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Tetanus toxin interaction with human erythrocytes. II. Kinetic properties of toxin association and evidence for a ganglioside-toxin macromolecular complex formation

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The properties of tetanus toxin interaction with human erythrocytes supplemented with disialo- and trisialo-gangliosides have been investigated. Binding of toxin is linear with time for 1 h and is 3–4-fold higher at 37°C than at 4°C during incubation of long duration. It exhibits saturation at toxin concentrations between 0.1 and 1 μg/ml; however, it is nonsaturable between 1 and up to 50 μg/ml. It is effectively prevented by free gangliosides and antibodies or by pretreatment with sialidase but is unaffected by a number of closely related ligands including toxoid and toxin fragments. NaCl (1 M) removes a great portion (86%) of cell-associated toxin while Triton X-100 extracts an additional fraction (30%) of the salt-resistant cell-bound toxin. The residual sequestred toxin after detergent extraction is sensitive to proteolytic degradation. The trypsin-stable fraction (1.5%) is biotoxic and may be indicative of internalization of toxin. A macromolecular complex of about 700 kDa containing toxin and gangliosides has been isolated and characterized by Sephacryl S-300 gel permeation chromatography, SDS-gel electrophoresis, immunoprecipitability and biotoxicity. This complex is obtained only in ganglioside-supplemented cells and not when free ^{3}H-labeled G_{D1b} is reacted with ^{125}I-labeled toxin in solution in the absence of cells. The hydrophobicity properties acquired as a result of ganglioside-toxin interaction, presumably at the cell surface, suggest a conformational change of the toxin which may enable its penetration into the bilayer.

Introduction

Uptake of tetanus toxin by peripheral nerve endings is believed to be the first step in a series of reactions which include retrograde axonal transport, transynaptic migration and, ultimately, inhibition of neurotransmitter release (Refs. 1–4; and for a recent review see Ref. 5). Using cultured nerve cells, we have recently studied the first step in this series of events and proposed a three-stage interaction between the toxin and the neuronal plasma membrane [6]. Basically, this interaction was initiated by a low ionic strength, energy-independent and reversible binding. It was then followed by a tight, salt-insensitive and detergent nonextractable toxin-cell association which ultimately resulted in an energy-dependent internalization process, operationally defined by the resistance of the toxin-membrane complex to sialidase treatment [6]. The initial binding step was shown to require the presence of sialosyl residues on the

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cell surface, although a trypsin-sensitive binding component was also noted [7]. As a result of these studies, we suggested that gangliosides act as shuttle vehicles to transfer the toxin from the outer to the inner cellular compartment [6]. This working hypothesis is now tested in human erythrocytes supplemented with gangliosides, as detailed in the previous report [8]. Using these cells, we characterize the interaction of the toxin with the ganglioside-supplemented erythrocyte and describe the isolation of a macromolecular complex consisting of toxin-ganglioside components. The relevance of this complex for the sequestration of tetanus toxin by neuronal membranes naturally enriched in polysialogangliosides is discussed.

Materials and Methods

Human erythrocytes. Human erythrocytes were separated from outdated blood and washed as previously described [8].

Ganglioside incorporation. Ganglioside incorporation was performed essentially as previously described [8]. Briefly, washed human erythrocytes adjusted to a concentration of 5% were incubated with 0.1 or 0.2 mg/ml G_{1b} ganglioside mixture (composed of G_{T1b} and G_{D1b} at a molar ratio of 77 and 21%, respectively) for 2 h at 37°C in 0.05 M Na_2HPO_4 and 0.15 M NaCl adjusted to pH 5.4 with phosphoric acid. In some experiments, crude bovine brain ganglioside extract (ICN Pharmaceuticals, Cleveland, OH) or purified G_{T1b} or ^{3}H-labeled G_{D1b} [8] were employed. The cells were rinsed three times with 0.25% bovine serum albumin or ovalbumin (Sigma, St. Louis, MO) in phosphate-buffered saline (pH 7.4) and resuspended in 20 mM Tris-acetate (pH 7.4) in 0.3 M mannitol buffer for subsequent binding studies.

Binding of ^{125}I-labeled tetanus toxin. Toxin was purified by a ganglioside affinity column and iodinated as described [9]. Ganglioside-supplemented erythrocytes, in Tris-mannitol buffer were incubated with $1 \cdot 10^{-9}$ M ^{125}I-labeled tetanus toxin after affinity purification [9] in the presence of 0.1% gelatin and 0.1% ovalbumin in Eppendorf microfuge tubes in 0.3 ml final volume. Following a brief centrifugation ($1000 \times g$, 2 min), the radioactive medium was removed and the cell pellet was rinsed twice with Tris-mannitol buffer followed by three rinses, 1 ml each, with phosphate-buffered saline containing 0.1% gelatin. The combined washes and the erythrocyte pellets defined as salt-sensitive and salt-insensitive cell-associated toxin, respectively, were counted in a Kontron gamma counter at greater than 92% efficiency. Summation of these two fractions represent total cell-associated radioactivity. Nonspecific binding, defined as the amount of toxin bound to either untreated or to ganglioside-treated cells, after chloroform/methanol extraction was subtracted from all data presented. Experiments were repeated at least twice and were averages of 4–6 reaction mixtures ± S.E.

Gel chromatography. Cell-associated toxin was extracted with 1% Triton X-100 (from Rohem and Haas) in phosphate-buffered saline for 15 min at 4°C. Samples were centrifuged for 20 min at $25\,000 \times g$ to separate Triton-extractable from nonextractable fraction. The latter was solubilized by 1% sodium dodecyl sulfate (SDS). Separation and molecular weight estimation of these fractions was performed by gel permeation on a column of Sephacryl S-300 (Pharmacia, Uppsala, Sweden) containing 50 ml gel, equilibrated and eluted with 0.1 M Na_2SO_4, 0.01 M Tris-H_2SO_4, 10% glycerol, 0.1% gelatin and 0.05% Triton X-100, as detailed elsewhere [8]. The column was calibrated with protein markers which included: Dextran blue (Pharmacia), thyroglobulin 669 kDa (Sigma), rabbit immunoglobulin 150 kDa (Miles-Yeda) and bovine serum albumin, 67 kDa (Pharmacia) was performed as previously described [10] using 1 cm diameter columns filled with 10 ml gel and equilibrated by 2 M Tris-HCl buffer (pH 7.4). Samples from the eluted fractions were tested for biotoxicity [9] or immunoadsorbed with 1 unit/ml equine tetanus antitoxin (Sclavo, Wayne, NJ) and precipitated by *Staphylococcus aureus* membrane preparations (The Enzyme Center, Boston, MA).

Gel electrophoresis . Disc/SDS-polyacrylamide gel electrophoresis was done in 0.1 M Tris-HCl (pH 7.1) using 5% acrylamide in 10 cm long tubes, as previously described [10]. The gels were sliced and 3-mm slices were taken for radioactivity determination. Molecular weights were estimated using a calibration kit purchased from Pharmacia.

Results

Binding characteristics of ^{125}I-labeled tetanus toxin to ganglioside-enriched human erythrocytes

In the previous report [8], we have shown that binding of tetanus toxin to erythrocytes is most effective under low ionic strength conditions, while physiologic salt concentrations cause release of a substantial portion of the bound toxin. Both types of toxin-cell association reflect the dependency of the process on the cell surface ganglioside.

A typical time-course and temperature dependency of the salt-resistant toxin uptake to ganglioside-supplemented cells is illustrated in Fig. 1. Binding is considerably greater at 37°C than at 4°C and is proportional with time up to 1 h and then reaches a plateau which is maintained for about 4 h. The amount of tetanus toxin association as a function of toxin concentration is shown in Fig. 2. Evidently, at a low ligand concentration (below 50 ng/ml) there is practically no toxin binding while between 0.1 and 1 μg/ml a plateau is observed. Above 1 μg/ml, binding increases up to at least 50 μg/ml toxin. A similar biphasic interaction of the toxin has been shown with nerve cell cultures [11] and thyroid [12] and nerve [13] membranes.

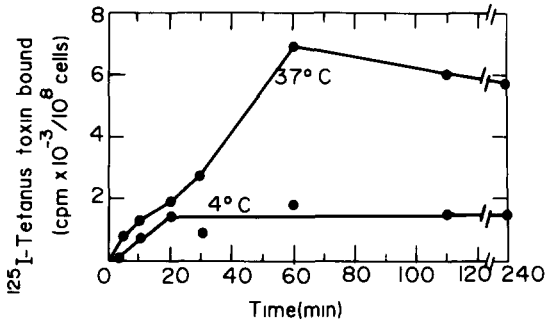

Fig 1 Time-course and temperature-dependent ^{125}I-labeled tetanus toxin binding to ganglioside-supplemented erythrocytes Washed human red blood cells were incubated with 0 1 mg/ml G_{1b} ganglioside mixture for 2 h at 37°C as described under Materials and Methods After removal of unbound ganglioside by phosphate-buffered saline (pH 7.4), cells were incubated with ^{125}I-labeled tetanus toxin (65000 cpm/tube) in Tris-mannitol buffer at 4 or 37°C for the times designated Cell-associated radioactivity was determined after three rinses with phosphate-buffered saline as described under Materials and Methods. Values of the salt-resistant cell-associated toxin represent average of triplicates

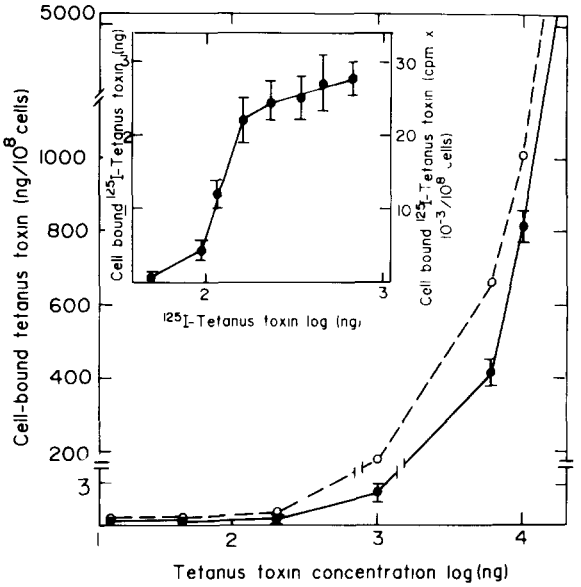

Fig. 2 Concentration-dependent uptake of ^{125}I-labeled tetanus toxin in ganglioside-supplemented human erythrocytes G_{1b} ganglioside-supplemented cells were prepared as described for Fig. 1. After removal of unbound ganglioside by phosphate-buffered saline (pH 7.4), cells were incubated for 1 h at 37°C with increasing amounts of ^{125}I-labeled tetanus toxin (inset) or with a constant amount of labeled toxin (10^5 cpm) and increasing doses of unlabeled tetanus toxin At the end of incubation, cells were rinsed twice in Tris-mannitol buffer and washed with phosphate-buffered saline to collect the salt-sensitive (○) fraction The residual, cell-associated radioactivity represents the salt-resistant binding (●). Values are averages of three experiments.

The optimum pH for toxin binding was established to be around pH 5.0 with a shoulder around pH 7.0. Binding studies were performed usually at neutral pH to avoid damage of erythrocytes at extreme pH during long incubation periods.

Displacement of cell-associated tetanus toxin by various agents

A number of substances have been tested for their ability to prevent or displace the ganglioside-dependent binding of the toxin with the erythrocytes. As summarized in Table I, to exclude fragment B, none of the toxin derivatives, each added at a relatively high concentration, could prevent or displace the already cell-bound toxin. The toxin derivatives in fact, slightly stimulated binding. Substances known to interact with gangliosides, such as thyrotropin, were slightly stimula-

TABLE I

THE ABILITY OF DIFFERENT LIGANDS AND BIOLOGICAL SUBSTANCES TO PREVENT OR DISPLACE ^{125}I-LABELED TOXIN ASSOCIATION WITH GANGLIOSIDE-SUPPLEMENTED ERYTHROCYTES

Ganglioside-supplemented cells prepared as described in Fig 1 were subjected for 30 min with the various substances prior to addition of toxin Incubation with toxin was continued for 2 h in the presence of the additives For displacement, toxin-treated cells after phosphate-buffered saline wash were subjected for 1 h to the various additives, and residual cell-associated radioactivity was counted Fragments B and C prepared by Helting and Zwisler [38] were kindly provided by Dr. W. Habig, Bethesda. Tetanus toxoid, 1900 flocculation units/mg was purchased from Connaught Laboratories (Canada) DEAE-cellulose-purified antitoxin monoclonal antibody was prepared by us Partially purified human fibroblast interferon was obtained from Dr. M Ravel, Rehovot Values are averages of two to three experiments with less than 10% error and are expressed as percentages of control

Substance tested	^{125}I-labeled tetanus toxin-bound (salt-resistant) (% of control)	
	Prevention	Displacement
Toxin and derivatives		
Tetanus toxin (0.1 mg/ml)	105	85
Fragment B (0.05 mg/ml)	122	52
Fragment C (0.1 mg/ml)	130	93
Toxoid (0.2 mg/ml)	e145	89
Antibodies		
Equine antitoxin (20 μg/ml)	33	74
Mouse monoclonal antitoxin (50 μg/ml)	20	82
Bioactive ligands		
Thyrotropin (0.1 mg/ml)	124	95
Interferon (0.2·10 U/ml)	92	85
Gangliosides		
G_{D1b} (0 01 mg/ml)	42	36
(0.1 mg/ml)	0	20
G_{T1b}/G_{D1b} (0.1 mg/ml)	0	25

tory while interferon showed no marked effect. As anticipated, antitoxin antibodies neutralized the toxin in solution and abolished binding. They were ineffective, however, in releasing the bound toxin. G_{D1b} and G_{T1b} added to the medium in the presence of toxin prevented binding and also remarkably displaced cell-bound toxin.

To investigate further the properties of toxin-cell association, a number of detergents were applied. As shown in Table II, after treatment of cells with 0.15 M NaCl, nearly 81% of the toxin is removed. Higher concentration of NaCl (1 M) removes an additional 5% of the labeled toxin. Treatment with Triton X-100 extracts about 30% of the salt-insensitive cell-associated toxin. This value remains the same even after 30 min incubation with the detergent. Nonidet NP40 or β-octyl glucoside were less effective (15–20%) in extracting the cell-bound

TABLE II

THE EFFECT OF SUCCESSIVE TREATMENT BY VARIOUS AGENTS ON THE AMOUNT OF CELL-ASSOCIATED TOXIN

G_{1b} ganglioside-supplemented cells (10^{10}/ml) were incubated with $5.1 \cdot 10^7$ cpm ^{125}I-labeled tetanus toxin ($5 \cdot 10^7$ cpm) for 1 h at 37°C Cells were successively treated with the reagents (noted in numerical order) and the pellets were counted after centrifugation Values are averages of duplicates with less than 10% error The first three reagents also included 0.1% gelatin

Treatment with	Cell-bound toxin (cpm/10^{10} cells) ($\times 10^{-3}$)	Initial binding (%)
1 Tris-mannitol buffer	1198	100
2 0.15 M NaCl	226	18 9
3 1.0 M NaCl	167	13.9
4 1% Triton X-100	115	9.5
5 1% SDS	12	1.0

toxin. Addition of SDS virtually solubilized the residual, Triton-insoluble pellet and also extracted the remaining cell-associated toxin. All toxin fractions released by the treatments noted in Table II exhibited similar toxicity levels compared to the original iodinated tetanus toxin (results not shown). The data presented so far suggest that the tight toxin-erythrocyte association is analogous to the sequestration process of tetanus toxin observed in neuronal cell cultures [11,14].

The sensitivity of the sequestered toxin to proteolytic degradation has been examined. As shown in Table III, about 82% of the cell-associated toxin remaining after NaCl (0.15 M) wash is sensitive to trypsin. After treatment with either 1 M NaCl or 1% Triton X-100, 28 and 43% of the initial radioactivity, respectively, is still bound to the erythrocytes. Treatment with trypsin after either of these extraction steps caused release of the label from the pellet, indicating external exposure of the protein. The remaining cell pellet radioactivity reaches nearly equal levels (about 800 cpm/10^8 cells). This residual, labeled fraction (1.5%) retains its biotoxicity.

The effect of trypsin and sialidase on the ganglioside-dependent interaction of toxin with the cells is shown in Table IV. Pretreatment of cells with trypsin has no remarkable effect of subsequent binding of toxin to either ganglioside-supplemented or nonsupplemented cells. Trypsin treatment after toxin binding released more than 84% the cell-associated toxin. In addition, there is 4-fold more trypsin-insensitive toxin bound to ganglioside-supplemented than nonsupplemented cells. Like the previous experiment, most of the cell-associated toxin is accessible to trypsin. Pretreatment of cells with sialidase, reduces toxin binding by 75 and 43% in ganglioside-supplemented and nonsupplemented cells, respectively. Treatment of cells with sialidase after incubation with tetanus toxin does not release the bound toxin, suggesting, in analogy to nerve cells [11], a temperature-dependent, tight interaction of the toxin with a sialosyl residue. This firm associated could also be due to toxin-lipid interaction in the bilayer not mediated by a ganglioside component [14]. The amount of toxin bound to cells nonsupplemented with gangliosides is also reduced by 43% after pretreatment with sialidase. In contrast to cells supplemented with gangliosides, 61% of the cell-bound toxin is removed after treating the control cells with sialidase. This would suggest a small but significant toxin-binding activity which is sialidase-sensitive and trypsin-insensitive.

The properties of toxin-erythrocyte interaction following hypotonic shock is shown in Table V. G_{T1b}-supplemented erythrocytes were incubated with tetanus toxin and then subjected to either low ionic strength Tris-mannitol buffer or to phosphate-buffered saline. The cells were then treated with a hypotonic solution and then washed with phosphate-buffered saline. Evidently, osmotically shocked cells preloaded with toxin in low ionic strength Tris-mannitol buffer retain twice as much toxin compared to cells which have been treated with 0.15 M NaCl prior to cell lysis under the same conditions. This raises the possibility that as a result of the osmotic shock, inverted ghosts were

TABLE III

EFFECT OF PROTEOLYSIS ON CELL-BOUND TOXIN AFTER SUCCESSIVE TREATMENT OF CELLS

After each stage, as described in Table II, cells were treated with 0 1 mg/ml trypsin (Calbiochem) in 5 mM EDTA for 20 min at 37°C and after centrifugation, pellets and supernatants were counted. Values expressed as cpm/10^8 cells are means ± S E of three experiments

Treatment with	Cell-bound toxin		Residual radioactivity (%)
	Before proteolysis	After proteolysis	
0.15 M NaCl	4425 ± 190	805 ± 235	18 2
1.0 M NaCl	2570 ± 340	730 ± 130	28 4
1% Triton X-100	2000 ± 350	860 ± 55	43.0
1% SDS	790 ± 240	570 ± 40	72 2

TABLE IV

EFFECT OF TRYPSIN AND SIALIDASE ON GANGLIOSIDE-DEPENDENT TETANUS TOXIN ASSOCIATION WITH ERYTHROCYTES

Cells treated with gangliosides as described in Fig 1 were incubated with 30 mU *Vibrio cholera* neuraminidase (Boehringerwerke) or 0 25% trypsin (Calbiochem) for 1 h or 15 min, respectively, at 37°C before or after incubation with ^{125}I-labeled tetanus toxin (50000 cpm/tube). The salt-insensitive toxin fraction was determined as described in Fig 1 Values expressed as cpm/10^8 cells are averages ± S D of three experiments

Enzyme erythrocytes	^{125}I-labeled tetanus toxin bound			
	Trypsin		Sialidase	
	cpm/10^8 cells	% bound	cpm/10^8 cells	% bound
Control				
Untreated	1120 ± 125	100	1300 ± 120	100
Before	1050 ± 100	94	740 ± 125	57
After	125 ± 100	11	510 ± 180	39
Gangliosides				
Untreated	3200 ± 270	100	3750 ± 125	100
Before	3500 ± 375	109	950 ± 70	25
After	500 ± 190	16	4125 ± 310	110

TABLE V

EFFECT OF HYPOTHONIC SHOCK ON ERYTHROCYTE-BOUND ^{125}I-LABELED TETANUS TOXIN

Cells were supplemented with 0.1 mg/ml pure G_{T1b} or with a mixture of bovine brain gangliosides (BBG) (0.2 mg/ml) and after 2 h at 37°C, excess ganglioside was removed as described under Fig 1 ^{125}I-labeled tetanus toxin (50000 cpm/tube) was added for 30 min at 37°C Unbound toxin was removed with either 0.1% gelatin in phosphate-buffered saline (PBS) or with 0 3 M mannitol and cells were counted (total) and subsequently treated with 20 mM Tris-acetate (pH 7.4) for 10 min at 4°C After a brief centrifugation at 20000 × *g*, the residual pellet was washed twice with phosphate-buffered saline and cell pellet was counted Values are means of three experiments and are expressed as cpm × 10^{-3} per 10^8 cells

Ganglioside supplement	PBS wash		Mannitol wash	
	Total	Cell-bound	Total	Cell-bound
G_{T1b}	8 8 ± 0.2	3 ± 0 3	3 4 ± 0 4	1 6 ± 0 1
BBG	4 9 ± 0.6	1.6 ± 0 2	2.0 ± 0 2	0 9 ± 0 1
Nonsupplemented	2 2 ± 0.3	1 1 ± 0 2	1 3 ± 0.2	0 8 ± 0 1

formed [15]. These ghosts may contain the toxin inside.

Chromatographic and electrophoretic behavior of ^{125}I-labeled tetanus toxin following interaction with ganglioside-supplemented cells

The chromatographic properties of the Triton X-100-extractable and nonextractable cell-associated toxin was investigated. Gel permeation chromatography was selected as it became apparent that the increased resistance to sialidase may be due to the formation of a toxin macromolecular complex which could mask the bound ganglioside. In order to verify this hypothesis, we subjected the Triton X-100-soluble and SDS-extractable radioactivity to gel permeation chromatography on Sephacryl S-300. As shown in Fig. 3, two radioactive peaks, one of which corresponding to a molecular weight of about 650000–700000 and a second peak of molecular weight of approx. 150000–200000, could be detected in either the Triton-extractable or SDS-extractable fractions. Both peaks were toxic and could be immunoprecipitated by tetanus toxin antibodies, the lower

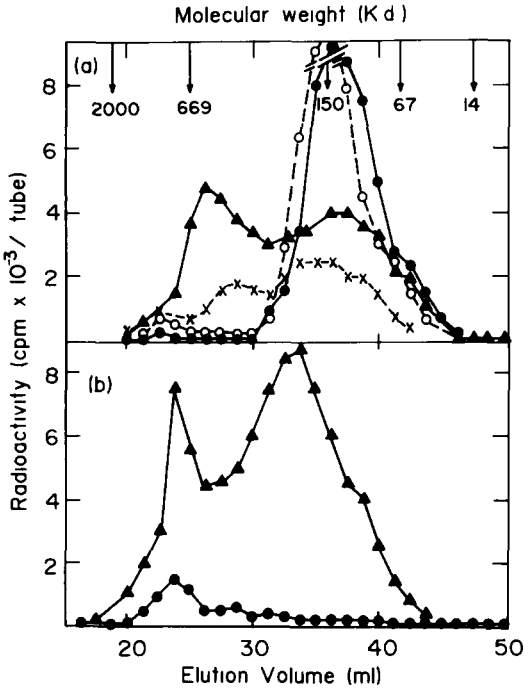

Fig. 3 Sephacryl S-300 gel filtration chromatography of Triton X-100 (panel a) and SDS (panel b) cell-associated tetanus toxin Ganglioside supplemented-cells were incubated with ^{125}I-labeled tetanus toxin using conditions similar to those described in Fig. 1. The salt-insensitive cell-bound toxin was subjected to 1% Triton X-100 followed by 1% SDS as described under Materials and Methods. (Panel a) Gel filtration pattern of Triton X-100-extractable cell-associated toxin (▲) and its corresponding immunoprecipitable radioactivity (×) The patterns of untreated toxin (○) and that of salt-sensitive toxin (●) are shown for comparison (Panel b) Gel filtration pattern of SDS-extractable toxin fraction remaining after Triton extraction of ganglioside-supplemented (▲) and nonsupplemented cells (●) Molecular weight markers (×10^3) included Dextran blue (2000), thyroglobulin (669), rabbit IgG (150), ovalbumin (67) and cytochrome *c* (14).

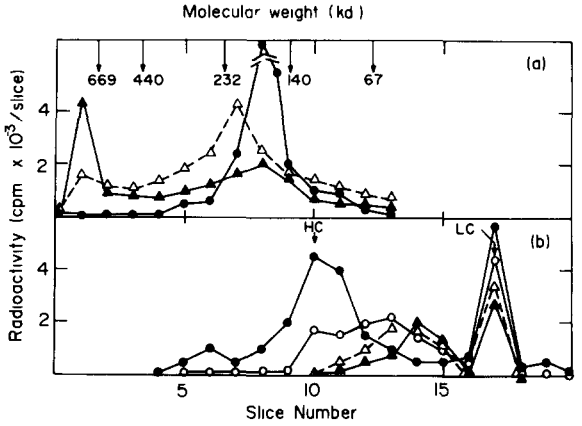

Fig. 4. SDS-polyacrylamide gel electrophoresis under regular (a) or denaturating (reductive) conditions (b) of ^{125}I-labeled tetanus toxin and different detergent extracts of cell-associated toxin Following electrophoresis in the presence of 1% SDS in 0.1 M Tris-HCl, gels were fixed, stained and sliced in 3-mm slices and the ^{125}I content was determined (Panel a) Unreacted ^{125}I-labeled tetanus toxin (2 10^4 cpm, ●), Triton extract (▲) and SDS extract (△) of cell-associated toxin obtained exactly as described in Fig 3 (Panel b) β-Mercaptoethanol-treated samples of Triton (▲) and SDS (△) extracted cell-associated toxin Reduced boiled (○) or unboiled (●) tetanus toxin is shown for comparison. HC, heavy chain; LC, light chain

molecular weight peak being more effectively precipitated than the higher molecular weight peak. SDS gel electrophoresis of the Triton X-100- and SDS-extractable cell-associated toxin reveal the presence of a high macromolecular weight complex with low mobility into the gel under nonreducing conditions (Fig. 4, panel a) and the presence of a fraction with an electrophoretic mobility similar to that of the native toxin. Under reducing conditions (Fig. 4, panel b), both extracts exhibit two radioactive, 50 and 100 kDa subunits, similar to the reported molecular weights of the light and heavy chains of tetanus toxin [16,17].

To clarify further the involvement of gangliosides in binding/sequestration of tetanus toxin to the cell surface, erythrocytes preincubated with ^{3}H-labeled G_{D1b} were subsequently exposed to ^{125}I-labeled tetanus toxin and then the various Triton-extractable and SDS-extractable fractions were analyzed by Sephacryl S-300 gel permeation chromatography (Fig. 5). Precalibration of the column with either ^{3}H-labeled G_{D1b} or ^{125}I-labeled tetanus toxin showed a predicted elution pattern for a high molecular weight polypeptide and a low molecular weight ganglioside (Fig. 5, upper panel). A mixture of the two labeled substances results in a single peak of ^{125}I-labeled tetanus toxin and two discrete peaks of ^{3}H-labeled G_{D1b} each corresponding to a high and a low molecular weight fraction. This experiment demonstrates that under the conditions used for gel chromatography there are no homotypic toxin-toxin or ganglioside-ganglioside micellar associations. There are, however, heterotypic associations between the toxin and the

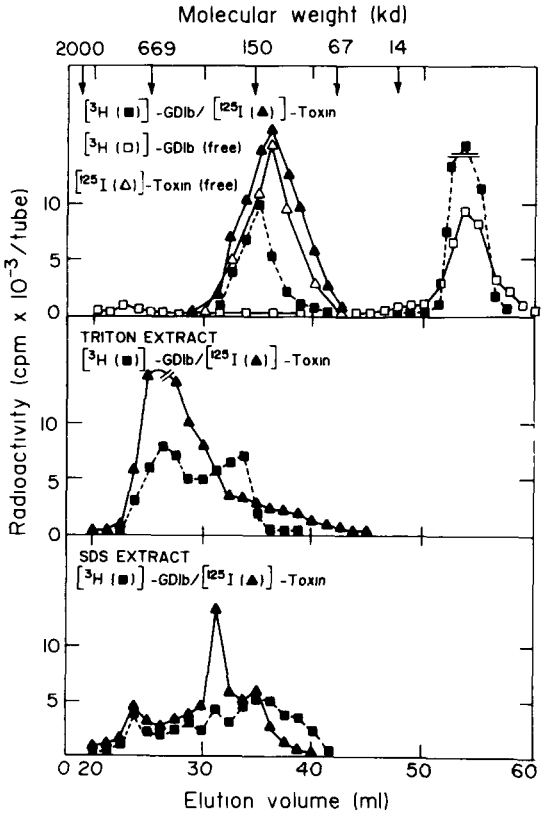

Fig 5 Sephacryl S-300 chromatography of toxin/G_{D1b} mixture (upper panel), Triton-extractable (mid panel) and SDS-extractable (lower panel) cell-associated tetanus toxin Cells were supplemented with ^{3}H-labeled G_{D1b} as described under Materials and Methods and then subjected to ^{125}I-labeled tetanus toxin for 2 h at 37°C The salt-insensitive cell-bound toxin was subjected to 1% Triton X-100 followed by 1% SDS The extracts were chromatographed as described in Fig. 3 and ^{3}H-labeled G_{D1b} and ^{125}I-labeled tetanus toxin radioactivity was determined. The spill of gamma into beta counting was appropriately corrected For the upper panel, ^{125}I-labeled toxin (1 10^{6} cpm) and ^{2}H-labeled G_{D1b} (6 10^{6} cpm) were incubated for 30 min at 37°C prior to application

G_{D1b} ganglioside which are mediated by the erythrocyte. This is shown in the middle panel of Fig. 5 which illustrates the radioactivity distribution of ^{3}H-labeled G_{D1b} and ^{125}I-labeled tetanus toxin extracted from erythrocytes by Triton X-100 and separated on the Sephacryl column. Evidently, a major peak of ^{125}I radioactivity is eluted at about 700 kDa, whereas two distinct tritium-labeled peaks can be found, one of which is coeluted with the tetanus toxin. This indicates that upon interaction with cells supplemented with gangliosides, a remarkable shift in the chromatographic behavior of the labeled toxin toward a higher molecular weight form occurs. The elution profile of the SDS cellular extract is shown in the lower panel of Fig. 5. This extract is devoid of a high molecular weight toxin complex. It contains a major peak of about 200 kDa which lacks ^{3}H-labeled G_{D1b} but may contain a cell surface constituent. This molecular complex may result from the dissociation of the toxin-ganglioside 700 kDa complex after addition of SDS. Extraction of the ^{3}H-labeled G_{D1b}/^{125}I-labeled toxin complex by chloroform/methanol leads to a total separation of the two labeled compounds. The tritium label is entirely recovered as ^{3}H-labeled G_{D1b} upon thin-layer chromatography (data not shown).

Additional characterization of the macromolecular complex was obtained by assessing its behavior on Phenyl-Sepharose hydrophobic chromatography. As shown in Fig. 6, the toxin-ganglioside complex prepared in the absence of cells and

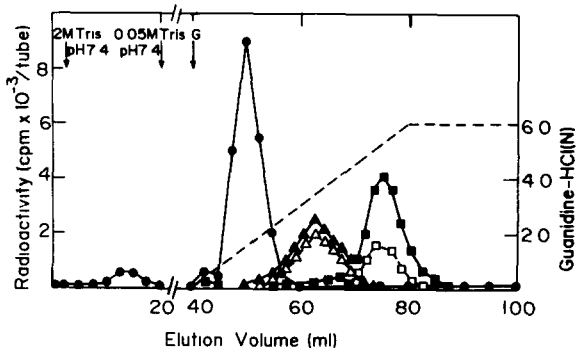

Fig. 6. Hydrophobic chromatography of cell-associated toxin on Phenyl-Sepharose CL-4B. Cell-associated toxin obtained as detailed in Fig. 4 was chromatographed on a Phenyl-Sepharose column equilibrated with 2.0 M Tris-HCl buffer (pH 7.4). After sample application (100 μl), the column was washed with the equilibrium buffer (first arrow) followed by a gradual decreasing of Tris buffer molarity to 0.05 M (second arrow). The column was then eluted with a linear gradient of increasing molarity of guanidine-HCl (G) in 0 05 M Tris-HCl buffer (pH 7.4) at a constant flow-rate of 8 ml/tube. 2-ml fractions were collected and radioactivity was determined. Samples subjected for separation were: free ^{125}I-labeled tetanus toxin (●); ^{125}I-labeled toxin (▲)/^{3}H-labeled G_{D1b} (△) complex isolated as in the middle panel of Fig. 5 (700 kDa); ^{125}I-labeled tetanus toxin (■)/^{3}H-labeled G_{D1b} (□) complex isolated as in the upper panel of Fig. 5 (150 kDa).

isolated by Sephacryl (150 kDa) and further subjected to Phenyl-Sepharose chromatography exhibits the highest hydrophobicity on the column, the peak being eluted at 5 M guanidine-HCl. In contrast, the toxin-ganglioside complex isolated from the erythrocyte membrane (700 kDa by Sephacryl analysis) displays an intermediate hydrophobicity, the peak being eluted at about 2.5 M guanidine-HCl. The elution of free toxin in a low hydrophobicity region is consistent with the high polarity of the toxin as reported by Robinson et al. [18] and low hydrophobicity as determined by reversed-charge electrophoresis [19]. On the other hand, the altered behavior of the complexed toxin as evident by this technique emphasizes the possibility of a conformational change on toxin structure following binding to a specific ganglioside. Such a change could provide the means to overcome the lipid barrier for penetration of the toxin into the cell.

The biotoxicity of the high molecular weight complex was studied by subcutaneous injections of equivalent doses of radioactive material of both native toxin and the macromolecular (700 and 200 kDa) complexes isolated from these columns. All preparations showed similar symptoms of tetanus toxicity.

Discussion

In the accompanying report, we have studied the optimal conditions which promote insertion of G_{D1b} and G_{T1b} gangliosides into the erythrocyte membrane to facilitate subsequent binding of tetanus toxin to cells This report documents some of the properties of the toxin-cell association and suggests the formation of a toxin-ganglioside macromolecular complex. The formation of this complex, which to the best of our knowledge is now reported for the first time, is supported both by pharmacokinetic and by biochemical data. First, we show that binding of tetanus toxin to cells is multiphasic with respect to the toxin concentration. Binding is composed of basically three distinct components: (i) a low or near zero binding component predominant at toxin concentrations below 50 ng/ml, (ii) a saturable component achieved between 0.1 and 1 μg/ml toxin with an apparent maximum binding of 3.0 ng toxin/10^8 cells and (iii) a nonsaturable component at concentrations of toxin above 1 μg/ml toxin (Fig. 2). The nonsaturable component presumably accounts for the poor displacement of labeled toxin by excess, unlabeled toxin as we demonstrated in this report (Table I). Similar observations have been made with crude ganglioside extracts [20], central nervous system preparations [20–23] and nerve cell cultures [24] pretreated with excess unlabeled toxin. This phenomenon of negative cooperativity can be explained by differences in the affinities of various gangliosides (i.e., G_{D1b} or G_{T1b}) toward the toxin They may also result from lowering the affinity constant of the ligand following binding. Alternatively, they can be explained by the formation of discrete ganglioside clusters or domains in the bilayer [25] as proposed for the interaction of G_{M_1} with cholera toxin [26] or following Sendai virus interaction with membrane gangliosides [27]

The specificity of the toxin-ganglioside receptor interaction is demonstrated by the inability of a number of ligands to displace the toxin (Table I). In contrast, exogenous gangliosides are very effective in displacing cell-bound toxin, suggesting equal affinities between free and cellular-associated gangliosides in competing for the toxin. The remaining cell-associated toxin after ganglioside displacement and the time-course and temperature-stimulated associated (Fig. 1) all point to a tight toxin-cell interaction analogous to brain cells [11].

Addition of Triton X-100 solubilizes a substantial portion (30%) of the labeled toxin, while the remaining portion stays most probably in association with cytoskeletal elements [11,28]. The isolation of a macromolecular complex of an apparent molecular weight of 700 000 from the latter fraction by gel permeation chromatography is also compatible with toxin sequestration by cells. This complex is formed whenever cell-bound but not free ganglioside interacts with tetanus toxin. The molecular nature and the possible involvement of a nonganglioside cellular component is currently under investigation. A similar aggregation phenomenon has been reported in the case of botulinum toxin and G_{Q1b} [29] and serum albumin and G_{M1} [30] gangliosides. These interactions may depend on a number of variables such as concentration and purity of the ganglioside involved, the ionic strength or the existence of other lipids

The requirement of gangliosides to initiate binding of tetanus toxin to the plasma membrane of the erythrocyte is strongly suggested in this and in the accompanying report. In spite of that, the subsequent steps which result in toxin sequestration and internalization remain a puzzle. These steps may involve a rearrangement of the ganglioside in the bilayer [25] due to changes in the protein structure and aggregation or both. The formation of the high molecular weight complex maybe a clue to unravel this phenomena.

Finally, the ability of tetanus toxin to transfer ions by forming channels in lipid bilayers [31] or of toxin fragments to enter into liposomes at low pH [32] are compatible with these possibilities. We have recently extended the experiments of Clowes et al. [31] and demonstrated that tetanus toxin at neutral pH can generate cation-permeable channels. The formation of these channels is absolutely dependent on the presence of gangliosides [33]. Studies carried in the planar lipid bilayer with G_{M1} and cholera toxin have led to similar conclusions [34], indicating that the toxin may, at least in model systems, span the bilayer and induce channel formation [35]. Conformational changes in the polypeptide chain as a prerequisite for interaction or penetration has been suggested in the case of cholera toxin (see Ref. 36 for a recent review) and was also proposed as a mode of action for several glycoprotein hormones [37]. In this context, the principal role of the membrane ganglioside may be to narrow the gap and bridge between the water-soluble toxin and the lipid bilayer to favor movement of the ligand into the bilayer The macromolecular complex between toxin and ganglioside isolated from cells displays properties consistent with this hypothesis. The present studies provide the basis for further experiments aimed at understanding the molecular mechanism for insertion of proteins into the lipid bilayer

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