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Ultrastructural localization of cell membrane G_{M1} ganglioside by cholera toxin

(immunoelectron microscopy/membrane receptor/*Vibrio cholerae* sialidase/ganglioside titration)

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ABSTRACT An immunoelectron microscopic method is described for sensitive high-resolution visualization of tissue-bound cholera toxin. The principle is to incubate cells or tissue sections with toxin and then to localize the bound toxin with toxin-specific peroxidase (donor:hydrogen-peroxide oxidoreductase; EC 1.11.1.7)-conjugated antibody and enzyme substrate. Thin sections are examined for electron-opaque precipitates in a transmission electron microscope. Because of the specific binding of the toxin to membrane ganglioside G_{M1}, the method can be used for ultrastructural localization of this ganglioside. Semiquantitative data are obtained by titration of the limiting concentration of cholera toxin producing specific precipitates. The specificity of the method was controlled in various ways, including analyses of the correlation between the immunoelectron microscopy results and determinations of ganglioside G_{M1} in tissues with different ganglioside concentrations, tissues hydrolyzed with *Vibrio cholerae* sialidase, tissues in which exogenous G_{M1} has been incorporated, and lipid-extracted tissues. The immunoelectron microscopic method demonstrates that membrane G_{M1} ganglioside is positioned on the external side exclusively. Cell-bound toxin remains in its original location on the plasma membrane surface of cells below 18°, but appears to be redistributed both laterally and vertically in the membrane of cells incubated at 37° for 30 min or longer. The results of this method indicate that in the central nervous system G_{M1} is concentrated in the pre- and postsynaptic membranes of the synaptic terminals; a further increase in reactivity of these structures after hydrolysis of the nervous tissue with *V. cholerae* sialidase suggests that higher gangliosides of the same series are particularly increased in the pre- and postsynaptic junctions.

Cholera toxin is a structurally and immunologically well-characterized oligomeric protein produced by *Vibrio cholerae* (1). It causes the diarrhea in cholera by activating adenylate cyclase in the intestinal mucosa (2) after binding to receptors on the epithelial cell surface (3). The toxin is a ubiquitous activator of plasma membrane adenylate cyclase in mammalian tissues, indicating that the receptors for the toxin are present on most or all cell types (1).

Recent studies have strongly indicated that the ganglioside galactosyl-*N*-acetylgalactosaminyl - (sialyl) - galactosylglucosylceramide (G_{M1}) is the membrane receptor for cholera toxin: (*i*) G_{M1} specifically binds and inactivates cholera toxin in equimolar proportions (4). (*ii*) A direct correlation between the cellular G_{M1} content and the number of toxin receptors exists in intestinal cells of various species (3). (*iii*) Chemical modifications of cholera toxin affect binding to cells and to isolated G_{M1} in parallel (J. Holmgren, I. Lönnroth, and L. Svennerholm, unpublished). (*iv*) Incorporation of exogenous G_{M1} into the membrane of target cells increases the number of binding receptors as well as the biologic responsiveness to toxin (3, 5). (*v*) Binding of cholera toxin to cell membranes specifically prevents the tritiation of membrane G_{M1} by means of galactose oxidase followed by reduction with sodium [^{3}H]borohydride (6).

In the present communication we describe an immunoelectron microscopic (IEM) method for sensitive high-resolution visualization of tissue-bound cholera toxin. The previously shown very high specificity of the toxin in binding to the ganglioside G_{M1} and the correlation found in the present study between the results of IEM and chemical G_{M1} analyses indicate that the described method will give new possibilities for ultrastructural localization of the ganglioside G_{M1} in various tissues.

MATERIALS AND METHODS

Highly purified cholera toxin, prepared by R. Finkelstein (7), was supplied by C. Miller, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA. The gangliosides G_{M1} and G_{M2} [*N*-acetylgalactosylaminyl-(sialyl)-galactosylglucosylceramide] were purified from human brains (8). Antiserum to cholera toxin was prepared in rabbits by subcutaneous immunization; it contained precipitating antibodies to both the L and H subunits of the toxin (9). The immunoglobulin of this antiserum was recovered by repeated precipitation with 37% saturated ammonium sulfate and conjugated to peroxidase (donor:hydrogen-peroxide oxidoreductase; EC 1.11.1.7). The best labeling was obtained by incubating the immunoglobulin from 200 μl of antiserum with 12.5 mg of peroxidase (Sigma Chemical Co., St. Louis, MO, USA; type VI) and 0.07% purified glutaraldehyde (Merck AG, Darmstadt, W. Germany) in a total volume of 0.25 ml of Tris buffer for 2 hr at room temperature under continuous shaking. The residual nonconjugated peroxidase was separated from the antibody–enzyme complex by gel filtration through a column of Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden).

Several different tissues and cell types were used. Thymocytes were prepared by squeezing thymic organs from DBA 1/J or C57Bl mice through a stainless steel grid. Human blood cells were obtained by puncture of a cubital vein. YAC lymphoma cells were collected from ascites fluid of A/Sn mice, in which the cell line was propagated. Freshly prepared pieces of small intestine from rabbits, rats, and adult human beings were carefully freed from adherent fat and connective tissue and dissected to specimens with a luminal surface of 0.5–2 mm^2. Small pieces of retina, cerebrum, and cerebellum of rats and rabbits were prepared immediately after sacrifice.

The standard procedure was as follows: The tissue specimens or cell suspensions were, after rinsing, incubated for 15 min at 18 ± 1° with 10 nM cholera toxin in a phosphate-buffered

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Abbreviations: Ganglioside nomenclature is according to Svennerholm (17): G_{M1}, galactosyl-*N*-acetylgalactosaminyl-(sialyl)-galactosylglucosylceramide. IEM, immunoelectron microscopy.

balanced salt-solution supplemented with 0.5% bovine serum albumin. The specimens were carefully rinsed with phosphate-buffered saline and immersed for 30 min in the peroxidase-conjugated anti-cholera toxin immunoglobulin. They were then rinsed again in phosphate-buffered saline and fixed for 1 hr in a cacodylate-buffered solution, pH 7.2, containing 2% glutaraldehyde. After careful rinsing, the specimens were processed for demonstration of peroxidase activity by incubation at room temperature for 30 min in a cacodylate-buffered solution of 1 mM 3.3-diaminobenzidine (Sigma) and 3 mM hydrogen peroxide (Merck AG). To increase the contrast of the osmiophilic reaction product, the specimens were postfixed in osmium tetroxide. After dehydration and embedding in Epon, thin sections (20–40 nm) were prepared with a diamond knife in an LKB Ultrotome III ultramicrotome and examined in a Siemens transmission electron microscope. Sections were examined unstained as well as after double-staining with uranyl acetate and lead citrate. Multiple sections representative for the tissue specimens were always examined. Evaluation comprised the number of precipitates as well as their size, electron opacity, and distribution; 200–1000 cells were inspected and all readings were done by the same investigator. In case of absence of precipitates with the standard procedure, the toxin concentration was increased 10- and 100-fold, and the incubation time was also extended to 60 min. For quantitation of positive reactions, serial toxin dilutions down to 1 pM were tested.

Redistribution of cell-bound toxin was studied with an incubation temperature of 37° and incubation times of 5, 15, 30, 45, 60, and 90 min. In these studies the cells were fixed in ice-cold buffered formalin/picric acid immediately after the incubation with toxin to prevent further redistribution of cell-bound toxin and also to enable reaction of toxin present intracellularly or buried within the plasma membrane with the antibody–peroxidase conjugate.

Sialidase hydrolyses were performed on tissue specimens at 37° for 30 min with 5 or 25 units of *Vibrio cholerae* sialidase per ml (Behring Werke AG, W. Germany) in 50 mM borate/0.2% gelatin buffer, at pH 7.5, containing 0.1 M NaCl and 10 mM $CaCl_2$. Portions of the specimens were used for IEM studies of cholera toxin binding and other portions for chemical analyses.

The content of ganglioside sialic acid, assayed as *N*-acetylneuraminic acid, and the ganglioside pattern were determined before and after sialidase treatment (10, 11). Free sialic acid was assayed with a thiobarbiturate method (12).

Binding of ^{125}I-labeled cholera toxin to cells was studied as described (3).

RESULTS

Structural Localization of Cell-Bound Toxin. When studied in the light microscope after the standard incubation at 18° with, in order, cholera toxin, anti-toxin immunoglobulin conjugated with peroxidase, diaminobenzidine, and osmium tetroxide, the contour of various cells appeared brown at variable intensities. On examination by electron microscopy, opaque precipitates were observed along the plasma membrane of cells. As studied with dispersed cells, binding of toxin always seemed to be completed within 5 min at 18°, whereas with tissue blocks incubation for up to 15 min with toxin was needed to reveal the full binding pattern, probably because of accessibility problems. A decrease in temperature to 4° had no appreciable effect on the extent of binding of cholera toxin or the localization of the toxin-induced precipitates. They were always confined to the outside of the plasma membrane (Fig. 1*A*), even after pro-

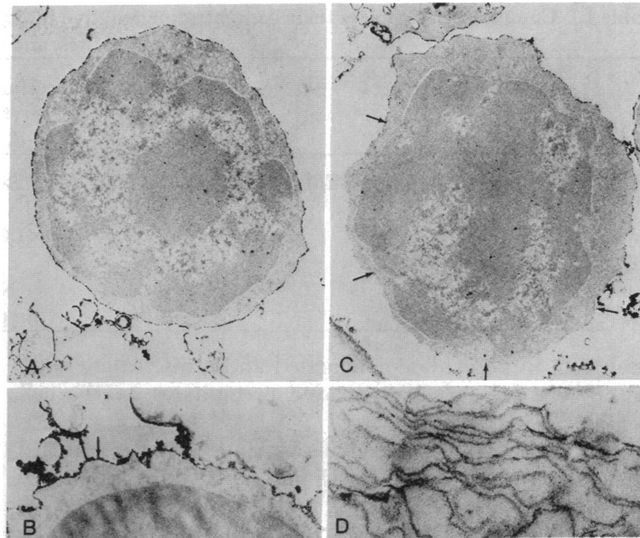

FIG. 1. (*A*) Mouse thymic cell incubated for 30 min in 1 nM cholera toxin at 4° and then processed for IEM. The precipitations indicating toxin binding sites are studded along the entire plasma membrane of the thymus cell. (× 11,900.) (*B*) Higher magnification of a mouse thymic cell and cell fragments treated as in *A*. Cholera toxin binds only to sites present on the external surface of cell fragments (arrows). (× 24,000.) (*C*) Mouse thymic cell incubated for 30 min in 1 nM cholera toxin at 37° and then treated as the cell in *A*. Most of the precipitates reflecting bound cholera toxin have been concentrated to the upper right half of the cell indicating cap formation, while the areas marked by arrows lack signs of activity. (× 11,900.) (*D*) Ghosts of human erythrocytes treated with 0.1 μM cholera toxin for 30 min at 4° and then processed. There are no specific precipitates along the membrane fragments. (× 33,000.)

longed incubation with toxin and fixation of the cells before incubation with the peroxidase-labeled antibody. Also, when cell membrane fragments were incubated with toxin at low temperature, the IEM staining was exclusively on the external side (Fig. 1*B*).

In contrast, when incubations were performed at 37° and the cells were fixed before addition of the antibody–enzyme complex, rearrangements of the receptor-bound toxin in the membrane were indicated. With brief incubation, 5 or 15 min, of cells and tissues with toxin, the precipitates remained located on the outer surface of the membrane. After incubation for 30 min at 37°, a lateral redistribution of the membrane-bound toxin was evident, including accumulation in "caps" (Fig. 1*C*). In addition, precipitates were now also present deeper in the membrane. These redistributions were further accentuated by increasing the incubation time of toxin with cells for up to 90 min. There were no electron-opaque reaction products inside the intact cells except in pinocytotic vesicles.

Specificity for Cholera Toxin. Several observations indicate the specificity of the IEM technique in demonstrating cell-associated cholera toxin.

(*i*) Incubation of tissue with either the peroxidase–antibody conjugate or with peroxidase only without prior exposure of the tissue to cholera toxin resulted in a faint diffuse background staining. This background was readily distinguished from the "specific" reaction products which had a much higher electron opacity. Similar faint background staining was obtained when peroxidase-labeled antiserum to the brain-specific protein S-100 replaced the conjugated anti-cholera toxin immunoglobulin in studies with specimens of small intestine.

(*ii*) Toxin-exposed tissue pieces and cells were incubated for 30 min with nonconjugated antiserum to cholera toxin and rinsed with phosphate-buffered saline prior to the standard

Table 1. Comparison of cholera toxin concentration required for formation of precipitates in the IEM method, the number of cholera toxin binding sites, and the G_{M1} concentration

	Human erythrocytes	Human small intestine mucosal cells	Mouse thymocytes	Mouse YAC lymphoma cells
Cholera toxin concentration for IEM precipitates, M	$>10^{-6}$	10^{-9}	10^{-9}–10^{-10}	10^{-10}–10^{-11}
Cholera toxin binding sites per cell	<1,000	15,000	140,000	1,400,000
G_{M1} concentration, molecules per cell	Not detected	90,000	870,000	Not analyzed

incubation in peroxidase-conjugated antiserum. There were no, or very few, electron-opaque precipitates on the cell membranes examined.

(*iii*) Preincubation of the cholera toxin used in the standard procedure with 5 μM G_{M1} for 15 min reduced the staining of the cells to the background level. Preincubation with 5 μM G_{M2} did not reduce the staining.

Correlation with Determinations of Receptor Activity and G_{M1} Concentration. The ability of the IEM method to recognize differences in G_{M1} concentration between various tissues or to register an induced increase or decrease in this number within the same tissue was examined. This was done by comparing the IEM results with chemical determinations of the G_{M1} concentration or with estimations of G_{M1} receptors by binding of radiolabeled cholera toxin. The studies included cells and tissues with widely different G_{M1} concentrations, tissues hydrolyzed with *V. cholerae* sialidase to increase the G_{M1} content, cells in which exogenous G_{M1} had been incorporated, and ganglioside-depleted tissues.

(*i*) *Native cells and tissues*. The number of binding sites for cholera toxin differs largely among human erythrocytes, human small intestine mucosal cells, mouse thymocytes, and mouse YAC lymphoma cells as determined with radiolabeled cholera toxin (Table 1). These cells were examined with the IEM technique, using serial 1:3 dilutions of cholera toxin in concentrations ranging from 10^{-6} to 10^{-12} M. The lowest concentrations that induced formation of distinct precipitates on cell membranes was determined. There was a good correlation between the inverse concentration of toxin required for formation of distinct precipitates in the IEM technique, the number of cholera toxin binding sites, and the concentration of G_{M1} (Table 1). Six G_{M1} molecules correspond to one binding site for toxin on the cell membrane.

The IEM results with human erythrocytes have special significance from the point of specificity. These cells do not contain measurable quantities of G_{M1}, but relatively large amounts of the closely allied ganglioside sialosyllactoneotetraosylceramide (13). This latter ganglioside does not fix or inactivate cholera toxin (4). When intact human erythrocytes were examined by the IEM method, no precipitates were found along the plasma membrane even at a toxin concentration of 1 μM. Neither were any reactions produced when erythrocyte ghosts were examined (Fig. 1*D*).

When the clinical target tissue for cholera toxin, the small intestine, was examined by the IEM technique, the microvilli lining the luminal surface showed electron-opaque precipitates having a patchy distribution. The lateral and basal membranes of these high, columnar cells had a smaller number of precipitates. The most prominent reaction was seen in caveolar membranes and in the junctional complexes between adjacent epithelial cells, especially in the region of zonulae occludens. Varying, mostly low, activity could also be detected in the cell coat. The cell membrane of the goblet cells with its few short and clumpy microvilli demonstrated similar or even more pronounced reactivity than that of the epithelial cells.

The rabbit retina has a low proportion of G_{M1} (ref. 13 and Table 2). When examined by the IEM method, there was only a moderate staining reaction in the nonsialidase-treated tissue at a cholera toxin concentration of 1 nM. This staining was located on the inner segments of the photoreceptors and on the synapses of the plexiform layers. Few and faint precipitates were observed in the cell membranes of ganglion and glial cells (Fig. 2*A*).

Sections of cerebrum and cerebellum of rats and rabbits, the tissues of which are very rich in gangliosides, including G_{M1} (10, 11), showed, when tested at cholera toxin concentrations down to 0.1 nM, numerous precipitates concentrated on the opposite pre- and postsynaptic membranes of the synaptic terminals. At this toxin concentration, precipitates were almost lacking on other parts of neuronal and neuroglial membranes (Fig. 3). It should be stressed that the described pattern refers to the first cell layer not injured by the preparation of the tissue block, i.e.,

Table 2. Effect of sialidase treatment on G_{M1} concentration and cholera toxin concentration required for distinct precipitates by the IEM technique

	Total gangliosides, nmol NeuAc/ μmol lipid-*P*	NeuAc released, nmol/ μmol lipid-*P*	G_{M1}, nmol/ μmol lipid-*P*	Toxin concentration required for precipitates, nM
Thymocytes				
Control	2.0	—	0.5	0.5
Sialidase, 5 units	—	11	—	—
Sialidase, 25 units	—	15	1.0	0.2
Retina				
Control	42	—	2.0	3.0
Sialidase, 5 units	—	12	5	—
Sialidase, 25 units	—	25	12	0.5

The results are the mean value of at least three different experiments. NeuAc, *N*-acetylneuraminic acid.

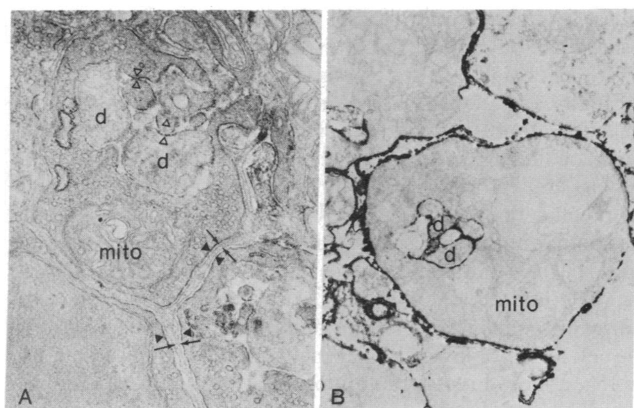

FIG. 2. (*A*) Electron micrograph of the external plexiform layer of a rabbit retina incubated *in vitro* for 30 min in 1 nM cholera toxin at 18° and then processed for IEM. The membranes of the nerve cell processes (filled arrowheads) are studded by larger amounts of precipitates than the neuroglial cell membranes (arrows). The pre- and postsynaptic membranes (open arrowheads) are about equally stained. There is a rod cell nucleus in the lower left corner and processes of horizontal cells (d) in the center of the complex synapses of the rod cells. Mito, mitochondrion. (× 15,000.) (*B*) Rabbit retina treated with sialidase (25 international units/ml) at 37° for 30 min and then processed as in *A*. There is a general marked increase in the number of binding sites after the enzyme treatment in all membranes. (× 16,000.)

the outermost cells are excluded. These latter cells were very strongly stained even on further dilution of toxin, indicating that they absorb much toxin. In preliminary experiments with enriched fractions of nerve cell bodies in suspension and of synaptosomes, much stronger staining reactions than those produced on the corresponding structures in the tissue blocks were obtained; as in the intact tissue, the synaptosomes had much greater reactivity than the nerve cell bodies (unpublished data).

(*ii*) *Sialidase hydrolysis*. *V. cholerae* sialidase increased the number of binding sites for cholera toxin significantly on mouse thymic cells, as determined with ^{125}I-labeled toxin. Thus, treatment of 10^7 thymocytes at 37° for 30 min with 5 and 25 units of sialidase increased the number of binding sites 1.3 and 2.0 times, respectively. The mouse thymus cells also showed an increased concentration of G_{M1} after sialidase treatment (Table 2). This change was recognized by the IEM technique, so that, by the standard procedure, the cells showed an increased number of electron-opaque precipitates. Furthermore, the same number of precipitates as on the control incubated cells were obtained with one further 1:3 dilution of toxin on the cells treated with 25 units of sialidase.

Sialidase treatment of rabbit retina produced a severalfold increase in ganglioside G_{M1} concentration (Table 2). On evaluation by the IEM technique, using a cholera toxin concentration of 1 nM the sialidase-treated retinal tissue showed an increased number of precipitates (Fig. 2*B*). Furthermore, for a staining intensity similar to that on untreated retina, a 3 to 10 times less cholera toxin concentration was sufficient (Table 2). Incubation of pieces of rat cerebrum and cerebellum with *V. cholerae* sialidase increased their IEM staining reactivity also, especially in the pre- and postsynaptic junctions.

In the human, bovine, and porcine intestine, the ganglioside pattern did not change after treatment with *V. cholerae* sialidase and no free sialic acid was released (3), nor did sialidase treatment produce any appreciable change in receptor activity of the intact rabbit small bowel mucosa (3). In agreement with these observations, the staining patterns of human and rabbit intestines incubated with sialidase were unchanged when examined by the IEM method.

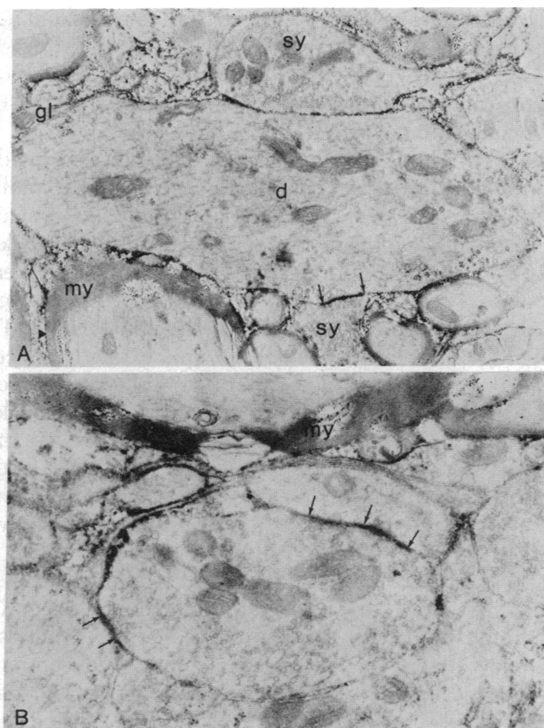

FIG. 3. (*A*) Electron micrograph of rat cerebral cortex after incubation of a tissue slice with 10 nM cholera toxin for 15 min and then further processed. Precipitates are distributed along the membranes of nerve and glial (gl) cells. There is a large dendrite (d) in the center. The synapses (sy) show an increased number of precipitates in the synaptic clefts (arrows). Note the large number of precipitates along myelin sheaths (my) and the lack of penetration in between the membranes of the myelin. (× 17,000.) (*B*) Electron micrograph of a slice of cerebral cortex incubated in 0.5 nM cholera toxin for 15 min. The pre- and postsynaptic membranes (arrows) are still covered by numerous precipitates, while most adjacent membranes lack such precipitates. (× 23,000.)

(*iii*) *G_{M1} incorporation*. Human, porcine, and rabbit intestines incorporate added G_{M1}. The incorporated ganglioside has the ability to bind cholera toxin and to increase the intestinal secretory response (3). When pieces of rabbit small intestine were preincubated with 5 μM G_{M1} in a phosphate-buffered saline for 30 min and rinsed before the subsequent incubations with cholera toxin and the other reagents, the staining reaction of the surface membrane, including the microvilli of the intestinal cells, increased. Furthermore, the distribution of precipitates was changed from a patchy pattern to an even distribution. Similar results were obtained when retinal tissues were preincubated with G_{M1}, i.e., the neuronal cell surfaces became studded with precipitates on both the pre- and postsynaptic membranes in the plexiform layers.

(*iv*) *Lipid extraction*. Extraction of the lipids in rabbit small intestine, retina, and cerebellum with chloroform/methanol 2:1 (vol/vol) before the incubation reduced the IEM staining to the background level.

DISCUSSION

The IEM technique described enables ultrastructural visualization of cholera toxin during its interaction with cells and tissues, including the intestine. The resolution is about 100 times that of immunofluorescence and about 10 times that of autoradiography. The high affinity in the binding of cholera toxin

to its cell membrane receptor (3, 14, 15) and the excellent sensitivity of immunoenzyme methods (16) allow the IEM studies to be performed with biologically relevant, low concentrations of the toxin, in the order of 1 nM for the rabbit intestine and 0.1 nM for mouse thymocytes (3, 15).

We believe that the described IEM technique provides unique possibilities for localization of G_{M1} ultrastructurally. Although Cuatrecasas (14) reported that not only G_{M1}, but also certain other gangliosides and glycoproteins, could bind cholera toxin and also interfere with the binding of toxin to cell membranes, there is, in our opinion, strong evidence that the toxin binds only to membrane G_{M1} ganglioside on intact cells. The complete loss of binding capacity after lipid extraction of cells and tissues excludes substances other than lipids as receptors. The only lipids, beside G_{M1}, showing any reactivity with cholera toxin in our previous studies (4), G_{D1a} and G_{A1}, had less than 0.20% the activity of G_{M1}, and such minor activity could be due to contamination with G_{M1}. The absence of staining noted with human erythrocytes that lack G_{M1} supports the specificity of the method for G_{M1}.

The IEM results correlate very well with other estimates of receptor activity or G_{M1} concentration for cells in suspension. This is found both with cells differing in their ganglioside content and with cells in which a change in G_{M1} concentration has been experimentally produced. With tissues, diffusion problems and absorption of toxin to cells on the surface of the tissue pieces seem to complicate the quantitation of G_{M1} by the IEM method, as indicated by the findings with sections of cerebrum in comparison with isolated synaptosomes and nerve cell bodies. One should be aware, therefore, that IEM results obtained with different tissues may not be directly compared in terms of G_{M1} content. For instance, although retina has a much higher G_{M1} content than human intestinal mucosa, the intestinal brushborder is stained at a lower concentration than are the retinal ganglion cells. The major reason for this is probably the difference in diffusion gradient, i.e., the examined intestinal structures have been exposed to almost the actual concentration of cholera toxin used in the incubation medium, whereas the cells in the retina have met a toxin concentration much lower than the nominal one. These circumstances do not detract, however, from the usefulness of the IEM method in providing sensitive qualitative information about the distribution of G_{M1} in various tissue structures. Furthermore, when the aforementioned problems are avoided by comparing within a particular tissue adjacent cells or structures, the IEM titration results probably give a good reflection of the relative G_{M1} content. This is indicated by the increased staining after preincubation of tissue sections with G_{M1} and especially by the results after incubation of the tissues with *V. cholerae* sialidase.

The gangliosides are components of the cell membranes of all cells, but are particularly abundant in the neurons. Several gangliosides have the same basic carbohydrate structure as ganglioside G_{M1} (8). By enzymatic hydrolysis with *V. cholerae* sialidase, these di-, tri-, tetra-, and pentasialogangliosides can be degraded to ganglioside G_{M1}, which is resistant to the sialidase hydrolysis. In the present study we have examined cells and tissues by the IEM method before and after sialidase hydrolysis and found a close correlation between the increase in staining and the chemically quantified increase in G_{M1}. With the semiquantitative IEM technique developed, it thus appears possible to determine the localization of both ganglioside G_{M1} and the oligosialogangliosides of the same series. This approach will be important in the studies of the gangliosides in nervous tissue, since 80–90% of the brain gangliosides can be isolated as G_{M1} after sialidase hydrolysis (17).

Finally, the IEM method seems to provide new possibilities for elucidating the apparently very complex interactions between cholera toxin and cells which occur after the binding to the G_{M1} receptors (18). The precipitates reflecting the cell-bound cholera toxin were initially located on the outside of the plasma membrane. It is particularly important that when the inside of the cell membrane was directly exposed to toxin (e.g., the experiment depicted in Fig. 1*B*), it was not stained, indicating an exclusive external orientation of ganglioside G_{M1} in the cell membrane. On longer incubation at 37°, but not at 4° or 18°, a lateral redistribution of toxin on the membrane was noted, confirming previous immunofluorescence findings (15, 19, 20). In addition, provided that the cells were fixed after their interaction with the toxin so that the subsequently added antibody–enzyme complex could reach beyond the cell surface, precipitates were now also seen deeper in the membrane. These observations suggest that the binding of cholera toxin to G_{M1} receptors at the cell surface is followed by lateral as well as vertical migration of the toxin within the membrane. The vertical migration particularly might be functionally important in bringing the toxin with its effector subunit H in position to activate adenylate cyclase at the inside of the cell membrane (18).

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