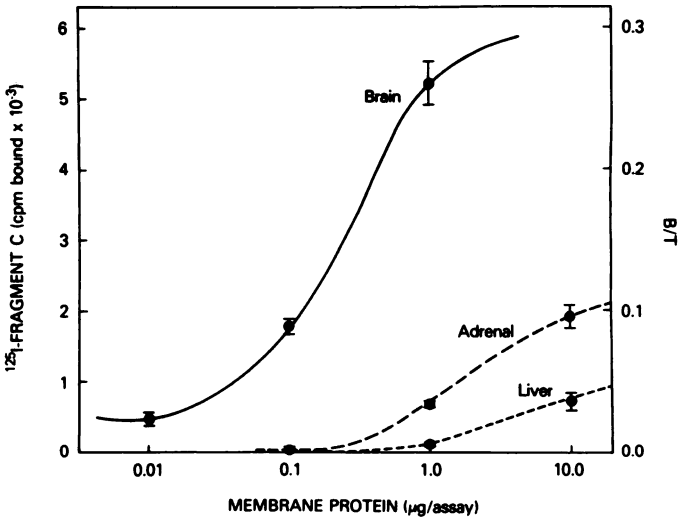

FIG. 5. *Binding of ^{125}I-labeled fragment C to guinea pig tissue membranes*
Each *point* represents the mean ± standard error for three determinations.

Table 2 shows the regional distribution of ^{125}I-labeled tetanus toxin binding in the central nervous system of rats. The highest binding was observed in cerebellum and frontal cortex, the lowest in medulla oblongata and spinal cord. However, the differences in binding are rather small: the distribution of binding sites through the areas that we have examined varies less than 3-fold.

Pharmacological specificity of the tetanus toxin binding site. We have used a variety of neurotropic agents to probe the specificity of the tetanus toxin binding site. Ligands were directly added in the standard binding assay for ^{125}I-labeled tetanus toxin as described under Materials and Methods. Two final concentrations were used (10^{-7} and 10^{-5} M) except when otherwise stated (Table 3). None of the tested compounds significantly affected toxin binding.

We have also studied the effect of the toxin on [^{3}H]-diazepam binding to rat brain membranes as well as the effect of toxin on the GABA and halide-mediated stimulation of [^{3}H]diazepam binding (19). No effects were

TABLE 2

Binding of ^{125}I-labeled tetanus toxin to rat neural tissue

Sample	% Bound
Expt. 1[a]	
Cerebellum	11.4 ± 0.3
Frontal cortex	10.4 ± 0.8
Hypothalamus	9.1 ± 0.5
Striatum	8.1 ± 0.5
Medulla oblongata	4.3 ± 0.5
Expt. 2[b]	
Whole brain	27
Spinal cord	12

[a] Binding experiments were performed with 0.02 μg of membrane protein per assay, and 60,000 cpm of toxin were added; results of three assays per tissue are shown.

[b] Binding experiments were performed with 0.1 μg of membrane protein per assay, and 50,000 cpm of toxin were added; results of two assays per tissue are shown.

TABLE 3

Effect of selected compounds on ^{125}I-labeled tetanus toxin binding

^{125}I-Labeled tetanus toxin (40,000 cpm) was incubated with rat brain membranes (0.02 μg) and the indicated concentrations of the ligands. The value for the filter blank, i.e., no membranes, was 222 ± 6 cpm. Values for counts per minute bound represent the mean ± standard error for three determinations.

Ligand	M	cpm bound	M	cpm bound
Unlabeled toxin	0	6730 ± 681	10^{-7}	1231
(control)	10^{-8}	2545	10^{-6}	579
Glycine	10^{-7}	6601 ± 469	10^{-5}	6701 ± 534
GABA	10^{-7}	6730 ± 346	10^{-5}	6538 ± 306
Diazepam	10^{-7}	7120 ± 96	10^{-5}	6236 ± 475
Serotonin	10^{-7}	7667 ± 265	10^{-5}	7280 ± 370
Epinephrine	—	—	10^{-5}	6296 ± 676
Norepinephrine	10^{-7}	7232 ± 371	10^{-5}	5302 ± 544
Succinylcholine	10^{-7}	6444 ± 236	10^{-5}	5862 ± 557
Vinblastine	10^{-7}	5880 ± 281	10^{-5}	6242 ± 312
Colchicine	—	—	10^{-5}	6456 ± 750

detected at concentrations of the toxin up to 1 μM (data not shown).

DISCUSSION

The relatively low concentration of brain membranes used in the present study (0.02 μg of membrane protein per assay) was chosen to increase the specificity of the binding observed as well as to decrease the effect of membrane-membrane interactions. At 0.02 μg of membrane protein, the receptor concentration is approximately 1 nM; since this concentration is less than the K_D, the values obtained should be meaningful. As shown in Fig. 5, binding of fragment C to adrenal or liver membranes was not detectable at these low concentrations. The concentration of brain membranes has previously been shown to influence the salt, pH, and temperature optima for binding (17). Competitive binding studies (using unlabeled toxin to displace ^{125}I-labeled toxin from rat brain membranes) performed with 0.02 or 0.5 μg of membrane per assay indicated a 10-fold higher K_D at the latter concentration (data not shown). Thus, the conditions chosen to study binding *in vitro* can influence the data obtained.

In the present study we have extended previously reported (5) similarities in the binding of ^{125}I-labeled tetanus toxin and ^{125}I-labeled fragment C to rat brain membrane preparations. The binding of the holotoxin or fragment C is a saturable process with affinity constants in the nanomolar range. This is similar to the value recently reported for binding of this toxin to bovine cortex membranes (20). However, the estimated concentration of binding sites is unusually high and seems unlikely to represent the small population of critical receptors that might be expected to mediate the molecular action of such a potent toxic agent. On the other hand, the bioeffect should be a function of not only the affinity constant but also the concentrations of toxin and receptor. It may be that this large receptor population is crucial to the high potency (0.6 fmole will kill a mouse) of tetanus toxin. This large receptor population is consistent with the brain content of the gangliosides G_{D1b} and G_{T1} (21), previously shown to interact best with tetanus toxin (12, 22) and fragment C (14). Recent experimental data have questioned the essentiality of these gangliosides as functional receptors for tetanus toxin (23), although no alternative receptor structures have yet been identified.

An additional consideration is the fact that tetanus toxin is transported and perhaps concentrated by the process of retrograde axonal transport. Toxin taken up at peripheral nerve endings moves toward the neuroaxis. Thus, high-affinity binding *in vitro* may represent binding not only with a receptor but also with a specific transport site. Whether our data represent interaction with a pharmacokinetic and/or pharmacodynamic site is unclear.

Fragment C and the holotoxin appear to compete for the same site on the membrane, as was demonstrated by the parallelism of their mutual displacement curves. Each of these ligands was better able to displace itself than the heterologous ligand. This action might reflect the effects of high concentrations of ligand on receptor conformation and affinity; alternatively, it suggests that a slightly different recognition mechanism (possibly a nonshared subsite) might be involved in the binding of the two proteins. Fragment C is not toxic, but it does undergo retrograde axonal transport (14). This exemplifies the difficulty in tracing a direct relationship between the ^{125}I-labeled toxin binding site and the receptor mediating the toxic action.

The tetanus toxin binding site in the central nervous system is unevenly distributed, but no sharp differences are observed between the highest and the lowest binding area (Table 2). Toxin binding is greatest in regions rich in gray matter, as has been reported by others (20, 24). Although neural tissue shows the best binding ability for fragment C, a substantial amount of binding can also be detected in other tissues when high concentrations of membranes are used. Previous data have also shown that thyroid membranes have specific binding sites for the toxin and fragment C (5, 12) and, indeed, that toxin has an effect on thyroid function *in vivo* (25).

None of the various chemical agents applied to these membranes could block toxin binding. Of particular interest are colchicine and vinblastine, because these two agents can block both the secretion of neurotransmitters at the presynaptic level and inhibit the retrograde axonal transport of tetanus toxin (26–28). Their inhibition of retrograde transport is thought to be mediated by effects on the microtubular system. As shown in Table 3 they do not reverse the binding of labeled toxin to the membrane site.

In addition, toxin binding was not affected by either diazepam, a drug reported to be beneficial in controlling the convulsions of advanced tetanus in man (7), nor serotonin, a compound reported to bind to gangliosides (29). It is recognized that a common binding site for toxin and drug might not be detected if it occurs at only a few critical receptors which are not recognized as a discrete group in these binding assay. We plan to extend these studies to cell cultures in which the effects of selected drugs on both internalization and binding can be evaluated.

Although the binding site for tetanus toxin shows a high degree of specificity when probed with this variety of drugs and neurotransmitters, previous data have shown that the binding of ^{125}I-labeled tetanus toxin can be significantly perturbed by thyroid-stimulating hormone or cholera toxin (12). Furthermore, cholera toxin and, to a lesser extent, thyrotropin are able to undergo retrograde axonal transport under experimental conditions similar to those used for tetanus toxin (17).

The extent of the similarities in the binding of toxin and fragment C lends encouragement to the study of various hybrids of fragment C. For example, a fragment C-antifragment B hybrid might be useful in delivering antibody to therapeutically useful sites not normally accessible to antibody. The feasibility of constructing functional hybrid proteins has been widely documented. In fact, the converse type of experiment has shown that the toxicity of toxin-antibody complexes can be directed to certain cell types by virtue of the antibody specificity (30).

REFERENCES

1. Matsuda, M., and M. Yoneda. Isolation and purification of two antigenically active, complementary polypeptide fragment of tetanus neurotoxin. *Infect. Immun.* **12**:1147–1153 (1975).
2. Helting, T. B., and O. Zwisler. Structure of tetanus toxin. I. Breakdown of the toxin molecule and discrimination between polypeptide fragments. *J. Biol. Chem.* **252**:187–193 (1977).
3. van Heyningen, S. Binding of ganglioside by the chains of tetanus toxin. *F. E. B. S. Lett.* **68**:5–7 (1976).
4. Helting, T. B., O. Zwisler, and H. Wiegandt. Structure of tetanus toxin. II. Toxin binding to ganglioside. *J. Biol. Chem.* **252**:194–198 (1977).
5. Morris, N. P., E. Consiglio, L. D. Kohn, W. H. Habig, M. C. Hardegree, and T. B. Helting. Interaction of fragments B and C of tetanus toxin with neural and thyroid membranes and with gangliosides. *J. Biol. Chem.* **255**:6071–6076 (1980).
6. Wassermann, A., and T. Takaki. Ueber tetanusantitoxische eigenschaften des normalen centralnervensystems. *Biol. Klin. Wochenschr.* **35**:5–6 (1898).
7. Habermann, E. Tetanus, in *Handbook of Clinical Neurology* (P. J. Vinker and G. W. Bruyn, eds.), Vol. 33, Part 1. Elsevier/North-Holland, New York, 491–547 (1978).
8. Mellanby, J., and G. George. Cephalic tetanus in rats, in *Proc. 4th. Int. Conf. Tetanus*. Lyon, Foundation Merieux, 201–204 (1975).
9. McGeer, P. L., and E. G. McGeer. Use of the neurotoxin agents kainic acid and tetanus toxin in the extrapyramidal system. *Adv. Cytopharmacol.* **3**:437–446 (1979).
10. Osborne, R. H., and H. F. Bradford. Tetanus toxin inhibits amino acid release from nerve endings in vitro. *Nature* (*Lond.*) **244**:157–158 (1973).
11. Bigalke, H., I. Heller, B. Bizzini, and E. Habermann. Tetanus toxin and botulinum A toxin inhibit release and uptake of various transmitters as studied with particulate preparations from rat brain and spinal cord. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **316**:244–251 (1981).
12. Ledley, F. D., G. Lee, L. D. Kohn, W. H. Habig, and M. C. Hardegree. Tetanus toxin interaction with thyroid plasma membranes: implications for structure and function of tetanus toxin receptors and potential pathophysiological significant. *J. Biol. Chem.* **252**:4049–4055 (1977).
13. Bolton, A. E., and W. M. Hunter. The labeling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J.* **133**:529–539 (1973).
14. An der Lan, B., W. H. Habig, M. C. Hardegree, and A. Chrambach. Heterogeneity of ^{125}I-labeled tetanus toxin in isoelectric focusing on polyacrylamide gel and polyacrylamide gel electrophoresis. *Arch. Biochem. Biophys.* **200**:206–215 (1980).
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
16. Pellegrino, L. J., and A. J. Cushman. *A Stereotoxic Atlas of Rat Brain*. Appleton, New York (1967).
17. Lee, G., E. F. Grollman, S. Dyer, F. Beguinot, L. D. Kohn, W. H. Habig, and M. C. Hardegree. Tetanus toxin and thyrotropin interactions with rat brain membrane preparations. *J. Biol. Chem.* **254**:3826–3832 (1979).
18. Rodbard, D. Mathematics of hormone-receptor interaction, in *Receptors for Reproductive Hormones* (B. W. O'Malley and A. R. Means, eds.). Plenum, New York, 289–362 (1973).
19. Tallman, J. F., J. W. Thomas, and D. W. Gallagher. GABAergic modulation of benzodiazepine binding site sensitivity. *Nature* (*Lond.*) **274**:383–384 (1978).
20. Rogers, T. B., and S. H. Snyder. High affinity binding of tetanus toxin to mammalian brain membranes. *J. Biol. Chem.* **256**:2402–2407 (1981).
21. Suzuki, K. The pattern of mammalian brain gangliosides. III. Regional and developmental differences. *J. Neurochem.* **12**:969–979 (1965).
22. van Heyningen, W. E., and J. Mellanby. Tetanus Toxin, in *Microbial Toxins* (S. Kadis, T. C. Montie, and S. J. Ajl, eds.), Vol. IIA. Academic Press, New York, 69–108 (1971).
23. Habermann, E., H. Bigalke, and I. Heller. Inhibition of synaptosomal uptake by tetanus and botulinum A toxin: partial dissociation of fixation and effect of tetanus toxin. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **316**:135–142 (1981).
24. Habermann, E. Interaction of labeled tetanus toxin and toxoid with substructures of rat brain and spinal cord in vitro. *Naunyn-Schmiedebergs Arch. Pharmacol.* **276**:341–359 (1973).
25. Habig, W. H., E. F. Grollman, F. D. Ledley, M. F. Meldolesi, S. M. Aloj, M. C. Hardegree, and L. D. Kohn. Tetanus toxin interactions with the thyroid: decreased toxin binding to membranes from a thyroid tumor with a thyrotropin receptor defect and in vivo stimulation of thyroid function. *Endocrinology* **102**:844–851 (1978).
26. Kryzhanovskii, G. N., Y. G. Sandalov, V. I. Rodina, V. V. Rozhanets, and R. N. Glebov. Action of tetanus toxin and colchicine on synaptic membranes of the rat cerebral cortex. *Bull. Exp. Biol. Med.* **83**:139–142 (1977).
27. Erdmann, G., H. Wiegand, and H. H. Wellhoner. Intraaxonal and extraaxonal transport of ^{125}I-tetanus toxin in early local tetanus. *Naunyn-Schmiedebergs Arch. Pharmacol.* **290**:357–373 (1975).
28. Nicklas, W. J., S. Puszkin, and S. Berl. Effect of vinblastine and colchicine on uptake and release of putative transmitters by synaptosomes and on brain actomysin-like protein. *J. Neurochem.* **20**:109–121 (1973).
29. van Heyningen, W. E. The fixation of tetanus toxin, strychnine serotonin and other substances by ganglioside. *J. Gen. Microbiol.* **31**:357–387 (1963).
30. Youle, R. J., and D. M. Neville, Jr. Anti-Thy 1.2 monoclonal antibody linked to ricin is a potent cell-type-specific toxin. *Proc. Natl. Acad. Sci. U. S. A.* **77**:5483–5486 (1980).

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