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Gangliosides do not move from apical to basolateral plasma membrane in cultured epithelial cells

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Both qualitative and quantitative approaches were used to ascertain whether gangliosides, incorporated into the apical plasma membrane of cultured epithelial cells from kidney of toad (A6) and dog (MDCK), were able to redistribute past the tight junctions to the basolateral membrane. The apical surfaces of confluent epithelia were exposed to rhodaminyl gangliosides and the distribution of the inserted gangliosides was assessed qualitatively by fluorescence microscopy. All of the fluorescence was confined to the apical surface for at least 1 h after the fluorescent gangliosides had become incorporated; none appeared on the basolateral surface. These observations were confirmed by incubating the cells with anti-rhodamine antibodies and ^{125}I-labeled protein A. In order to quantitate further the ganglioside distribution, binding assays were performed using ^{125}I-labeled cholera toxin, which binds specifically to ganglioside G_{M1}. Exogenous G_{M1} added to the apical membrane was not detected on the basolateral membrane 4 h after its incorporation even though there was extensive disappearance of the inserted ganglioside, presumably through endocytosis. To directly examine the behaviour of endogenous gangliosides, the apical surface of the epithelial cells was exposed to bacterial neuraminidase, which hydrolyzes more complex gangliosides to G_{M1}. The cells exhibited a 10-fold increase in binding of cholera toxin to their apical surface, but no increase in binding to their basolateral surface. Thus, no cellular pathways for movement from apical to basolateral plasma membrane appear to be available for implanted or endogenous gangliosides.

Introduction

A special property of epithelia is the presence of apical and basolateral surfaces, the two membrane domains being separated by tight junctions formed between adjacent cells. The plasma membrane components of epithelial cells are asymmetrically distributed between the apical and basolateral surfaces [1–4]. This polarity is essential for epithelial transport function. The mechanism of development and maintenance of this polarity, however, is not well understood. In a previous study [5], the latter problem was approached by selectively labeling with a fluorescent probe one surface of a confluent monolayer of epithelial cells grown in culture, and determing whether the probe

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Abbreviations: G_{M1}, galactosyl(β1 → 3)*N*-acetylgalactosylaminyl(β1 → 4)[*N*-acetylneuraminyl(α2 → 3)]galactosyl(β1 → 4)glucosyl(β1 → 1′)ceramide; VSV, vesicular stomatitis virus; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

could move past the region of the tight junction to the surface not labeled initially. The study focused on synthetic lipid probes, which diffuse freely in the membrane. It was found that the ability of a lipid probe to pass the tight junction is correlated with its ability to flip-flop to the inner leaflet of the bilayer of the plasma membrane. Here we examine whether gangliosides, which are natural constituents of the cell membrane, and do not spontaneously flip-flop in artificial bilayers [6], behave similarly. It has been shown that cells can take up exogenous gangliosides [7–18]. These gangliosides are inserted into the plasma membrane [11–13] and preserve their functional properties since they can serve as receptors for toxins [7,10,12,16], virus [9], and fibronectin [14,15,18], and can promote neuritogenesis of neuroblastoma cells [17]. In addition, the inserted gangliosides are free to move in the plane of the membrane [13,15,16] and have a diffusion coefficient of 10^{-8} cm^2/s, similar to other lipid probes [15]. In the present study, we used fluorescent derivatives of gangliosides [19] and cholera toxin, which binds specifically to ganglioside G_{M1} [10], as probes to measure the ability of gangliosides to redistribute from the apical to the basolateral surface of cultured kidney epithelial cells.

Materials and Methods

Materials. Bovine brain gangliosides were obtained from ICN Nutritional Biochemicals (Cleveland, OH). Rhodamine-labeled gangliosides were prepared as described previously [19] and purified to remove free fluorophore. Cholera toxin, its B subunit and *Vibrio cholerae* sialidase were obtained from Calbiochem-Behring (La Jolla, CA). ^{125}I-labeled cholera toxin was prepared using the chloramine-T procedure [20]; 70% of the iodotoxin specifically bound to membranes [21]. ^{125}I-protein A (2–10 $\mu Ci/\mu g$) was from New England Nuclear (Boston, MA). A monoclonal antibody (17D7) to A6 surface protein was a gift from Dr. J. Turner (University of Toronto). Anti-rhodamine antibodies were prepared as described previously [16].

Cell cultures. Continuous epithelial cell lines, A6 derived from kidney of *Xenopus laevis* (ATTC CCL 102), and MDCK, Madin-Darby canine kidney cells (ATCC CCL 34), were obtained from the American Type Culture Collection (Rockville, MD). Culture conditions were as described [5] except that for A6 cells, the bicarbonate concentration of the growth medium was reduced to 8 mM and the gas phase was 1% CO_2 in air. The cells were plated on glass coverslips or on plastic tissue-culture dishes 7–10 days prior to an experiment, at which time all epithelia were confluent and forming domes. In experiments, A6 cells were rinsed with A6 Ringer solution and were incubated with CL-2 medium [22]. MDCK cells were rinsed with Dulbecco's phosphate-buffered saline and were incubated with Dulbecco's modified Eagle's medium. In the following text, we refer to the rinsing and incubation solutions as 'buffer' and 'medium', respectively. As epithelial cells grow with their basolateral surfaces attached to the culture dish and tight junctions are a barrier to flow between the cells, only the apical surface of a confluent epithelium is exposed to and bathed by solutions added to the dish.

Fluorescent labeling of cells. Cells were rinsed extensively with serum-free medium and incubated with fluorescent gangliosides at a final concentration of 30 $\mu g/ml$ for 1 h at 27°C for A6 and 37°C for MDCK epithelia. The cells were rinsed twice with buffer and examined under fluorescence and phase-contrast optics using a Zeiss Universal microscope.

Binding assays

The principles of the two methods for measuring binding are shown in Fig. 1.

Method 1. For this method (which, developed in our laboratory by R.J. Turner, is similar to that used by Pesonen and Simons [23]), cells were grown in 16-mm-diameter wells of a Cluster 24 (Costar Cambridge, MA). To insert G_{M1} or rhodaminyl gangliosides, we incubated the epithelia in medium containing the gangliosides as detailed in the legends to the figures and tables and rinsed the cells three times with buffer. To measure binding of ^{125}I-labeled cholera toxin, 0.5 ml of buffer containing approx. 0.5 nM iodotoxin and 0.1% bovine serum albumin was added to each well. Nonspecific binding was determined in the presence of 200 nM unlabeled toxin as described previously [21] and represented 5–10% of total binding. After 60 min at 4°C. the cells were

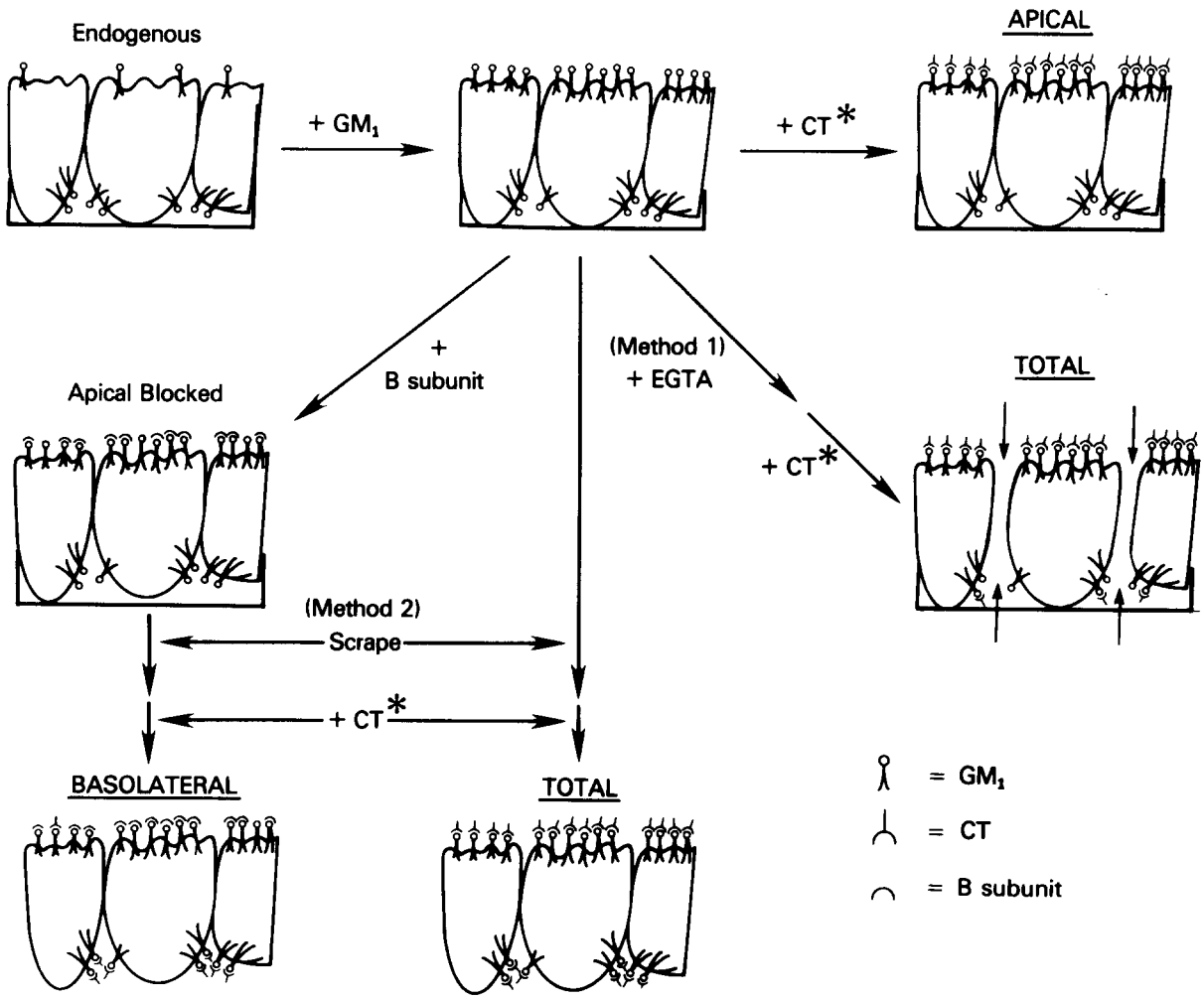

Fig. 1. Schematic diagram of the binding assay. See text for explanation.

rinsed three times with ice-cold buffer containing 0.1% bovine serum albumin. The cells then were rinsed with ice-cold buffer and treated overnight in 1 M NaOH to dissolve the cells. The contents of each well were counted in a Beckmann gamma counter and portions were taken for protein determination [24]. In other experiments measuring rhodaminyl gangliosides or endogenous surface proteins, cells were incubated with anti-rhodamine antibodies (7 μg/dish) or monoclonal antibody 17D7 (diluted 1 : 100) in 100 μl of buffer for 2 h at 4°C, rinsed and incubated with ^{125}I-protein A (0.025 μCi) in 0.5 ml of buffer for 30 min at 23°C. The cells then were rinsed and assayed for radioactivity and protein as described above. To measure total (apical + basolateral) surface binding, the tight junctions were disrupted by incubating the epithelia in Ca^{2+}- and Mg^{2+}-free buffer containing 1 mM EGTA without (A6) or with (MDCK) 0.1% trypsin for 15 min at 23°C prior to the binding assay.

Method 2. Binding of ^{125}I-labeled cholera toxin to the apical surface of the epithelia was measured as described under method 1. For measurement of total (apical + basolateral) binding, we first scraped the cells from the well using a Teflon policeman and then transferred the suspension of small epithelial sheets to a 12 × 75 mm test tube. The suspension was incubated in 0.5 ml of buffer containing ^{125}I-labeled cholera toxin (approx. 0.5

nM) and 0.1% bovine serum albumin for 1 h at 0°C with agitation to maintain the sheets in suspension. After adding 3 ml of ice-cold buffer containing 1% bovine serum albumin, the tubes were centrifuged and the supernatants removed by aspiration. The cells were washed twice more by this procedure, dissolved in 1 M NaOH and analyzed for radioactivity and protein as described above. Nonspecific binding was determined by including 200 nM unlabeled toxin in the assay. To measure basolateral binding, we first blocked apical binding sites by incubating the epithelia in wells with 200 nM unlabeled cholera toxin B subunit in medium for 90 (A6) or 30 (MDCK) min at 4°C. The cells then were rinsed, scraped and assayed in suspension for ^{125}I-labeled cholera toxin binding as described above.

Results

Distribution of fluorescent gangliosides on domes of A6 cells

We observed that the apical surfaces of confluent cultures of A6 epithelial cells which had been incubated with rhodaminyl gangliosides and rinsed, became fluorescent (Fig. 2). The surfaces were brightly stained and little fluorescence was noted inside the cells. In order to determine whether fluorescent gangliosides, which were added to the apical surface of the cells, passed the tight junction to the basolateral surface, we examined domes. It has been found that the most convenient technique for viewing the location of the fluorescent probe was to focus the microscope part way up a dome of cells so that apical and basolateral surfaces of individual cells are in focus simultaneously [5]. If the entire plasma membrane is labeled, each cell at a given cross-section of the dome defined by the plane of focus of the microscope will appear as a complete ring of fluorescence. Such rings are observed when apical surfaces are labeled with lipid probes that penetrate the tight junctions [5]. If only the apical membrane is labeled, incomplete fluorescent rings, or arcs, are seen [5]. Fig. 2 shows domes of A6 cells labeled with rhodaminyl gangliosides; only arcs of the apical membranes are fluorescent. Thus, the fluorescent gangliosides did not pass the tight junctions to the basolateral membranes during the incubation time.

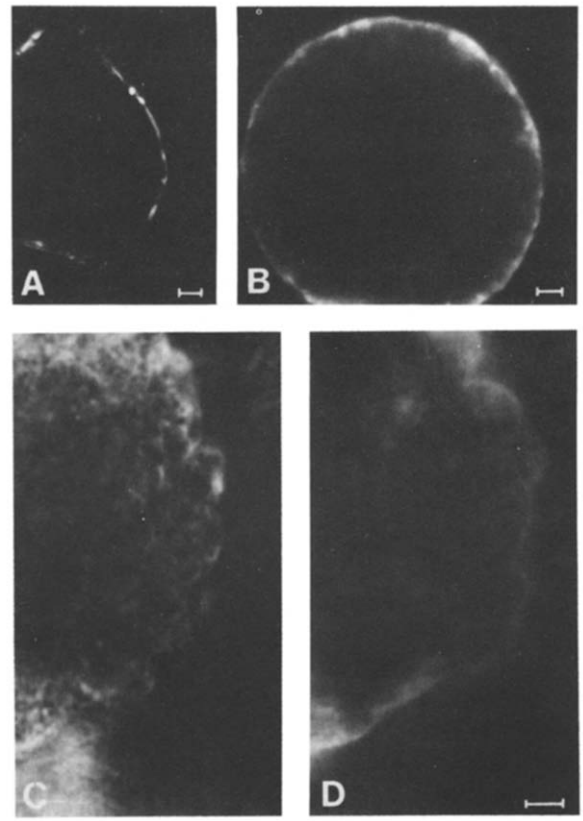

Fig. 2. Fluorescent micrographs of domes of A6 epithelia labeled with rhodaminyl gangliosides. Confluent epithelia forming domes were incubated with 30 μg/ml of rhodaminyl gangliosides for 30 (A) or 60 (B, C, D) min at 27°C, rinsed and examined for surface fluorescence on domes as described in Materials and Methods. The plane of the focus is part way up the dome and labeling of only the apical surface appears as a series of arcs (see text). (C) Bright-field photomicrograph of the dome in (B). Bar = 10 μm.

Quantitative determination of ganglioside distribution – method 1

The above studies using fluorescence microscopy showed that fluorescent gangliosides were unable to pass tight junctions even after an hour. In order to obtain more information about this phenomenon and to evaluate the behaviour of unmodified gangliosides inserted into the apical membrane, we turned to a more quantitative determination. We used two different methods to assay apical and basolateral surface gangliosides. In the first method, we measured apical binding of cholera toxin or antibodies to attached, intact epithelia. To obtain total binding, we opened the

tight junctions by trypsinization and/or chelation of Ca^{2+}.

As shown in Table I, we measured 6390 cpm/dish of specific toxin binding to endogenous G_{M1} on the apical surface of A6 cells. After opening the tight junctions by chelation, iodotoxin binding to endogenous receptors increased slightly over apical surface binding. Thus, there is some endogenous G_{M1} on the basolateral surface (approx. 19% of the total). As a control, we used a monoclonal antibody that binds to a protein antigen that is present on both apical and basolateral surfaces. The binding of the antibody as measured with ^{125}I-protein A increased from 2100 ± 87 cpm/dish to 2950 ± 272 cpm/dish after opening the tight junctions. (Binding in the absence of antibody was 211 ± 12 and 234 ± 12 before and after EGTA treatment, respectively.) From these latter results, we conclude that basolateral binding can be detected with method 1.

Upon incubation with 1.5 μM G_{M1} for 2 h, followed by rinsing, binding of iodotoxin to the apical surface increased 3.5-fold. After the G_{M1}-treated cells had been incubated for an additional 4 h at 27°C, binding to the apical surface decreased 52%. Based on studies with other cells [12,15,25–27], the decrease was due most likely to internalization of the G_{M1}. When the tight junctions were opened by chelation and total toxin binding was measured, the extent of binding was similar to that observed prior to opening the tight junctions. Thus, most of the toxin binding was to the apical surface and none of the incorporated G_{M1} appeared to move to the basolateral surface even after 4 h.

Similar experiments were performed with MDCK cells (Table I). In this case, labeling of the apical surface with exogenous G_{M1} yielded a 31-fold increase in toxin binding over endogenous (the more extensive incorporation of G_{M1} by MDCK cells compared to A6 cells may be due in part to incubation of the former with G_{M1} at 37°C, whereas the latter were incubated at 27°C, their normal growth temperature), essentially all of it on the apical surface as total binding after opening of the tight junctions by trypsin and chelation was not increased. After 4 h of incubation in medium, there was a 60% decrease in toxin binding to the G_{M1}-treated cells, but no increase in binding to the basolateral surface. Thus, as was observed with A6 cells, exogenous G_{M1} incorporated into the apical membrane of MDCK cells may undergo internalization but is unable to redistribute to the basolateral surface. The absence of increased toxin binding to the basolateral surface is consistent with the lack of either cell-surface or intracellular pathways for gangliosides to move between the two membrane domains.

Table II shows the data for MDCK cells labeled with rhodaminyl gangliosides, using anti-rhoda-

TABLE I

DISTRIBUTION OF CHOLERA TOXIN RECEPTORS ON CONFLUENT EPITHELIA AFTER EXPOSURE OF THE APICAL SURFACE TO EXOGENOUS G_{M1}

Confluent epithelia were incubated without or with 1.5 μM G_{M1} for 1.5 h at 27°C (A6) or 37°C (MDCK). The cells were rinsed extensively and assayed for binding of ^{125}I-labeled cholera toxin before and after treatment with EGTA (A6) or EGTA and trypsin (MDCK) to open tight junctions as described in Material and Methods (Fig. 1, method 1). In addition, some of the cultures were incubated in fresh medium for 4 h before the toxin binding assay. Values have been corrected for nonspecific binding as determined in the presence of 200 nM unlabeled toxin and are the means ± S.D. of triplicate determinations. Similar results were obtained in two additional experiments.

Cells	Junctions	^{125}I-Labeled cholera toxin bound (cpm/dish)		
		control	G_{M1}-treated	G_{M1}-treated after 4 h
A6	intact	6390 ± 360	22120 ± 920	10740 ± 150
A6	open	7915 ± 1300	19920 ± 1400	10530 ± 470
MDCK	intact	697 ± 11	21930 ± 3550	8790 ± 342
MDCK	open	804 ± 2	21120 ± 1695	8825 ± 323

TABLE II

DISTRIBUTION OF RHODAMINYL GANGLIOSIDES ON CONFLUENT MDCK EPITHELIA

Confluent MDCK epithelia were incubated with rhodaminyl gangliosides (30 μg/ml) for 1.5 h at 37°C and rinsed extensively. After the cells were treated without or with EGTA and trypsin to open tight junctions, they were incubated with rabbit anti-rhodamine antibodies followed by ^{125}I-protein A as described in Materials and Methods (Fig. 1, method 1). In addition, some of the cultures were incubated in fresh medium for 4 h before the binding assay. Values are the means ± S.D. of triplicate determinations. Less than 650 cpm/dish were detected when normal rabbit serum was used. Similar results were obtained in an additional experiment.

Treatment	^{125}I-Labeled protein A bound (cpm/dish)	
	intact junctions	open junctions
None	646 ± 53	362 ± 24
Rhodaminyl gangliosides	14310 ± 496	14680 ± 848
Rhodaminyl gangliosides then 4 h incubation	7200 ± 825	7770 ± 526

mine antibodies and ^{125}I-protein A to quantify their distribution. Initially, all of the rhodaminyl gangliosides were restricted to the apical surface. After 4 h, the amount of rhodaminyl gangliosides on the apical surface decreased by 50% with no increase on the basolateral surface.

Quantitative determination of ganglioside distribution – method 2

In order to measure more accurately the amount of toxin binding to the basolateral surface, we modified the assay (Fig. 1, method 2). Toxin binding to the apical surface was measured as in method 1, and total binding was assayed by scraping the epithelia from the wells and incubating them with iodotoxin in suspension. We directly measured binding to the basolateral surface by first blocking the toxin receptors on the apical surface with unlabeled B subunit of cholera toxin before scraping and suspending the cells. The results for A6 cells are shown in Table III. There was more toxin binding to endogenous G_{M1} on the basolateral surface than on the apical surface. Thus, method 2 appears to be more sensitive than method 1, which showed that binding to apical surface endogenous receptors represented 81% of the total binding to endogenous receptors. (Method 2 also was more sensitive than method 1 for detecting endogenous G_{M1} on the basolateral surface of MDCK cells (compare Table I with Table IV).)

TABLE III

BINDING OF CHOLERA TOXIN TO APICAL AND BASOLATERAL SURFACES OF CONTROL AND G_{M1}-TREATED A6 EPITHELIAL CELLS

Confluent A6 epithelia were incubated without or with 2 μM G_{M1} for 3 h at 27°C, rinsed extensively and assayed for ^{125}I-labeled cholera toxin binding as outlined in Fig. 1, method 2. Values are the means ± S.D. of quadruplicate determinations and have been corrected for nonspecific binding as determined in the presence of 200 nM unlabeled cholera toxin. Assays at different ^{125}I-labeled toxin concentrations gave essentially the same cpm bound, indicating that saturation of the binding sites had been reached. The amount of protein per dish was 145 ± 19 μg. Similar results were obtained in five additional experiments.

Cell surface	^{125}I-Labeled cholera toxin bound (cpm/dish)	
	control cells	G_{M1}-treated cells
Apical [a]	2000 ± 290	21600 ± 3700
Apical + B-subunit [b]	–	2900 ± 800
Basolateral [c]	8100 ± 2000	9600 ± 1500
Total [d]	10000 ± 2100	32700 ± 5200

[a] Binding of toxin to cells while attached to the dish.

[b] Cells were preincubated with 200 nM B subunit of the toxin before binding of iodotoxin to cells attached to the dish was determined.

[c] Cells attached to the dish were preincubated with 200 nM B subunit, rinsed, scraped and assayed in suspension for iodotoxin binding.

[d] Binding of toxin to cells scraped and maintained in suspension.

When A6 cells were incubated with exogenous G_{M1}, binding of iodotoxin to the apical surface increased 10-fold whereas binding to the basolateral surface remained similar to that observed with cells not treated with G_{M1} (Table III). Basolateral binding was either estimated by the difference between total and apical binding or directly measured on cells whose apical surface receptors had been blocked by prior exposure to the B subunit of cholera toxin. The latter effectively reduced binding of iodotoxin to the apical surface by 87%. Values obtained for binding to the basolateral surface by both procedures were similar; thus, the assay was quantitatively consistent.

TABLE IV

BINDING OF CHOLERA TOXIN TO APICAL AND BASOLATERAL SURFACES OF CONTROL AND G_{M1}-TREATED MDCK EPITHELIAL CELLS

Details are the same as those in Table III except that confluent MDCK epithelia were incubated without or with 1 μM G_{M1} for 1 h at 37°C, rinsed extensively and assayed for ^{125}I-labeled cholera toxin binding as outlined in Fig. 1, method 2. Values are the means ± S.D. of quadruplicate determinations and have been corrected for nonspecific binding as determined in the presence of 200 nM unlabeled cholera toxin. The amount of protein per dish was 122 ± 4 μg. Similar results were obtained in three additional experiments.

Cell surface	^{125}I-Labeled cholera toxin bound (cpm/dish)	
	control cells	G_{M1}-treated cells
Apical	1130 ± 37	16600 ± 1750
Apical + B-subunit	34 ± 4	637 ± 60
Basolateral	517 ± 35	2160 ± 613
Total	1670 ± 490	17500 ± 2495

Similar results were obtained with MDCK cells using method 2 (Table IV). Even though the level of G_{M1} (as measured by toxin binding) increased 15-fold on the apical surface of the G_{M1}-treated cells, there was no evidence for the exogenous G_{M1} inserted in the apical membrane redistributing to the basolateral membrane. In contrast to A6 cells, there appeared to be more endogenous G_{M1} on the apical than on the basolateral surface of MDCK cells. If the area of the basolateral membrane is 4-times greater than that of apical membrane, then G_{M1} may be asymmetrically distributed in MDCK cells, as are many membrane proteins [1–4].

Behaviour of endogenous gangliosides

In the above studies, we were able to demonstrate that exogenous gangliosides inserted into the apical membrane did not pass the tight junctions. It was of interest to determine whether endogenous gangliosides behaved in the same way. For this purpose, we incubated intact confluent cultures of A6 epithelia with *Vibrio cholerae* sialidase for 2 h at 27°C (Table V). When we assayed the cells for binding of ^{125}I-labeled cholera toxin, there was a 10-fold increase in toxin receptors on the apical surface, which is consistent with a corresponding increase in endogenous apical G_{M1} [21].

TABLE V

BINDING OF CHOLERA TOXIN TO APICAL AND BASOLATERAL SURFACES OF CONTROL AND SIALIDASE-TREATED A6 EPITHELIAL CELLS

Details are the same as those in Table III except that confluent A6 epithelia were incubated without or with *V. cholerae* sialidase (0.1 U/ml) for 2 h at 27°C in CL-2 medium (0.3 ml/well), rinsed extensively and assayed for ^{125}I-labeled cholera toxin binding as outlined in Fig. 1, method 2. Similar results were obtained in a separate experiment.

Cell surface	^{125}I-Labeled cholera toxin bound (cpm/dish)	
	control cells	sialidase-treated cells
Apical	3790 ± 435	36150 ± 3120
Apical + B-subunit	1410 ± 255	7230 ± 1410
Basolateral	6450 ± 450	13800 ± 1610
Total	8330 ± 1030	39230 ± 7860

As was described above, binding of iodotoxin to the basolateral surface was estimated either by the difference between total and apical binding or directly after blocking the apical receptors with cholera toxin B subunit. The latter did not completely block all of the apical receptors. When this is taken into account, there appeared to be no significant increase in toxin binding to the basolateral surface after sialidase treatment. Thus, endogenous G_{M1} behaves as exogenous G_{M1} inserted into the apical membrane and is unable to pass the tight junctions to the basolateral membrane.

Discussion

We have shown that fluorescent gangliosides are taken up by the apical membranes of confluent kidney epithelial cells but appear unable to pass the tight junctions to the basolateral membranes. We also used two different binding assays to quantitatively assess movement of gangliosides from the apical to the basolateral plasma membrane. The first assay (method 1) is similar to the one used by Pesonen and Simons to determine such movement of the G protein of vesicular stomatitis virus [23]. In that assay, to make basolateral membranes accessible to the binding ligand, the tight junctions were opened by chelating Ca^{2+}. One potential problem with this assay is that the binding ligand may not diffuse well through the

opened tight junctions or along the basolateral surface where the epithelium is still attached to the culture dish and basolateral binding may be underestimated. In the new assay (method 2), we scraped the epithelia from the culture dishes and then measured total binding to small sheets of the cells maintained in suspension. With this assay, we found more binding of cholera toxin to endogenous G_{M1} on the basolateral surface than we found with the first assay. In addition, we directly measured binding of cholera toxin to the basolateral surface by first blocking the toxin receptors on the apical surface with the B subunit of cholera toxin.

Both methods yielded qualitatively similar results. When either unmodified G_{M1} or rhodaminyl gangliosides were implanted in the apical surface of epithelia, there was no evidence of any movement of the exogenous gangliosides to the basolateral surface either during the initial period of uptake or after additional incubation of 4 h. During this latter period, there was an extensive disappearance from the apical membrane of the incorporated gangliosides, presumably due to internalization. Others have reported that exogenous gangliosides inserted into the plasma membrane of various cells also underwent endocytosis [12,15,25–27]. Thus, exogenous G_{M1} incorporated into G_{M1}-deficient cells was internalized as detected by loss of cholera toxin binding [25,26] and by quantitative electron microscope autoradiography [12]. The G_{M1} also was metabolized by the cells and the metabolic products obtained were consistent with the ganglioside entering the Golgi apparatus and lysosomes [26,27].

We believe that the use of exogenous gangliosides as probes for the behavior of endogenous gangliosides is valid. Exogenous gangliosides appear to be functionally inserted into the plasma membrane where they have been shown to serve as receptors for bacterial toxins [10] and viruses [9]. In addition, spin-labeled [13] and fluorescently-labeled [15,16] gangliosides were observed to be properly inserted into the lipid bilayer of the plasma membrane and free to diffuse as are other lipids. Fluorescent G_{M1} derivatives preserved their ability to function as cell surface receptors for cholera toxin [28]. It is unlikely that the exogenous gangliosides were simply adsorbed to other apical membrane components as they were not released from the cell surface by chelation and trypsinization. Furthermore, we were able to demonstrate that endogenous gangliosides in the apical membrane of the epithelial cells were unable to move to the basolateral membrane. Thus, cells exposed on their apical surfaces for 2 h to bacterial sialidase exhibited a large increase in apical receptors for cholera toxin. It is well known that *V. cholerae* sialidase converts more complex gangliosides to G_{M1} (see Ref. 21). There was no increase in binding of the toxin to the basolateral surfaces of the sialidase-treated cells. Thus, the newly created, but endogenous G_{M1} failed to diffuse past the tight junctions.

In a previous study with fluorescent lipid probes, the ability of the probe to pass the tight junction correlated with its ability to flip-flop from one leaflet of the lipid bilayer to the other [5]. Felgner et al. observed that gangliosides incorporated into the outer leaflet of phospholipid vesicles did not spontaneously flip-flop to the inner leaflet [6]. Our observations that gangliosides in the outer leaflet of the bilayer do not move from apical to basolateral plasma membranes by diffusing past tight junctions are consistent with theirs. Furthermore, our results lend support to the idea that lipids must be in the inner leaflet of the plasma membrane bilayer to diffuse past the region of the tight junction.

Van Meer and Simons have observed a polarity in the distribution of different phospholipids in viruses budding from apical compared to basolateral plasma membranes of MDCK cells [29]. Since phospholipids are asymmetrically distributed between inner and outer leaflets of the plasma membrane bilayer [30], the barrier in the region of the tight junction would serve to maintain the polarity of the lipid distribution. We also found that endogenous G_{M1} appears to be asymmetrically distributed per unit membrane between apical and basolateral plasma membranes of MDCK but perhaps not A6 cells. The nature of the outer leaflet diffusion barrier in the region of the tight junction remains to be determined.

It has been suggested that the maintenance of epithelial polarity is based on selective sorting mechanisms. Matlin et al. [31] have shown that there is an intracellular sorting route from apical to basolateral plasma membrane for the G protein

of the envelope of VSV. In infected epithelial cells, the G protein is synthesized in the endoplasmic reticulum and follows a sorting pathway to the basolateral plasma membrane where it is inserted. It has been used as a model basolateral membrane protein in studies of epithelial polarity. When G protein is experimentally fused into the apical plasma membrane of MDCK epithelia, it is endocytosed and routed to the basolateral plasma membrane [23,31]. Such a pathway does not appear to exist for exogenous gangliosides inserted into the apical plasma membrane. Despite extensive disappearance of the implanted gangliosides from the apical plasma membrane, they did not appear on the basolateral surface. Thus, our data indicate that intracellular pathways from apical to basolateral membrane may not be available to glycolipids as they are to glycoproteins.

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