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Topical Review

Role of Membrane Gangliosides in the Binding and Action of Bacterial Toxins

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Summary. Gangliosides are complex glycosphingolipids that contain from one to several residues of sialic acid. They are present in the plasma membrane of vertebrate cells with their oligosaccharide chains exposed to the external environment. They have been implicated as cell surface receptors and several bacterial toxins have been shown to interact with them. Cholera toxin, which mediates its effects on cells by activating adenylate cyclase, bind with high affinity and specificity to ganglioside G_{M1}. Toxin-resistant cells which lack G_{M1} can be sensitized to cholera toxin by treating them with G_{M1}. Cholera toxin specifically protects G_{M1} from cell surface labeling procedures and only G_{M1} is recovered when toxin-receptor complexes are isolated by immunoadsorption. These results clearly demonstrate that G_{M1} is the specific and only receptor for cholera toxin. Although cholera toxin binds to G_{M1} on the external side of the plasma membrane, it activates adenylate cyclase on the cytoplasmic side of the membrane by ADP-ribosylation of the regulatory component of the cyclase. G_{M1} in addition to functioning as a binding site for the toxin appears to facilitate its transmembrane movement. The heat-labile enterotoxin of *E. coli* is very similar to cholera toxin in both form and function and can also use G_{M1} as a cell surface receptor. The potent neurotoxin, tetanus toxin, has a high affinity for gangliosides G_{D1b} and G_{T1b} and binds to neurons which contain these gangliosides. It is not yet clear whether these gangliosides are the physiological receptors for tetanus toxin. By applying the techniques that established G_{M1} as the receptor for cholera toxin, the role of gangliosides as receptors for tetanus toxin as well as physiological effectors may be elucidated.

Key words adenylate cyclase · ganglioside · toxins · cholera toxin · tetanus toxin

Gangliosides Are on the Cell Surface

Gangliosides are complex glycosphingolipids; they consist of a lipid moiety (ceramide) to which is attached an oligosaccharide chain containing at least one sialic acid residue (Fig. 1). They are characterized by their carbohydrate portions; the major gangliosides in mammalian brain have a neutral tetraoligosaccharide to which are attached from one to three sialic acids (Fig. 2). Gangliosides are synthesized in the Golgi apparatus and are predominently located in the plasma membrane with their oligosaccharide chains exposed on the cell surface [32][1]. As the ceramide portion is too short to span the bilayer, gangliosides appear to be confined to the outer half of the lipid bilayer [120]. Because of their orientation on the cell surface, gangliosides have been implicated in various recognition phenomena. In the present review, I will describe the role of gangliosides as receptors for several bacterial toxins.

Cholera Toxin: Form and Function

Of the various bacterial toxins that have been reported to interact with gangliosides, choleragen (cholera toxin) has been the most extensively studied, understood, and reviewed [2, 26, 27, 41, 55, 62, 79, 99]. The toxin is produced by *Vibrio cholerae* and elicits the characteristic watery diarrhea associated with cholera. Choleragen mediates its pathological effects by binding to specific receptors on the intestinal mucosal cell [12, 65, 129] and activating adenylate cyclase [74, 119]; the subsequent rise in cyclic AMP results in chloride and water secretion by the cells [25]. Choleragen also is a potent and persistent activator of adenylate cyclase in most vertebrate cells [2, 30, 41].

The toxin is a globular protein of 84,000 daltons composed of two structurally and functionally distinct components (Fig. 3). The B component consists of five identical polypeptides, binds with high affinity to cell surface receptors, and is nontoxic. The A component consists of two dissimilar polypeptides linked by a disulfide bond, does not bind to cells, and is nontoxic to intact cells.

[1] In several lines of cultured cells, around 80% of the total gangliosides were found to be on the cell surface [85].

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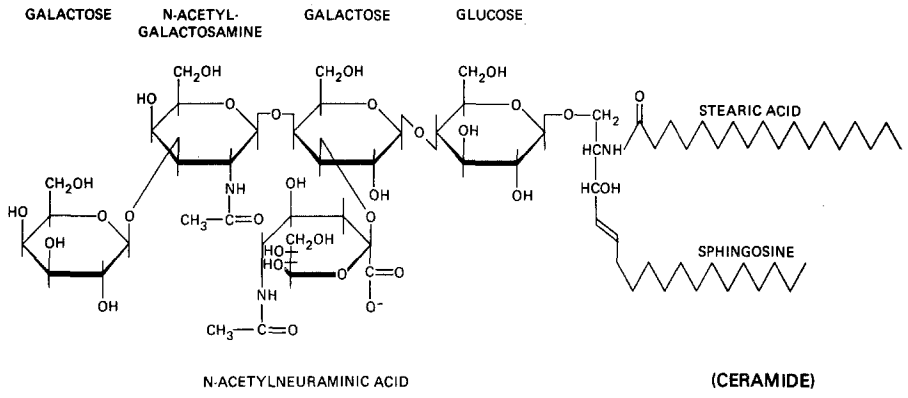

Fig. 1. Structure of monosialoganglioside G_{M1}

G_{M1} Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer
3
↑
2αNeuAc

G_{D1a} Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer
3 3
↑ ↑
2αNeuAc 2αNeuAc

G_{D1b} Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Cer
3
↑
2αNeuAc8← 2αNeuAc

G_{T1b} Galβ1→3GalNAcβ1→4Galβ1→ 4Glcβ1→Cer
3 3
↑ ↑
2αNeuAc 2αNeuAc8← 2αNeuAc

Fig. 2. Structures of major brain gangliosides. Nomenclature of gangliosides from Svennerholm [125]. *Abbreviations:* Cer, ceramide; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; NeuAc, N-acetylneuraminic acid

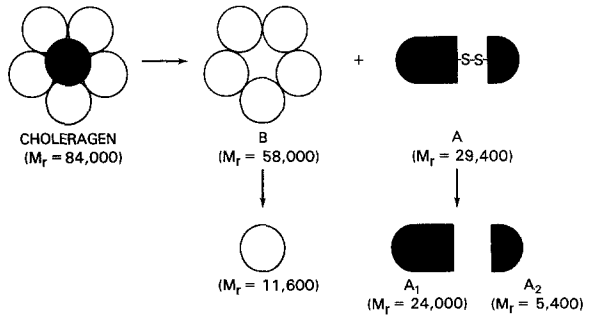

Fig. 3. Model of subunit structure of choleragen. Choleragen is composed of an A component and a pentameric B component. It is believed that the A component is attached to the B component through its A1 peptide. The structure of choleragen has been reviewed recently [62, 79]

Cholera Toxin Activates Adenylate Cyclase by ADP-Ribosylation

Choleragen and its A component, but not the B component, can activate adenylate cyclase in disrupted cells and membranes [40, 42, 89, 112]. Activation requires NAD and is enhanced by thiols which reduce the disulfide bond in the A component and release the A_1 peptide [40, 42, 89]. Moss and coworkers demonstrated that the A_1 peptide catalyzes the hydrolysis of NAD and the transfer of ADP-ribose to arginine and several proteins [97–99]. The cellular protein that is ADP-ribosylated by the toxin is a subunit of the guanine nucleotide binding component of adenylate cyclase [6, 43, 69] (Fig. 4). Also known as the regulatory component, it maintains adenylate cyclase in an activated state when GTP is bound to it [109]. Upon hydrolysis of the bound GTP or exchange with GDP, the cyclase reverts to an inactive state [7, 78, 109]. It is believed that ADP-ribosylation of the regulatory component inhibits GTP hydrolysis or exchange and maintains the cyclase is a persistently activated state [7, 78].

Ganglioside G_{M1} Is the Receptor for Cholera Toxin

Binding of choleragen to intact cells and membranes is rapid, saturable, and of high affinity [13, 30, 64, 129]. The nature of the toxin receptor was first suggested by van Heyningen et al., who observed that crude preparations of gangliosides inactivated choleragen [57]. Several groups then showed that ganglioside G_{M1} (Fig. 1) was the most potent inhibitor of toxin binding and action [13, 14, 66, 75, 122]. Cuatrecasas also demonstrated that rat adipocytes preincubated with G_{M1} bound increased amounts of choleragen and exhibited an enhanced response to the toxin [14]. In other studies, there was a correlation between toxin binding and G_{M1} content in several cell types [60, 65]. Finally, exposure of several cell lines to sodium butyrate caused a parallel increase in the number of toxin receptors and the amount of G_{M1} [29]. When butyrate-treated and G_{M1}-treated HeLa cells were compared, both types exhibited a similar increase in toxin binding and sensitivity (Fig. 5). Thus, exogenously incorporated G_{M1} was functionally equivalent to endogenously induced G_{M1} as a receptor for choleragen.

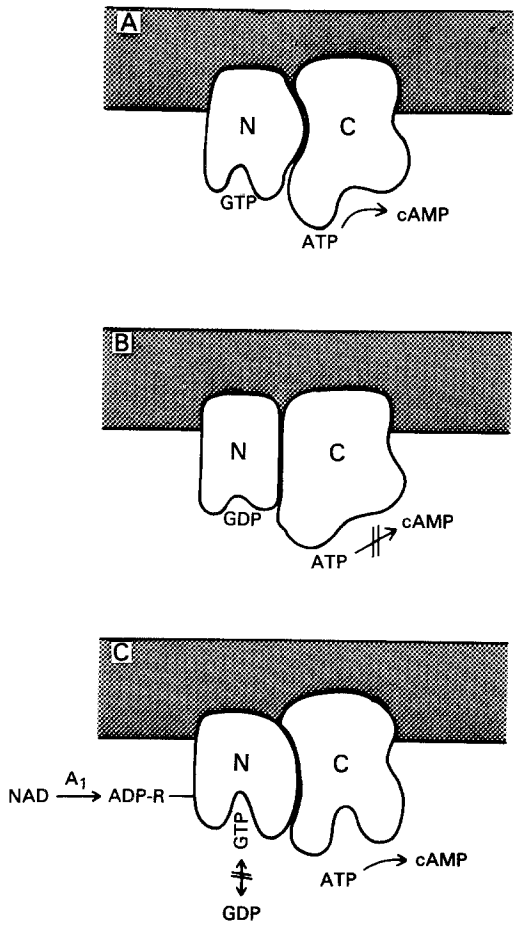

Fig. 4. Activation of adenylate cyclase by choleragen. (*A*): The regulatory component (*N*) when liganded with GTP stimulates the catalytic component (*C*) to convert ATP to cyclic AMP. (*B*): Upon hydrolysis of the bound GTP or exchange for GDP, adenylate cyclase reverts to an inactive state. (*C*): The A_1 peptide of choleragen catalyzes the transfer of ADP-ribose from NAD to *N*; the hydrolysis or exchange of GTP is inhibited and the cyclase remains activated

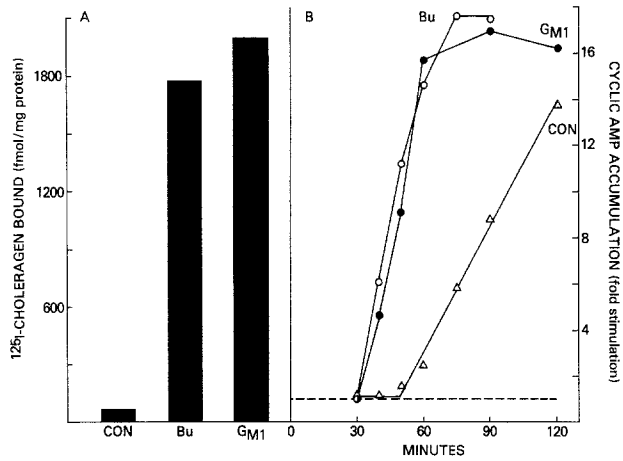

Fig. 5. Effect of treating HeLa cells with sodium butyrate or G_{M1} on binding and action of choleragen. HeLa cells were treated with no addition (*CON*, △), 5 mM sodium butyrate for 48 hr (*Bu*, ○) or 1 μM G_{M1} for 1 hr (G_{M1}, ●). The cells were washed and assayed for specific ^{125}I-choleragen binding (*A*) or accumulation of intracellular cyclic AMP in response to choleragen (*B*). Dotted line (-----) indicates basal levels of cyclic AMP. (Figure is reproduced from Fishman and Henneberry [33])

Is G_{M1} the Natural Receptor for Cholera Toxin?

Despite the substantial evidence implicating G_{M1} as the receptor for choleragen, several investigators have suggested that the native receptor for the toxin is more complex than G_{M1} [20, 72, 76, 77, 87]. To clarify the situation, studies were initiated with a line of transformed mouse fibroblasts (NCTC 2071) which had been adapted to grow in chemically defined medium[2]. These cells were unable to respond to choleragen and lacked any detectable G_{M1} [36, 89]. When the cells were cultured in medium containing [^{3}H]G_{M1}, the cells took up the ganglioside and responded to choleragen. A maximal response was observed when 100,000 molecules of G_{M1} had been incorporated per cell [89]. The cells were able to take up other gangliosides added to the medium but did not become sensitive to the toxin [36, 37]. Similar results were obtained with rat glioma C6 cells (Table 1). Only G_{M1} was effective in enhancing toxin binding and activation of adenylate cyclase. In addition, toxin binding increased in direct proportion to the amount of G_{M1} taken up by the cells; and, the more G_{M1} incorporated, the more rapidly adenylate cyclase became activated by choleragen [28, 37].

Several other lines of evidence support G_{M1} as the only receptor for choleragen. Exhaustive delipidation of cells and membranes completely removed all toxin binding activity [12, 13, 29], whereas proteases had no effect on toxin binding [12, 13, 27, 29]. Finally, lipid extracts of intestinal membranes were separated on thin-layer silica gel and the chromatogram overlayed with ^{125}I- choleragen. Toxin binding was detected only to material corresponding to G_{M1} (Fig. 6*A*). Intestinal membranes also were dissolved in sodium dodecyl sulfate (SDS) and separated by SDS-polyacrylamide gel electrophoresis. When the gel was overlayed with labeled choleragen, binding was detected only at the front of the gel where the lipids migrated (Fig. 6*B*).

[2] When NCTC 2071 cells were cultured in medium supplemented with fetal calf serum which contains gangliosides, the cells took up small amounts of G_{M1} from the serum and became sensitive to choleragen [31].

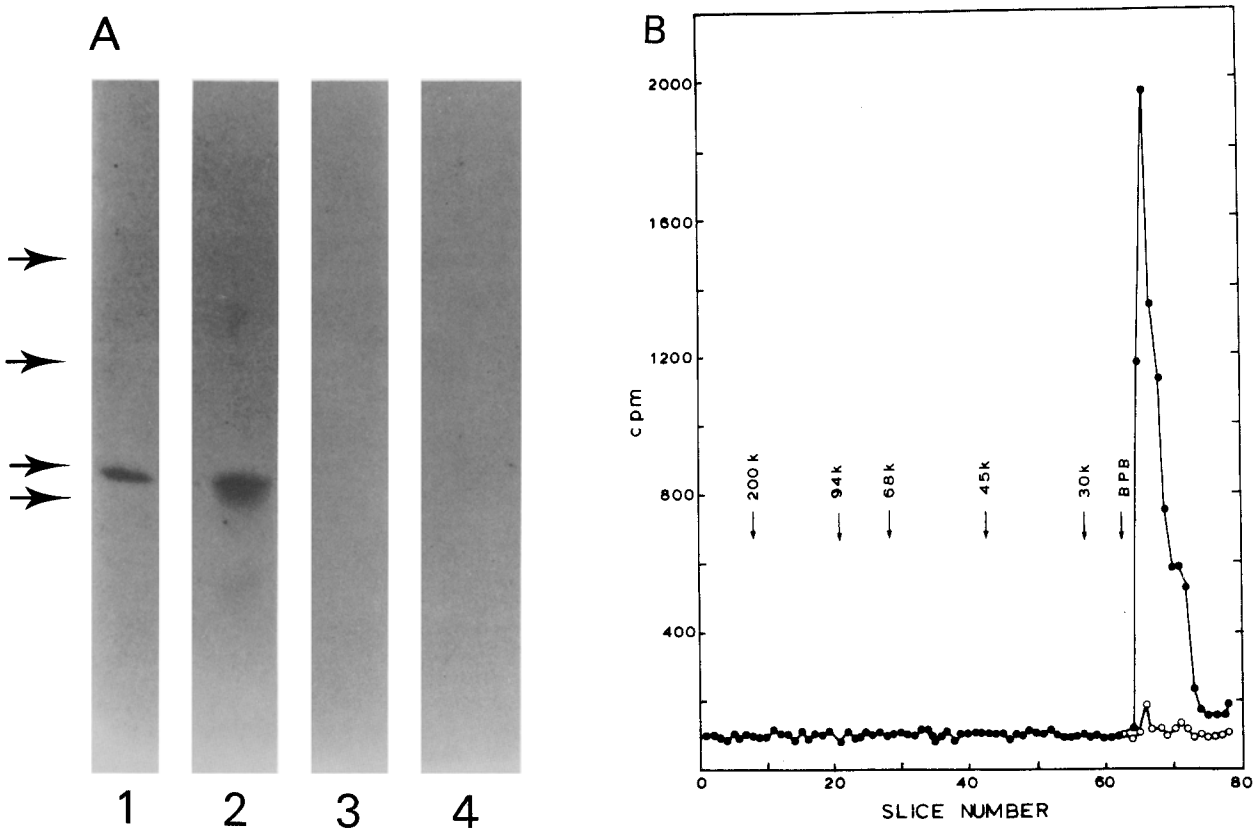

Fig. 6. Detection of the choleragen receptor in rat intestinal brush borders. (*A*): Total lipids were extracted from the brush borders and separated by thin-layer chromatography on a silica gel-coated plastic sheet. After drying the chromatogram, it was overlayed with ^{125}I-choleragen for 2 hr at 4 °C, washed, and analyzed for bound iodotoxin by autoradiography. Lane *1*, G_{M1}; lane *2*, total lipids from brush borders; lanes *3* and *4*, same as lanes *1* and *2* except incubated in the presence of excess unlabeled toxin. *Arrows* indicate mobilities of from top to bottom G_{M3}, G_{M2}, G_{M1}, and G_{D1a}. (*B*) Brush borders were dissolved in SDS and subjected to SDS-polyacrylamide gel electrophoresis. The gels were incubated with ^{125}I-choleragen in the presence (○) and absence (●) of excess unlabeled toxin, washed, sliced, and counted. *Arrows* indicate the positions of proteins of known molecular weight run on the same slab gel; BPB, bromophenol blue. (Figures are reproduced from Critchley, Magnani and Fishman [12])

Table 1. Treatment of rat glioma C6 cells with glycolipids: Effects on choleragen binding and action[a]

Glycolipid	Oligosaccharide structure	^{125}I-choleragen[b] bound	Cyclic AMP[c] accumulation
None		24	38
G_{A1}	Gal-GalNAc-Gal-Glc	22	34
G_{M3}	NeuNAc-Gal-Glc	18	38
G_{M2}	GalNAc-[NeuNAc]-Gal-Glc	24	46
G_{M1}	Gal-GalNAc-[NeuNAc]-Gal-Glc	1590	325
G_{D1a}	NeuNAc-Gal-GalNAc-[NeuNAc]-Gal-Glc	26	54
G_{D1b}	Gal-GalNAc-[NeuNAc-NeuNAc]-Gal-Glc	40	37

[a] Data from Fishman [28]. Cells were incubated with the indicated glycolipid (0.5 μM except G_{A1}, 0.1 μM) for 1 hr at 37 °C, washed, and incubated with 20 nM ^{125}I-choleragen for 30 min or 20 nM unlabeled toxin for 50 min at 37 °C. The cells were then assayed for bound iodotoxin and intracellular cyclic AMP. Uptake of each of the glycolipids, which were radiolabeled, was similar.

[b] Values in fmol/mg protein have been corrected for nonspecific binding as measured in the presence of 50-fold excess unlabeled choleragen.

[c] pmol/mg protein; in the absence of choleragen, cyclic AMP content was 30 pmol/mg protein.

Choleragen Directly Interacts with G_{M1} on the Cell Surface

When G_{M1}-treated NCTC 2071 cells were exposed to galactose oxidase followed by NaB^3H_4, ^{3}H was incorporated into G_{M1} [93]. When the cells were labeled in the presence of choleragen, incorporation of ^{3}H into G_{M1} was effectively blocked (Table 2). Similar results were obtained with cells that contain endogenous G_{M1} such as human fibro-

Table 2. Effect of choleragen on surface labeling of exogenous G_{M1} taken up by NCTC 2071 cells[a]

Oxidant	^3H in G_{M1} (dpm/mg protein $\times 10^{-3}$)	
	−Choleragen	+Choleragen
None	23	21
Sodium periodate	118	81
Galactose oxidase	160	20

[a] Data from Moss et al. [93]. G_{M1}-treated NCTC 2071 cells were incubated for 2 hr with and without choleragen, washed, and then treated with either sodium periodate or galactose oxidase. After washing the cells, they were exposed to NaB^3H_4, washed, and analyzed for ^3H incorporated into G_{M1}.

blasts [93], rat adipocytes [101], rat intestine [12] and murine neuroblastoma [85]. The surface-bound toxin specifically protected G_{M1} but not other glycolipids or glycoproteins from being labeled [12, 93]. Choleragen also protected surface G_{M1} from oxidation by $NaIO_4$, which oxidizes sialic acid residues (Table 2). These results clearly demonstrated that choleragen directly binds to G_{M1} on the cell surface.

Choleragen-receptor complexes also have been isolated by immunoadsorption procedures. Cells or membranes first were labeled by the galactose oxidase/NaB^3H_4 technique or by culturing them in medium containing [^3H]galactose, then incubated with choleragen and extracted with non-ionic detergents. The soluble toxin-receptor complexes were immunoadsorbed by anticholeragen antibodies and precipitated by fixed *S. aureus*. Using this procedure, G_{M1} was specifically recovered from murine fibroblasts and lymphoid cells [11], rat intestinal membranes [12], and murine neuroblastoma cells [52, 85].

Cholera Toxin Binds to G_{M1} in Model Systems

Choleragen is precipitated from solution by G_{M1}, forms a precipitation band with the ganglioside in agar diffusion gels, cosediments with it in the ultracentrifuge, and binds to G_{M1} that has been absorbed to plastic tubes [56, 61, 63, 66, 117, 122].

Choleragen also binds to the free oligosaccharide of G_{M1} [34, 67, 115]. Binding is multivalent with each toxin molecule binding to four to six oligosaccharide chains [34, 117]; presumably, there is one binding site on each of the five polypeptides of the B component. In the presence of G_{M1}-oligosaccharide, choleragen and its B component exhibited shifts in their fluorescence and circular dichroic spectra [34]. The fluorescence studies indicated that the single tryptophan residue on each peptide of the B component was being shifted to a more hydrophobic environment. Together with the circular dichroic data, these results are consistent with a perturbation or conformational change in the toxin molecule upon binding to its receptor. Choleragen also binds to G_{M1} incorporated into model lipid membranes [35, 90, 95, 107, 126–128]. Binding of the toxin causes a perturbation of the lipid bilayer [90, 95, 128] which is also caused by the B but not the A component [95].

When cholergen was bound to G_{M1}-liposomes, the A component remained accessible as it was reduced to A_1 and A_2 by thiols [126]. Using photoreactive radioactive lipids incorporated into liposomes containing G_{M1}, the ability of choleragen or its subunits to penetrate into the lipid bilayer was examined further [127]. The lipids were designed so that the photoreactive group was buried within the bilayer at different depths. When bound to these liposomes, choleragen or its subunits did not penetrate into the bilayer; the B and the A_2 peptides were labeled only by the most shallow probe and the A_1 peptide was not labeled by any of the probes. After reduction of the bound toxin with glutathione, A_1 was labeled by all three probes, labeling of A_2 by the shallow probe was increased and there was still no labeling of A_2 and B by the deeper probes. These studies confirm the work of Wisniesky and Bramwell, who used biological membranes (virus particles) and a photoreactive lipid probe buried in the outer monolayer of the viral membrane [131]. They found that A_1 but not B penetrated into the membrane in a time and temperature dependent manner.

Is there a Cytoskeletal Connection?

Several groups have reported that choleragen bound to lymphocytes underwent a lateral redistribution to form patches and caps [9, 106, 118]. These effects were observed with either fluorescently labeled antibodies, choleragen, or a G_{M1} derivative. The redistribution was observed at 37 but not 0 °C and was blocked by anticytoskeleton drugs as well as metabolic poisons. Capping was inhibited by high concentrations of choleragen and not observed in cells with high amounts of toxin receptors (either natural or by pretreating the cells with G_{M1}) [106]. G_{M1}-deficient leukemic lymphocytes preincubated with low concentrations of G_{M1} and then choleragen exhibited pronounced caps [106]. Taken together with the ability of choleragen to induce capping of the fluorescent G_{M1} derivative incorporated into lymphocytes, it is clear

Table 3. Detergent extraction of bound ^{125}I-choleragen from cells and membranes[a]

Cell line		Detergent-extracted choleragen (%)
Mouse neuroblastoma NB41A	*In situ*	11.3±0.75
	In suspension	17.3±0.70
	Membranes	27.4±2.9
Rat glioma C6	*In situ*	15.2±1.1
	Membranes	25.2±2.6
G_{M1}-treated C6[b]	*In situ*	21.2±0.35

[a] Data from Hagmann and Fishman [52]. Cells were incubated with ^{125}I-choleragen *in situ* or in suspension, washed, and extracted with 0.5% Triton X-100 for 5 min at 0 °C under conditions which leave the cytoskeleton intact. In addition, membranes were prepared from toxin-treated cells and extracted with 1% Triton X-100 for 15 min at 0 °C.

[b] Cells were incubated with G_{M1} for 1 hr at 37 °C.

toxin-G_{M1} complexes were forming caps. As the B component also induced capping [9, 106, 118], the phenomenon was not due to activation of adenylate cyclase but appeared to precede it [9].

Since the lipid portion of G_{M1} is too short to span the bilayer, these results suggested that G_{M1} is associated with some membrane protein which in turn spanned the bilayer and interacted with the cytoskeleton. This possibility is supported by additional evidence. Sahyoun et al. have recently reported that choleragen bound to rat erythrocytes was not readily extracted by non-ionic detergents and remained associated with the cytoskeletal residue [113]. Upon further solubilization of the cytoskeletal residue, the toxin appeared to be associated with a large macromolecular complex. Similar observations have been made by us and others [52, 124]. Less than 25% of the choleragen bound to cells and membranes was extracted by Triton X-100 under conditions that left the cytoskeleton intact (Table 3). Resistance to extraction was relatively independent of the amount of toxin bound, the number of toxin receptors per cell, and the temperature of binding [52]. When rat glioma C6 cells were incubated with [^{3}H] G_{M1}, over 70% of the ganglioside was extracted from the cells. When these cells were treated with choleragen, only 20% of the bound toxin was extracted and the amount of G_{M1} extracted was significantly reduced. Thus, toxin-G_{M1} complexes appeared to remain associated with cytoskeletal elements under conditions where the bulk of the plasma membrane lipids were removed by the detergent. In addition, this apparent association appeared to be induced by the toxin.

Multivalent Binding of Cholera Toxin to the Cell Surface

The multivalent binding between choleragen and the oligosaccharide of G_{M1} also appears to occur between the toxin and its receptor on the plasma membrane. Craig and Cuatrecasas observed that choleragen-treated lymphocytes bound to agarose beads containing covalently attached G_{M1} [9]. Choleragen also caused agglutination of G_{M1}-treated human erythrocytes and liposomes [107]. Under careful conditions, it was found that the ratio of G_{M1} content to choleragen bound in several cell lines was between 5 and 7 [85].

Cuatrecasas and coworkers suggested that multivalent binding to choleragen to its receptor and lateral redistribution of the toxin-receptor complex in the plane of the membrane were essential steps in its mechanisms of action [2, 9, 112]. In order to assess the relative contributions of these two processes to toxin action, the effect of G_{M1}-oligosaccharide on the activation of adenylate cyclase by choleragen was examined [30]. When cells with a low density of toxin receptors were incubated with toxin at 4 °C and then shifted to 37 °C in the presence the oligosaccharide, activation of adenylate cyclase was effectively inhibited (Table 4). This inhibition was not observed with G_{M1}-treated cells (Table 4) or cells with a high density of endogenous receptors [30]. The effect of the oligosaccharide was significantly reduced when the cells were incubated with toxin above 18 °C before adding the oligosaccharide. At 37 °C, the longer the delay between the addition of choleragen and that of the oligosaccharide, the smaller the inhibition was. These results were interpreted as follows: Multivalent binding of choleragen to cells with few receptors requires lateral movement of the receptors and is time and temperature dependent. Multivalent binding of the toxin to cells with many receptors is rapid and occurs at low temperatures where lateral diffusion is minimal. Thus, multivalent binding appears to be essential for toxin action but lateral redistribution may only be required to achieve multivalent binding of the toxin to cells with few receptors. These conclusions are supported by the ability of sodium azide to block choleragen activation of adenylate cyclase in control but not G_{M1}-treated human lymphocytes[3].

Choleragen Takes Its Time on Intact Cells

In contrast to the immediate rise in adenylate cyclase activity observed when membranes are incu-

[3] *Unpublished observations.*

Table 4. Inhibition of choleragen-activation of adenylate cyclase by G_{M1}-oligosaccharide and reversal by G_{M1}-treatment[a]

Cell line	G_{M1}-treatment	Toxin receptors (fold increase)	Inhibition of toxin-activation of adenylate cyclase by G_{M1}-oligosaccharide (%)
HeLa	–	1	88
	+	458	3
Rat glioma C6	–	1	85
	+	78	2
Human Lymphocytes	–	[b]	100
	+	–	29

[a] Data on HeLa and C6 cells from Fishman and Atikkan [30]. Cells were incubated with and without 1 μM G_{M1}, washed, and assayed for ^{125}I-choleragen binding. Other portions were incubated with and without choleragen for 10 min at 4 °C and then 2 hr at 37 °C in the presence and absence of G_{M1}-oligosaccharide. The cells then were assayed for adenylate cyclase activity.

[b] Not determined.

bated with A_1 peptide and NAD [22, 40], there is a distinct lag in activation of cyclase when intact cells are exposed to choleragen (*see* Fig. 5). The lag period has been studied extensively [3, 28] and appears to be highly dependent on temperature. At or below 15 °C, choleragen bound to intact cells did not activate adenylate cyclase even after 24 hr [28]. As the temperature was elevated, the lag period became shorter and the rate of activation increased. Incubating the cells with choleragen at 15 °C and then shifting them to a higher temperature did not reduce the lag period. Even under optimum conditions, the lag period was about 10 min in several cell lines that have a large number of toxin receptors[4]. Whereas choleragen exposed to cells in the presence of anticholeragen antibodies was inactive, the toxin once bound to the cells became resistant to the antibodies within 1 min at 37 °C [28, 42]. When the cells were incubated with choleragen at or below 15 °C for 15 min, exposed to antitoxin and shifted to 37 °C, the toxin remained inactive even though it remained bound to the cells [28]. These and other results indicate that the lag period represents some transmembrane process.

The Remaining Mystery in Cholera Toxin Action

It is generally accepted that this transmembrane process represents the mechanism whereby the A_1 peptide gains access to the regulatory component of adenylate cyclase. As indicated in Fig. 7, choleragen is initially bound through its B component to G_{M1} oligosaccharides on the external side of the plasma membrane. The regulatory component (designated as *N*) is located on the cytoplasmic side of the membrane with most of it extended into the cytoplasm [24, 73]. Several models have been proposed to explain the mechanism of A_1 entry into the cell.

1) Gill has hypothesized that the B subunits penetrate into the membrane and form a hydrophilic channel through which the A component can pass [39]. Once across the membrane, A is reduced to generate A_1 by cytoplasmic reductants such as glutathione, and A_1 now free in the cytoplasm can ADP-ribosylate *N*. Both the holotoxin and the B component can induce permeability changes in lipid bilayers containing G_{M1} which are consistent with the formation of pores or channels [95, 128]. The studies with photoreactive lipid probes, however, indicated that A_1 but not B was penetrating the membrane [127, 131].

2) Others have speculated that choleragen un-

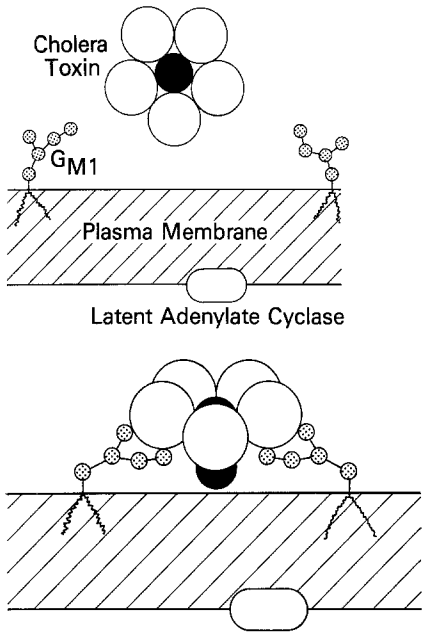

Fig. 7. Topography of G_{M1}, bound choleragen, and adenylate cyclase in the plasma membrane. (*Top*): G_{M1} is located on the outer layer of the plasma membrane with its ceramide portion buried in the membrane and its oligosaccharide exposed on the surface. The regulatory and catalytic components of adenylate cyclase are attached to the inner layer of the plasma membrane. (*Bottom*): After multivalent binding of the B component of choleragen to several G_{M1} oligosaccharides, the A component is still located on the cell surface and adenylate cyclase on the cytoplasmic side of the membrane. (Figures are reproduced from Fishman [27])

[4] S. Kassis, J. Hagmann, P.H. Fishman, *unpublished observations* on mouse neuroblastoma, Friend erythroleukemic and G_{M1}-treated rat glioma C6 cells.

dergoes receptor-mediated endocytosis, is processed in the lysosomes and recycled back to the plasma membranes [68, 82]. This idea was developed based on the ability of lysomotropic agents such as chloroquine, NH_4Cl, dansylcadeverine, and methylamine to inhibit the action of choleragen on intact cells [68, 82]. Choleragen undergoes partial endocytosis [53, 70] and is slowly degraded by intact cells [51, 91]. When the rate of degradation was compared with the rate of activation of adenylate cyclase, no degradation was detected until 1 hr, by which time adenylate cyclase had been completely activated [28*a*]. My own studies indicated that these agents were quite variable in their effects on toxin action and degradation[3]. With mouse neuroblastoma cells, chloroquine was the most effective inhibitor of toxin degradation (>90%) but only had a slight effect on activation of cyclase. Methylamine and NH_4Cl were moderately effective in inhibiting degradation; but, whereas methylamine partially blocked activation, NH_4Cl had no effect. Dansylcadaverine effectively inhibited both processes but also inhibited hormone-stimulated adenylate cyclase and proved to be very toxic to the neuroblastoma cells. It was nontoxic to human fibroblasts and did not inhibit hormone-stimulated adenylate cyclase. Both chloroquine and dansylcadaverine, however, had little or no effect on toxin action even though both compounds inhibited toxin degradation over 90% in the human fibroblasts. In addition to elevating the pH of the lysosomes and inhibiting lysosomal proteases, these agents also appear to have effects at the cell membrane by interfering with ligand-induced clustering of receptors and internalization of ligand-receptor complexes [38, 81, 104]. Thus, their site and mode of action in reference to choleragen is not clear.

3) A third model depicted the A component penetrating across the membrane [27, 32]. Multivalent binding of the B component to several receptors would induce a perturbation both in the toxin and the membrane. This would facilitate the dissociation of the A and B components and the penetration of the A component into the membrane. The ability of G_{M1}-oligosaccharide to perturb the toxin molecule [34], the ability of the toxin to perturb lipid bilayers [90, 128] and the ability of the A component to penetrate membranes as detected by photoreactive probes [127, 131] are all consistent with this model. The model was based on the assumption that the A component had hydrophobic regions which became exposed after the toxin bound to G_{M1} on the cell membrane. The A component has been reported to directly bind to fat cells [112] and liposomes [95]. Recently, Ward et al. [130] have reported that choleragen and its subunits are hydrophilic with no evidence of masked hydophobic regions. Thus, direct penetration of a hydrophilic protein across the membrane bilayer would not be a very efficient process. It has been pointed out that only a small number of choleragen molecules per cell are required to activate adenylate cyclase [28, 40–42] and that only a small number of the toxin molecules bound to a cell may be involved in the activation [40–42].

Are Membrane Proteins Involved in Cholera Toxin Translocation?

Cells exposed to cycloheximide or puromycin lost their ability to respond to choleragen but not beta-adrenergic agonists [51]. Inhibition of cyclase activation by the toxin was time and dose dependent and paralleled inhibition of protein sythesis. Cycloheximide-treated cells actually bound more choleragen than did control cells; thus, loss of toxin action was not due to loss of toxin receptors. In addition, adenylate cyclase was activated in membranes from cycloheximide-treated cells that were incubated with the A1 peptide and NAD. Exposure of the cells to cycloheximide also blocked their ability to degrade bound ^{125}I-choleragen. These results indicated that choleragen was able to bind to intact cells treated with cycloheximide but was unable to gain access to the inside of the cell and activate adenylate cyclase.

Since the A component has to be reduced to A_1 in order to activate adenylate cyclase in membranes [40], the possibility that A_1 was generated in intact cells and not in cells exposed to cycloheximide was explored. Cells that had been incubated with ^{125}I-choleragen at 4 and shifted to 37 °C for different times were lysed and dissolved in sodium dodecyl sulfate. The samples then were analyzed for the distribution of labeled toxin subunits by SDS-polyacrylamide gel electrophoresis [51]. After 15 min at 37 °C, small amounts of A_1 were detected in the control but not the cycloheximide-treated cells, and the amount of A_1 increased by 30 min. The relationship between generation of A_1 and activation of adenylate cyclase was compared in several cell lines[4]. Both processes exhibited the same lag time and time course, the same temperature dependence and the same inhibition by anticholeragen antibodies. Thus, A_1 appears to be the active form of choleragen in intact cells as well as in membranes and prior inhibition of protein synthesis prevents its formation.

A New Model for Cholera Toxin Penetration and Action

Based on the ability of cycloheximide to block choleragen action on intact cells as well as inhibit its degradation and conversion to A_1 peptide, it was proposed that a membrane protein was involved in the translocation of the A component across the membrane [51]. As indicated in Fig. 8, this model is similar to the third model described above except that the internalization of the A component is facilitated by the "translocator." Once into the membrane, A could be reduced to A_1 by a plasma membrane thiol:protein oxireductase [97]. It is also possible that treatment with cycloheximide results in a decrease in this enzyme, thus preventing the formation of A_1 and the activation of adenylate cyclase. It would not explain why the cycloheximide-treated cells were unable to degrade the toxin. There also appears to be a protein factor in the cytoplasm as well as the membranes that promotes the ADP-ribosylation of the regulatory component by the A_1 peptide [22, 116]. Inhibition of protein synthesis could reduce its level in the cells and thereby reduce cellular responsiveness to choleragen. This appears unlikely as cyclase was activated in membranes from cycloheximide-treated cells by A_1 and NAD. In addition, it would not explain the inhibition of A_1 generation and toxin degradation.

One aspect of the "translocator model" that is especially attractive is that the translocator presumably spans the membrane. Thus, it could explain the resistance of toxin-receptor complexes to detergent extraction [52, 113, 124] as well as the lateral redistribution of these complexes in lymphocytes [9, 106, 118]. The toxin-receptor-translocator would form a macromolecular complex that can associate with the cytoskeleton and remain behind after extraction of the cells with detergents. There are several possibilities as to how these complexes might be formed. As the B component is resistant to extraction and protects G_{M1} from being extracted [52], complex formation appears to be independent of the A component and requires G_{M1} to be bound to the B component. Multivalent binding of the B component to several G_{M1} molecules in the membrane could lead to an association with the translocator. This association could either be between the translocator and the B subunits or the ceramide moities of the ganglioside. The fact that choleragen bound to cells at 4 °C is detergent resistant implies that the translocator is already associated with the cytoskeleton [52]. As each toxin is multivalent, it could crosslink several translocators, and at 37 °C the macromolecular complexes now associated with the cytoskeleton could undergo patching and capping.

Escherichia coli Toxin, a Clone of Cholera Toxin

Certain strains of *E. coli* produce a heat-labile enterotoxin which is associated with "traveler's diarrhea" [46, 111]. The toxin (LT) appears to mediate its effects by activating adenylate cyclase [23]. The

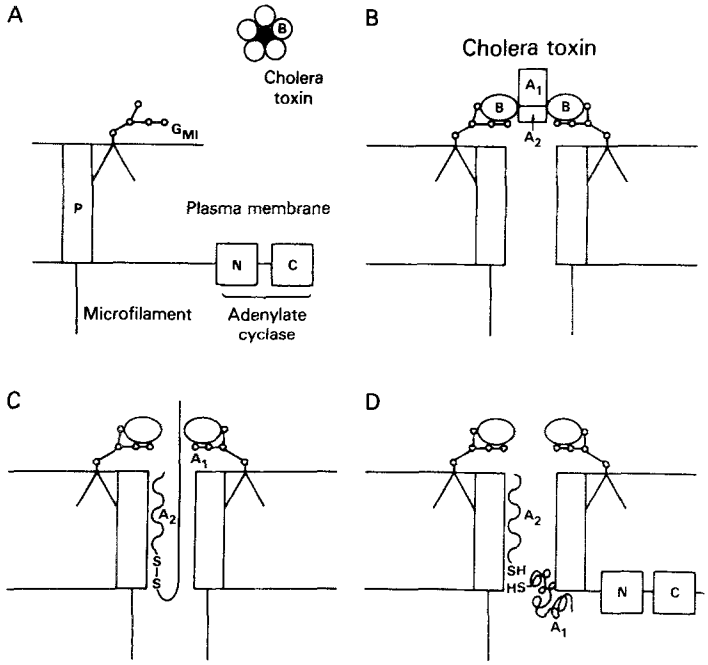

Fig. 8. Model of mechanism of action of choleragen on intact cells. (*A*): Various components are orientated as described in Fig. 7 except for transmembrane protein (*P*) associated with the cytoskeleton. (*B*) Multivalent binding of B component of choleragen to several G_{M1} molecules results in association of the toxin-G_{M1} complex with *P* and a conformational change in choleragen which allows A_2 peptide to interact with *P*. (*C*): *P* functions as a translocator and facilitates the transmembrane movement of the A component. (*D*): The A component is reduced to generate the A_1 peptide which can catalyze the ADP-ribosylation of *N* and the activation of adenylate cyclase. Model from Hagmann and Fishman [51, 52]

toxin is similar in size to choleragen and is composed of A and B subunits which appear structural and antigenically similar to the subunits of choleragen [15, 47, 108]. The LT A subunit, which has sequence homologies with choleragen A [121] and ADP-ribosyltransferase activity [96], activates adenylate cyclase and catalyzes the ADP-ribosylation of the regulatory component in membranes [44]. LT A is different from choleragen A in that it is a single peptide as isolated from the bacterium [94, 108]; ADP-ribosyltransferase activity requires reduction with thiols [44, 94, 96] and is enhanced by proteases which converted LT A to a peptide similar to the A_1 of choleragen [94].

LT can be inactivated by G_{M1} [20, 61, 105, 132], and its binding to G_{M1}-deficient cells is enhanced by treating the cells with G_{M1} but not other gangliosides [94]. LT and its B subunit can bind to G_{M1}-oligosaccharide which induced a blue-shift in their fluorescence spectra similar to that observed with cholera toxin [94]. Each LT molecule appears to bind several oligosaccharide molecules [94]. Thus, LT B appears to be similar to choleragen B in terms of structure, function, and receptor specificity. It is unclear from the literature as to whether G_{M1} is the native or only receptor for LT in the intestine. This is based on the ability of choleragen B to block choleragen action but not LT action [61, 105]. G_{M1}-deficient NCTC 2071 cells were observed to be unresponsive to LT but became responsive after they were treated with G_{M1} [92]. Thus, it is clear from this experiment that G_{M1} can function as a receptor for LT and mediate its effects on mammalian cells. Further work will be necessary in order to determine whether intestinal cells have an additional component that binds LT.

Tetanus Toxin

Tetanus toxin is the potent neurotoxin produced by *Clostridium tetani* [5, 48, 84]. The toxin is a protein of 150,000 mol wt and is composed of two polypeptide chains linked together by a disulfide bond [10, 83]. Reduction of the toxin separates it into heavy and light chains of 100,000 and 50,000 daltons each. Papain digestion of the holotoxin cleaves the heavy chain to release a 47,000-dalton peptide (fragment *C*) and the remainder of the heavy chain still linked to the light chain (fragment *B*) [100]. Neither of the two chains of tetanus toxin or the two fragments formed by papain are by themselves toxic. There is substantial evidence that the binding activity of the toxin is on the heavy chain in the fragment *C* region [5, 45, 54, 88].

Tetanus toxin appears to mediate its neurotoxic effects on the peripheral and central nervous system by blocking the release of neurotransmitters from inhibitory presynaptic membranes [5, 48, 84]. At the neuromuscular junction, release of acetylcholine is impaired; in the central nervous system, the toxin inhibits the release of glycine and GABA.

Van Heyningen and coworkers were the first to demonstrate an interaction between gangliosides and tetanus toxin [58, 59]. Crude fractions of brain gangliosides were able to fix the toxin and remove it from solution. It was subsequently shown that gangliosides with a disialyl group such as G_{D1b} and G_{T1b} (*see* Fig. 2) were the most effective in fixation of the toxin [56]. Tetanus toxin was shown to bind to these gangliosides absorbed to plastic tubes [63] or incorporated into lipid bilayers [8, 88]. Binding of ^{125}I-toxin to brain membranes was inhibited by gangliosides and G_{D1b} and G_{T1b} were the most effective [88, 110]. Rogers and Snyder demonstrated high affinity binding of ^{125}I-tetanus toxin to brain membranes ($K_d = 1.2$ nM) and inhibition of binding by G_{T1b} ($K_i = 6$ nM) and by G_{D1b} ($K_i = 10$ nM) [110]. Scatchard analysis indicated a single class of toxin binding sites (up to 700 pmol/mg of membrane protein). Interaction of gangliosides with the heavy chain and fragment *C* has also been described [5, 54, 88].

Tetanus toxin appears to bind specifically to neurons and has been used a marker for neurons in primary cultures [16–18, 86]. Whereas primary neuronal cultures bound the toxin, continuous cell lines did not [17]. The primary cultures contained G_{D1b} and G_{T1b}, whereas the continuous lines (oligodendroglioma, C6 glioma, neuroblastoma N2a and a neuroblastoma-glioma hybrid, NG108) did not. Prior exposure of the NG108 cells to gangliosides enhanced their ability to bind tetanus toxin [17]. Treatment of primary neuronal cells with neuraminidase, which hydrolyzes G_{D1b} and G_{T1b} to G_{M1}, reduced toxin binding [16]. Toxin binding also was diminished by treatment of bovine brain membranes with neuraminidase, whereas trypsin and chymotrypsin had no effect [110].

Although these studies support the concept that gangliosides are receptors for tetanus toxin, other studies do not. G_{T1} only partially blocked the retrograde axonal transport of tetanus toxin, whereas G_{M1} completely blocked cholera toxin transport [123]. G_{D1b} but not its free oligosaccharide was found to bind to tetanus toxin [53*a*]. In contrast to the results of Dimpfel et al. with neuroblastoma N2a cells [17], Zimmerman and Piffaretti reported binding of the toxin to these cells [133]. When the cells were induced to differentiate

by removal of serum, toxin binding was enhanced, Neuraminidase treatment eliminated toxin binding to growing but not to differentiated cells. The latter cells exhibited morphological changes when exposed to tetanus toxin, whereas the growing cells did not. The authors distinquished between effective and ineffective binding and proposed that gangliosides were not involved in effective binding of the toxin [133]. Habermann and coworkers have recently demonstrated that tetanus toxin inhibits the uptake of choline by rat brain synaptosomes and the release of acetylcholine and noradrenaline by the particles [4, 49, 50]. Prior treatment of the synaptosomes with neuraminidase eliminated G_{D1b} and G_{T1b}, reduced ^{125}I-toxin binding by 70%, but had no effect on toxin action.

These studies raised the possibility that gangliosides were not the functional receptors for tetanus toxin. The ability of neuraminidase to eliminate 70% of the toxin binding sites but not toxin effects suggests the possibility of spare or excess receptors. The maximal effects of the toxin were observed with 10–20 pmol of toxin per mg membrane protein [4, 49, 50], whereas toxin binding was reported to be 0.7 [110] to 4 [45] nmol per mg protein. *In vivo*, tetanus toxin is extremely potent and less than a fmol will kill a mouse. Thus, only a few molecules of toxin per neuron may be an effective dose. If there are a vast excess of toxin receptors, neuraminidase treatment could eliminate most of them as well as most of the G_{D1b} and G_{T1b} without preventing the few molecules of toxin that still bind from elicting a biological response.

Additional experiments similar to those done with cholera toxin may clarify the role of gangliosides in the binding and action of tetanus toxin. By using ganglioside-deficient cells that do not bind or respond to the toxin, one might be able to demonstrate that treatment of the cells with G_{T1b} and G_{D1b} elicited toxin binding and action. One obstacle is the fact that the mechanism of action of tetanus toxin has not been resolved. Thus, there is no biochemical effect such as activation of adenylate cyclase by cholera toxin that can be measured in intact cells. The high potency of tetanus toxin may mean that it acts catalytically as does cholera toxin. Although no enzyme activity has been ascribed to tetanus toxin, the heavy and light chains may represent binding and activity components analogous to cholera toxin and diphtheria toxin [103]. The latter toxin, when nicked by proteases and reduced by thiols, is separated into two peptide fragments. The *B* fragment contains the binding determinants and the *A* fragment is an ADP-ribosyltransferase [103].

In vivo, tetanus toxin binds to and is taken up by axonal terminals; the toxin then moves by intraaxonal retrograde transport; then toxin finally undergoes transsynaptic migration to presynaptic junctions where it blocks the release of inhibitory transmitters. In order to accomplish these movements, the toxin must be capable of traversing membranes. There is some evidence for a hydrophobic region on the heavy chain [130]. The entry of diphtheria toxin into cells is facilitated by low pH [21, 114]. At low pH, diphtheria toxin and toxin fragments form transmembrane channels or pores in model lipid bilayers [19, 71]. The *B* or binding fragment has two hydrophobic regions [80]. It is believed that after binding to the cell surface diphtheria toxin undergoes endocytosis; the endocytic vesicles fuse with lysosomes where the toxin becomes nicked and possibly reduced. The low pH then promotes the *B* fragment to form a transmembrane channel through which the *A* fragment can penetrate into the cytoplasm where it catalyzes the transfer of ADP-ribose from NAD to elongation factor 2 and inhibits protein synthesis. It is possible that the heavy chain of tetanus toxin has a similar function and mediates the transsynaptic movement of the light chain to its presynaptic site of action.

Future Directions

Several potent techniques have been developed to demonstrate that G_{M1} is the receptor for cholera toxin. They include: the incorporation of G_{M1} into G_{M1}-deficient cells; the protection by choleragen of G_{M1} from cell surface labeling; the immunoadsorption of G_{M1}-toxin complexes; and the detection of G_{M1} on thin-layer chromatograms and polyacrylamide gels by labeled toxin. They may prove useful in identifying gangliosides as receptors for other toxins, viruses, and possible physiological effectors. Although gangliosides are ubiquitous plasma membrane components of vertebrate cells, their normal function is unknown. They have been implicated as receptors for neurotransmitters, peptide hormones, and interferon [32, 56, 77]; but, more recent studies have not supported such a role [1, 102].

The mechanism by which choleragen as well as other bacterial toxins gain access to the interior of the cells also has to be determined. Although the transmembrane step remains unclear, G_{M1} appears to be involved in this process in addition to functioning as a binding site for choleragen. This conclusion is based on the ability of G_{M1}-oligosaccharide to perturb the structure of the *B*

component and the ability of the *B* component to perturb lipid bilayers containing G_{M1}. Thus, the choleragen-G_{M1} interaction may be a useful model for understanding how other toxins become internalized.

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