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Tetanus toxin interaction with human erythrocytes. I. Properties of polysialoganglioside association with the cell surface

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Human erythrocytes in suspension acquire gangliosides containing di- and trisialosyl residues added to the maintenance medium. This is reflected in the increased cell-associated sialic acid content and ability to bind ^{125}I-labeled tetanus toxin. A salt-sensitive and a salt-insensitive ganglioside-mediated toxin-cell surface association is detected which is reduced after sialidase treatment of ganglioside-supplemented cells. The salt-insensitive ganglioside-cell association is saturable after 2 h incubation in 0.3 M mannitol buffer and has an optimum at pH 5. The association process is higher at 37°C than at 4°C, depends on cell density, and is considerably higher in metabolically active cells compared to lysed cells. Pretreatment of cells with trypsin decreases the salt-resistant toxin association with ganglioside-supplemented cells. In contrast, glutaraldehyde-fixed cells treated with trypsin and supplemented with gangliosides bind more toxin which is insensitive to salt. Ganglioside-mediated tetanus toxin binding to the intact erythrocyte membrane can be utilized as a model system for studying the role of glycolipids in membrane function.

Introduction

Gangliosides are natural constituents of the outer leaflet of the mammalian plasma membrane [1]. The diversity of their carbohydrate residue has been implicated in determining the specificity with which they bind a variety of biologically active substances (for a review see Ref. 2). Artificial supplementation of mammalian and avian cells with G_{M1} ganglioside has previously been a useful technique in studying ganglioside function [3–6]. Gangliosides of the G_{1b} series have been particularly implicated in the binding [7–9] and possibly the sequestration and translocation of tetanus toxin by nerve cells in tissue culture [10–12]. Certain neural cell lines which lack these gangliosides and cannot bind effectively tetanus toxin do so after the addition of G_{D1b} or G_{T1b} species [13]. Following binding, a tight, sialidase-insensitive toxin-cell association was observed. This tight interaction may have some analogy to the ability of the toxin to react with gangliosides and translocate ions in planar lipid bilayers [14]. In spite of that, the molecular details by which gangliosides may promote the translocation of the toxin through the bilayer are not yet resolved.

The red blood cell has been selected as an experimental model with which to test the working hypothesis that gangliosides participate in the process of penetration and translocation of tetanus toxin, due to its relatively simple and well-characterized plasma membrane composition, and its ability to accumulate gangliosides from the extracellular medium [5,6,15]. An additional advantage is that unlike most nucleated mammalian cells, the red blood cell is practically devoid of

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endocytic activity. This report is concerned with the conditions which are necessary to acquire maximal uptake of disialo- and trisialo gangliosides in human erythrocytes by the criteria of binding of tetanus toxin.

Materials and Methods

Cells

Human erythrocytes obtained through the Hadassah Hospital Blood Bank, Jerusalem, were of outdated (4–7 weeks) A and O type blood group donors. The blood was diluted with 20 vol. phosphate-buffered saline (pH 7.4) (Ca^{2+}- and Mg^{2+}-free) and the cells were sedimented by centrifugation at $1000 \times g$ for 10 min at room temperature. The plasma and buffy coat were discarded and the cell pellet was washed four times and resuspended to the desired cell concentration with phosphate-buffered saline (pH 7.4). Erythrocyte ghosts were prepared from prewashed cells by the method of Dodge et al. [16] using a hypotonic solution consisting of 20 mM Tris-acetate buffer (pH 7.0). Cell lysis was achieved after 10 min incubation at room temperature and ghosts were collected and washed after brief centrifugations. Human type A, glutaraldehyde-fixed erythrocytes (Sigma, St. Louis, MO) were rinsed three times in phosphate-buffered saline (pH 7.4) prior to use.

Gangliosides

A partially purified ganglioside mixture containing G_{T1b} and G_{D1b} at molar percent of 77 and 21%, respectively, and trace amounts of G_{D3} (less than 2%) was kindly provided by Dr. T. Dinur, Department of Biochemistry, Hadassah Medical School, Jerusalem. This preparation was designated as G_{1b} ganglioside fraction and was used throughout the study. Pure G_{D1b} was kindly given by Dr. Hauttecouer from the Institut Pasteur, Paris. Tritium-labeled G_{D1b} was prepared by the galactose-oxidase procedure [17] using NaB^3H_4 (New England Nuclear, Boston, MA). The radioactively-labeled ganglioside stored in chloroform/methanol (2:1, v/v) at −20°C was better than 95% radiochemically pure and comigrated on TLC with an authentic G_{D1b} as standard. Before use, the organic solvents were removed by evaporation and ^3H-labeled G_{D1b} reconstituted with phosphate-buffered saline (pH 7.4).

Artificial addition of gangliosides

Unless otherwise stated, freshly washed human erythrocytes were incubated with various concentrations of gangliosides in phosphate-buffered saline (pH 7.4) as noted in the text. Free gangliosides were removed by three repetitive washings of cells by low-speed centrifugation (2 min at $1000 \times g$) in phosphate-buffered saline (pH 7.4) in the presence of 0.25% bovine serum albumin (fraction V, Sigma, St. Louis, MO). The ganglioside-enriched cell pellet after the last wash was resuspended in 0.02 M Tris-acetate (pH 7.4) containing 0.3 M mannitol. No apparent cell hemolysis or alterations in cell shape were detected by light microscopy and scanning electron microscopy.

Tetanus toxin

Purified tetanus toxin, homogeneous on sodium dodecyl sulfate polyacrylamide gel electrophoresis was kindly supplied by Dr. B. Bizzini from the Institut Pasteur, Paris [18]. The toxin was iodinated by the Bolton-Hunter procedure to a specific activity of approx. 1.0–1.5 mCi/mg as detailed by AnderLan et al. [19] and purified on a ganglioside-affinity column as recently described [20]. The purified toxin was diluted in the Tris-mannitol buffer supplemented with 0.2% gelatin and stored at 4°C in small aliquots. For binding experiments, the toxin was diluted further in the Tris-mannitol buffer to reach a final concentration of $1 \cdot 10^{-9}$ M.

Detection of cell-bound gangliosides

Chromatography. Cell-associated ganglioside content and composition was determined after extraction of the cell pellet with chloroform/methanol (2:1, v/v) as described elsewhere [10]. In brief, the lipid-containing fraction pooled from two consecutive extractions of cells was evaporated to dryness under air and redissolved in chloroform/methanol (2:1, v/v) solution. After brief centrifugation (2 min at $20000 \times g$), aliquots of the organic solvent containing a mixture of neutral, polar and glycolipids was subjected to TLC analysis using silica gel G precoated plates (Merck, Darmstadt). Gangliosides were visualized by resorcinol spray [21] or, when possible, by autoradiography. Lipid-bound sialic acid was determined by a slight modification of the procedure of Hahn et al. [22].

Binding of ^{125}I-labeled tetanus toxin. Aliquots of 0.1 ml of ganglioside-supplemented erythrocytes in Tris-mannitol buffer were added to Eppendorf microfuge tubes which have been pretreated with 1% ovalbumin to prevent nonspecific binding of toxin. When, 0.2 ml ^{125}I-labeled tetanus toxin ($(5–7)\cdot 10^5$ cpm/tube, 5–7 ng/tube) in Tris-mannitol buffer augmented with 0.1% ovalbumin and 0.1% gelatin was added. After 1 h incubation at 37°C, the reaction was terminated by centrifugation (2 min at $1000 \times g$) and the pellet was washed twice with 1 ml Tris-mannitol, low ionic strength buffer. The pellet representing the total cell-bound toxin was counted at greater than 92% efficiency (Kontron Gammamatic counter) and subsequently washed three times with 1 ml vol. phosphate-buffered saline (pH 7.4) containing 0.1% gelatin. The residual cell-associated radioactivity representing the salt-resistant toxin was counted as above. Nonspecific binding, defined as the amount of toxin bound to untreated cells or to ganglioside-supplemented cells extracted with chloroform/methanol (1 : 2, v/v), was subtracted from all data presented. Each experimental value is the mean of three or more reactions. Unless otherwise stated, experiments were repeated at least twice.

Miscellaneous procedures

Cell trypsinization. Mild proteolytic treatment of washed human erythrocytes either before or after ganglioside uptake was carried out in the presence of 0.25% trypsin (Sigma, St. Louis, MO) in phosphate-buffered saline (pH 7.8) for 30 min at 37°C. After addition of soybean trypsin inhibitor (1% final concentration, Sigma), erythrocytes were rinsed with phosphate-buffered saline (pH 7.4). No apparent hemolysis was encountered under these conditions.

Treatment with sialidase. Cell suspensions (5%) of untreated and ganglioside-enriched human erythrocytes prepared in Tris-mannitol buffer (pH 7.4) were treated with 0.05 U/ml of *Vibrio cholera* neuraminidase (Boehringwerke, Marburg) augmented with 2 mM $CaCl_2$. After 1 h incubation at 37°C, cells were rinsed twice with phosphate-buffered saline (pH 7.4) and subsequently incubated with ^{125}I-labeled tetanus toxin.

Results

TLC identification of cell-associated ganglioside

Human erythrocytes accumulate bovine brain gangliosides added to the incubation medium in a time-dependent manner as determined by the lipid-bound sialic acid associated with the cells. As shown in Fig. 1, after 1 h incubation in the presence of a G_{1b} ganglioside mixture, the sialic content of the erythrocytes is raised from a value of 0.75 nmol/10^8 cells to a plateau value of 2.84 nmol/10^8 cells. TLC analysis of the lipids isolated from the cells after different times of incubation show the presence of G_{T1b} and G_{D1b} gangliosides at ratios similar to that present in the incubation medium (Fig. 1, inset).

Binding of tetanus toxin as a criteria for identification and optimization of cell surface ganglioside content

The participation of G_{D1b} and G_{T1b} gangliosides in the binding (fixation) of tetanus toxin is

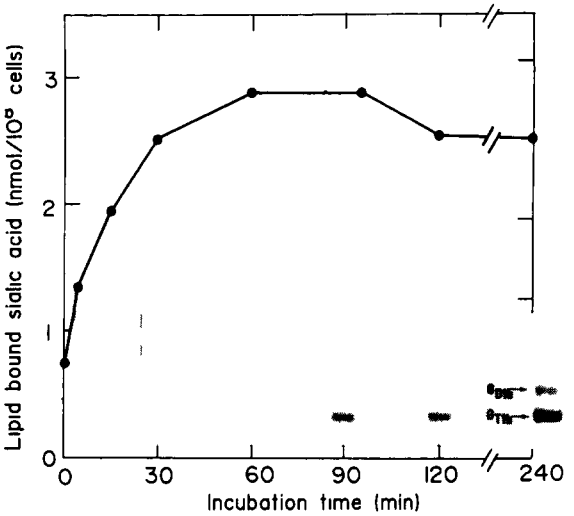

Fig 1 Time-course of lipid-bound sialic acid accumulation following ganglioside addition to human erythrocytes Human erythrocytes suspended in phosphate-buffered saline (pH 7.4) were incubated at 37°C with 0.2 mg/ml G_{1b} ganglioside preparation for times indicated. Lipid-bound sialic acid associated with the cells was determined as described under Materials and Methods Values, expressed as nmol sialic acid per 10^8 cells, are means of triplicate determinations. Parallel but not necessarily equivalent samples of lipid extracts were subjected to TLC analysis and visualized by resorcinol staining as described under Materials and Methods

well-acknowledged [8,9]. We have taken advantage of this property to investigate ganglioside incorporation into the cell surface by monitoring the levels of ^{125}I-labeled tetanus toxin that can be bound to the erythrocytes following ganglioside supplementation. The total amount of toxin bound to ganglioside-supplemented cells is a measure of the cell-surface-associated ganglioside. These gangliosides can be divided into two fractions with respect to binding of tetanus toxin. One fraction in which binding of toxin is salt-sensitive and a second salt-resistant fraction. The properties of these fractions have been examined into some details.

The total amount of toxin bound to cells depends on the concentration of the medium ganglioside used to supplement the cells. As shown in Fig. 2, this fraction approaches a plateau within 1 h at about 0.1 mg/ml exogenous ganglioside, but is not saturable even at 1 mg/ml when incubation

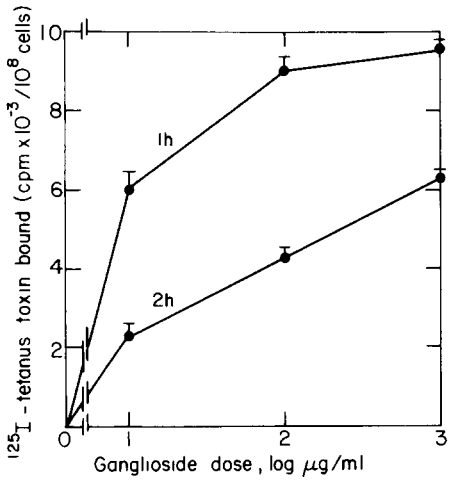

Fig 2 Concentration-dependent ganglioside accumulation by human erythrocytes determined by binding of ^{125}I-labeled tetanus toxin Cells were incubated with G_{1b} ganglioside preparation at various concentrations in phosphate-buffered saline (pH 7.4) at 37°C After 1 or 2 h incubation, unbound ganglioside was removed by three repetitive washes with phosphate-buffered saline (pH 7 4), and ^{125}I-labeled tetanus toxin (50000 cpm/10^8 cells) in Tris-mannitol buffer supplemented with 0.1% ovalbumin and 0 1% gelatin was added for 1 h at 37°C. At the end of incubation, excess toxin was removed by three sequential washes using the Tris-mannitol buffer as detailed under Materials and Methods The values expressed as cpm/10^8 cells represent the low ionic strength interaction of the toxin with the ganglioside-supplemented cells. The values are means of 3–5 samples

is performed for 2 h. Total toxin is remarkably higher after 1 h than after 2 h incubation. In contrast, the salt-resistant, cell-bound toxin, which cannot be removed by physiologic salt shows a clear time dependence as illustrated in Fig. 3. The amount of toxin bound to cells in a salt-resistant manner increases progressively with the time of incubation of the cells in ganglioside-supplemented medium for at least 2 h. Uptake of gangliosides into the salt-resistant cellular compartment is equally effective at all three concentrations studied. Thus, after 2 h incubation with 0.01 mg/ml, toxin binding is almost as high as that achieved in the presence of 0.1 mg and better than that observed at 1 mg/ml ganglioside.

Incorporation of G_{T1b} and G_{D1b} gangliosides by human erythrocyte is also dependent on the incubation temperature. As shown in Fig. 4, at low ganglioside concentration, the total amount of toxin that can be bound to the cells is significantly higher when ganglioside supplements are done at 4°C rather than at 37°C. There are no significant differences in the amount of total toxin bound to cells subjected to ganglioside concentrations greater than 0.3 mg/ml irrespective of the incubation temperature. In contrast, the ganglioside fraction which enables the salt-resistant cell toxin interaction is highly dependent on the temperature

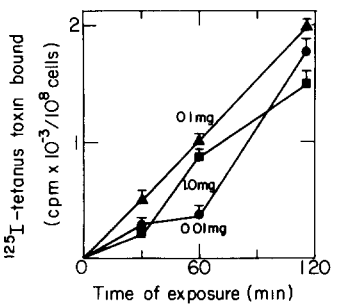

Fig 3 Time- and concentration-dependent accumulation of gangliosides by cells in the high ionic strength resistant cellular compartment determined by binding of ^{125}I-labeled tetanus toxin. Cells were incubated with toxin for 2 h at 37°C under similar conditions as described in Fig. 2, except that following toxin removal by the Tris-mannitol buffer, cells were further rinsed three times in phosphate-buffered saline (pH 7 4) augmented with 0.1% albumin. Values expressed as cpm/10^8 cells represent the high ionic strength interaction of the toxin with the ganglioside-supplemented cells and are means of 3–5 samples

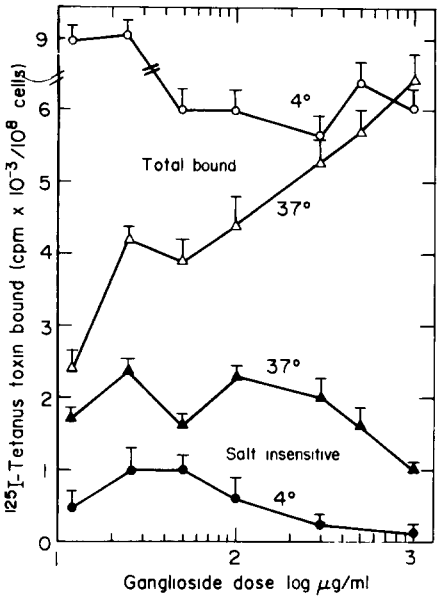

Fig. 4. Effect of temperature on ganglioside accumulation in erythrocytes determined by ^{125}I-labeled tetanus toxin binding Cells incubated for 2 h at either 4 or 37°C with various concentrations of G_{1b} gangliosides were rinsed off excess gangliosides and incubated further with ^{125}I-labeled tetanus toxin for 1 h at 37°C Total bound and salt-insensitive compartments represent the low ionic and high ionic strength cell-associated toxin, respectively, as described under Figs. 2 and 3. Each value is an average ± S.E. of four samples.

at which uptake takes place; it is substantially higher at 37°C than at 4°C over the entire range of substrate concentrations studied. It should be noted that ganglioside concentrations above 0.1 mg/ml, reduce the effective, salt-resistant tetanus toxin bound to cells. The reduced level of effective (salt-resistant) toxin bound to cells pretreated with gangliosides at 4°C suggest the importance of temperature and ganglioside concentration for the uptake of gangliosides.

Uptake of ^{3}H-labeled G_{D1b} ganglioside

The apparent molar concentration of gangliosides taken up by the cells and the amount of bound toxin has been studied directly with ^{3}H-labeled G_{D1b} and ^{125}I-labeled tetanus toxin. As shown in Table I, uptake of ^{3}H-labeled G_{D1b} is directly proportional to the amount of substrate added to the incubation medium. The ganglioside-mediated binding of tetanus toxin is

TABLE I

^{3}H-LABELED G_{D1b} UPTAKE AND ^{125}I-LABELED TETANUS TOXIN BINDING BY WASHED HUMAN ERYTHROCYTES

Human erythrocytes were incubated for 120 min with ^{3}H-labeled G_{D1b} in phosphate-buffered saline (pH 7.4), as detailed under Materials and Methods. Unbound ganglioside was removed by three sequential washes of cells in phosphate-buffered saline (pH 7.4) and ^{15}I-labeled tetanus toxin (133 fmol was added for 2 h at 37°C) At the end of incubation, the cells were washed three times with phosphate-buffered saline augmented with 0.1% albumin and cell-associated ^{125}I label was determined on the pellet The ^{3}H-labeled ganglioside was determined after Folch extraction [26] of the cell pellet Values are averages of triplicates.

G_{D1b} (pmol/10^8 cells)		Tetanus toxin bound (fmol/10^8 cells)	G_{D1b}/toxin (molar ratio) ($\times 10^3$)
added	incorporated		
2 10^1	2.9	0.2	15
$5 \cdot 10^3$	767.0	4.8	160
$5 \cdot 10^4$	8605.0	11 4	755

proportional to the amount of G_{D1b} taken up; at the lowest level of G_{D1b} (2.9 pmol/10^8 cells) there are 15000 molecules on the average which could bind one toxin molecule, while at the highest level of G_{D1b} (8.6 nmol/10^8 cells) there are nearly $0.8 \cdot 10^6$ ganglioside molecules per one toxin molecule. In other words, there is an excess of ganglioside molecules which are not directly involved in the binding process but may be still required in other aspects of this interaction. For comparison, in the central nervous system about $2.5 \cdot 10^9$ molecules per one toxin molecule have been estimated [23].

Effect of cell density, cell viability and diverse agents on ganglioside uptake

Uptake of gangliosides is also dependent on cell density. When normalized per unit cell density (1% cell suspension on the abscissa), at low cell density there is more ganglioside taken up into the total than into the salt-insensitive tight cellular compartment (Fig. 5). These differences are less pronounced as more erythrocytes compete for the same ganglioside concentrations. Thus, although ganglioside supplements in the medium are in large excess, the amount of total ganglioside acquired per cell is reduced as a function of in-

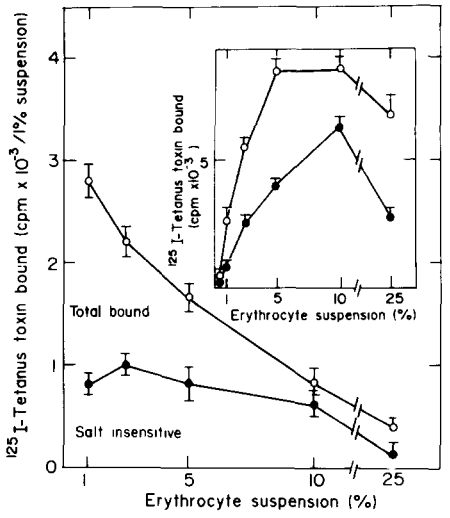

Fig 5 Effect of cell density on ganglioside accumulation measured by ^{125}I-labeled tetanus toxin binding. Washed erythrocytes were diluted in phosphate-buffered saline to the noted densities ($1 \cdot 10^8$ cells, 5% suspension) and incubated with 0 2 mg/ml G_{1b} ganglioside fraction for 2 h at 37°C. After ganglioside removal, ^{125}I-labeled tetanus toxin (1 10^5 cpm/tube) was added and cells were incubated for 2 h at 37°C. Total and salt-resistant cell-associated toxin was determined as described in the legend for Figs. 2 and 3 Values are expressed as cpm of toxin bound per 2 10^7 cells and represent averages ± S.E of 3–4 tubes. The inset illustrates the actual values obtained

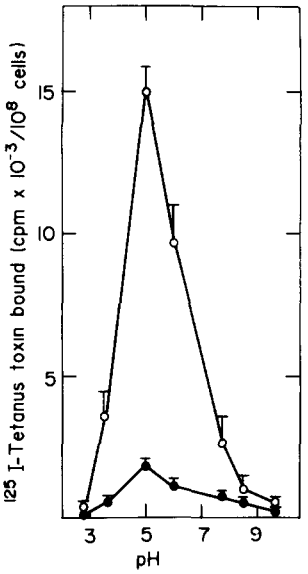

Fig 6 Effect of pH on ganglioside accumulation by erythrocytes estimated by ^{125}I-labeled tetanus toxin binding Experimental conditions for ganglioside uptake and subsequent tetanus toxin binding are similar to those described in Fig. 4 Tris base and acetic acid were used to prepare the various pH solutions Total (○) and salt-resistant (●) tetanus toxin binding was determined in triplicates using exactly the same experimental conditions as detailed in Fig 2

creasing cell density. In contrast, the salt-resistant fraction of cell-associated ganglioside is less affected by cell density; it retains similar values over a wide range of cell concentration.

Ganglioside uptake by human erythrocytes is dependent on pH, as shown in Fig. 6. Maximum ganglioside uptake occurred at approx. pH 5, while at pH 2.9 and pH 9.6 there was little ganglioside uptake. It should be noted that at these extreme conditions, changes in the shape of the erythrocytes were encountered. Also remarkable is the exceedingly high total toxin-binding activity at pH 5 contrasted to pH 4 and 7.6.

The effect of medium composition on accumulation of gangliosides is shown in Table II. Incubation of erythrocytes in Tris-mannitol buffer results in a higher ganglioside uptake into the salt-insensitive compartment compared to phosphate-buffered saline (pH 7.4). Addition of chelating agents, such as EDTA or divalent cations such as Ca^{2+}, had no noticeable effect on uptake. NaCl (50 mM) reduced

TABLE II

EFFECT OF MEDIUM COMPOSITION ON CELL SURFACE-GANGLIOSIDE ACCUMULATION BY ERYTHROCYTES

Cells were incubated with G_{1b} (0.2 mg/ml) fraction for 2 h at 37°C in the presence of either phosphate-buffered saline (PBS), Tris-mannitol or Tris-acetate/0.25 M sucrose (pH 7 4) (TAS) buffer. The salt-resistant ^{125}I-labeled toxin was determined as detailed in Fig. 3 and represents an average ± S E of triplicate tubes

Medium composition	Salt-resistant fraction	
	cpm/10^8 cells ($\times 10^{3-3}$)	% of control
PBS	1 9±0.1	100
Tris-mannitol	3 2±0.6	170
+5 mM EDTA	2 6±0.5	140
+5 mM $CaCl_2$	2 8±0.2	150
+50 mM NaCl	1 7±0.2	90
TAS	2 1±0 2	110
+5 mM Mg_2SO_4	2 3±0 5	120
50 mM NaCl	1 9±0 2	100

TABLE III

EFFECT OF CELL INTEGRITY ON UPTAKE OF GANGLIOSIDES

Washed human erythrocytes, intact or after hypotonic shock, were incubated with 0.2 mg/ml G_{1b} gangliosides under various experimental conditions (experiment A), for 2 h at 37°C). Free ganglioside was removed by rinsing with phosphate-buffered saline (PBS). Intact cells supplemented with gangliosides as in experiment A were osmotically shocked in 10 mM Tris buffer (experiment B) All cells were incubated with ^{125}I-labeled tetanus toxin under standard binding conditions as detailed in Fig 2 Values expressed as cpm $\times 10^{-3}$ per 10^8 cells are averages of triplicate determinations with S E values of less than 15%.

Experimental conditions	^{125}I-labeled tetanus toxin bound (cpm/10^8 cells) ($\times 10^{-3}$)			
	Intact cells		Lysed cells	
	total	salt-resistant	total	salt-resistant
Experiment A				
Tris-mannitol	10.8	4 4	6 0	< 0 05
Tris-mannitol + 5% glucose	8.5	5 7	5 0	0.10
PBS	9.5	1 9	6 5	0 10
Experiment B				
Hypotonic shock after mannitol	9.5	2.8	–	–

the fraction of ganglioside which is salt-resistant by 47%. Sucrose was less effective in substituting mannitol as the isotonic buffer during ganglioside uptake.

Intact or osmotically shocked human erythrocytes exhibit remarkable differences in their ability to incorporate gangliosides. As summarized in Table III, the former incorporate more gangliosides than the latter cells under all experimental conditions studied. There is little if any ganglioside uptake into the salt-resistant compartment in the osmotically shocked cells compared to the high values observed in intact cells. In contrast, 40 and 67% of the salt-resistant fraction is obtained in intact cells which have been supplemented with gangliosides in Tris-mannitol and Tris-mannitol + glucose buffers, respectively. Table III also shows that intact cells loaded with gangliosides in Tris-mannitol buffer ans subsequently lysed in Tris-acetate buffer (experiment B) lose more gangliosides than their unlysed counterparts. Thus, it would appear that the integrity of the erythrocyte membrane is necessary to incorporate or maintain the ganglioside fraction which is responsible for the tight binding of tetanus toxin.

The effect of sialidase pretreatment on the ability of intact cells to incorporate gangliosides is shown in Table IV. Binding of tetanus toxin is not changed when the enzyme is added before ganglioside addition. However, when ganglioside-supplemented cells are treated with sialidase prior to toxin addition, over 85 and 87% reduction in the total and the salt-resistant ganglioside fraction, respectively, is observed. This experiment indicates that nearly all ganglioside receptors for tetanus toxin acquired by the erythrocytes are susceptible to sialidase.

While pretreatment of cells with sialidase has no effect on ganglioside uptake, treatment of erythrocytes with trypsin greatly enhances gang-

TABLE IV

EFFECT OF SIALIDASE ON GANGLIOSIDE UPTAKE BY ERYTHROCYTES

Experimental details were similar to those described for Fig. 4, except that 0.1 mg/ml ganglioside was used. Values are expressed as means ± S.E. of four samples

Treatment	cpm/10^8 cells ($\times 10^{-3}$)	
	Total toxin	Salt-resistant
Control	44 ±3 4	17.1 ± 2.2
Sialidase before ganglioside addition	40 ±1 8	15.0 ± 0 9
Sialidase after ganglioside addition	6.2 ± 1.5	2 ±0.9

TABLE V

EFFECT OF TRYPSIN AND CELL FIXATION ON GANGLIOSIDE ACCUMULATION AS ESTIMATED BY ^{125}I-LABELLED TETANUS TOXIN BINDING

Glutaraldehyde-fixed human erythrocytes were treated with trypsin as noted under Materials and Methods Following treatment, cells were washed three times with phosphate-buffered saline and incubated with 0.1 mg/ml G_{1b} ganglioside for 2 h at 37°C. After removal of the unbound gangliosides, ^{125}I-labelled tetanus toxin (10^5 cpm/tube) was added to cells for 1 h at 37°C. Total toxin bound and salt-resistant cell-associated toxin was determined in quadruplicate tubes

Cell treatment	cpm/10^8 cells ($\times 10^{-3}$)			
	Non-fixed cells		Glutaraldehyde-fixed cells	
	Total	Salt-resistant	Total	Salt-resistant
Control	6.14±0.9	1.8±0.4	8.2±0.7	2.9±0.6
Trypsinized	6.25±0.7	1.4±0.4	15.9±1.2	6.1±0.6
Trypsinized + ganglioside	13.3 ±2.0	6.0±0.7	45.0±4.0	17.8±1.4
Control + ganglioside	21.0 ±2.3	12.9±1.0	44.8±3.8	12.9±2.1

lioside uptake, as shown in Table V. Glutaraldehyde-fixed cells which incorporate nearly 3-fold more gangliosides into the salt-resistant fraction compared to intact cells are sensitive (about 30%) to trypsin treatment. The fixed cells in general exhibit a higher capacity to bind toxin compared to unfixed cells, presumably reflecting some interaction of the toxin with nonganglioside constituents on the cell surface.

Discussion

This report examines various conditions which result in the uptake of G_{D1b} and G_{T1b} gangliosides by intact human erythrocytes as expressed by the ability of the cells to bind tetanus toxin. Normally these cells are practically devoid of such binding activity but acquire this capability in a time- concentration- and temperature-dependent manner, after exposure to these gangliosides.

Binding of tetanus toxin has enabled us to dissociate presumably between two ganglioside fractions based on the sensitivity toward physiological salt. Washing with physiological salt after supplementing cells with ganglioside is a routine procedure, yet after binding of the toxin, a substantial cell-associated toxin fraction could be removed by physiological salt. One possible explanation for this phenomenon is that gangliosides expose nonspecific toxin-binding sites once they become incorporated onto the cell surface. Alternatively, this remarkable sensitivity to physiologic salt which depends on temperature, time and cell integrity during ganglioside uptake, could reflect a preference of the incorporated ganglioside during exposure to toxin to ether retain its association with the cell or bind to the toxin and lose its association with the cell instead. This possibility seems more attractive in view of the excessive binding of total toxin when cells are supplemented with ganglioside at 4°C and pH 5 or even in fixed cells as we document in this study. The observation that the salt-sensitive toxin cannot be reutilized for additional binding to ganglioside (unpublished data) is consistent with a tighter ganglioside-toxin rather than ganglioside-cell association, at least for the salt-removable toxin fraction. In addition, the toxin itself can promote a tight interaction with the ganglioside as part of the mechanism of sequestration and internalization. At this time, we cannot establish whether the tight ganglioside-cell association is acquired prior to or after exposure of cells to tetanus toxin.

The physicochemical basis for the ganglioside uptake into cells is not yet known. The amphiphatic properties of gangliosides in aqueous solutions suggest the presence of monomers, oligomers as well as stable, high molecular weight micellar structures [24]. It is possible that each of these forms can associate with the erythrocyte membrane by a different mechanism. They may also be responsible for some of the intervariability encountered between assays. These arguments were also considered in the uptake of G_{M1} into the chick plasma membrane [6]. In this respect, a loose micellar ganglioside-plasma membrane interaction [24,25] could lead to a dissociation of the ganglioside-tetanus toxin complex from the cell by physiologic salt. In contrast, a stable cell-ganglioside association may be indicative of an interaction of the toxin with monomeric or oligomeric ganglioside forms, presumably correctly incorporated into the bilayer. The remarkable loss of toxin binding

to ganglioside-supplemented cells after treatment with sialidase (Table IV) is consistent with this possibility.

The present study also documents the fact that the amount of cell-associated gangliosides is much higher than that necessary to bind tetanus toxin (Fig. 1 and Table I). This discrepancy, also shown for the interaction of cholera toxin with G_{M1} [6], could be explained by either the existence of ganglioside domains on the membranes which are not accessible to tetanus toxin or to endocytic internalization of the ganglioside by the cells. The latter possibility has been discussed and excluded at least in the case of a mouse cell line supplemented with G_{M3} [5]. It is also unlikely to take place in erythrocytes which are normally devoid of endocytic mechanisms.

From the observation that pretreatment of cells with trypsin or fixation with glutaraldehyde, both of which significantly increase ganglioside uptake, we suggest that proteins present at the cell surface may interfere with the uptake process. This is in marked contrast to the inhibitory effect of trypsin on ganglioside uptake by cultured mouse cells [5] and may point out perhaps to some unique properties of the human erythrocyte membrane.

Finally, in contrast to fusion of membranes observed in chick erythrocytes after disialo- and trisialo-ganglioside addition [15], human erythrocytes encountered little or no damage after this treatment, as studied by scanning electron microscopy. The cells also showed no distinguishable changes in orientation or density of intramembrane particles, as revealed by freeze-fracture electron microscopy (unpublished data).

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