Direct Visualization of Redistribution and Capping of Fluorescent Gangliosides on Lymphocytes

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ABSTRACT Fluorescent derivatives of gangliosides were prepared by oxidizing the sialyl residues to aldehydes and reacting them with fluorescent hydrazides. When rhodaminyl gangliosides were incubated with lymphocytes, the cells incorporated them in a time- and temperature-dependent manner. Initially, the gangliosides were evenly distributed on the cell surface but were redistributed into patches and caps by antirhodamine antibodies. When the cells were then stained with a second antibody or protein A labeled with fluorescein, the fluorescein stain revealed the coincident movement of both the gangliosides and the antirhodamine antibodies. When the cells were treated with both rhodamine and Lucifer yellow CH-labeled gangliosides, the antirhodamine antibodies induced patching and capping of both fluorescent gangliosides but had no effect on cells incubated only with Lucifer yellow CH-labeled gangliosides. In addition, capping was observed on cells exposed to cholera toxin, antitoxin antibodies, and rhodamine-labeled protein A, indirectly showing the redistribution of endogenous ganglioside GM1, the cholera toxin receptor. By incorporating Lucifer yellow CH-labeled GM1 into the cells and inducing capping as above, we were able to demonstrate directly the coordinate redistribution of the fluorescent GM1 and the toxin. When the lymphocytes were stained first with Lucifer yellow CH-labeled exogenous ganglioside GM3, which is not a toxin receptor, there was co-capping of endogenous GM1 (rhodamine) and exogenous GM3 (Lucifer yellow CH). These results suggest that gangliosides may self-associate in the plasma membrane which may explain the basis for ganglioside redistribution and capping.

Gangliosides, which are sialic acid-containing glycosphingolipids, are constituents of the plasma membrane and are believed to be restricted to the outer leaflet of the bilayer (1–4). Because of this orientation, gangliosides have been implicated in a variety of cell surface events such as recognition phenomena and biotransduction of membrane-mediated information (1–9). The ability to introduce exogenous gangliosides into the plasma membrane of mammalian cells has provided a useful means to study the biological function(s) of these membrane components (4–10).

In previous studies, a simple method was developed to chemically modify gangliosides by covalent attachment of dinitrophenol or biotin groups to their sialyl residues (6, 7). The modified gangliosides were taken up by thymocytes and appeared to undergo lateral redistribution into patches and caps when the cells were exposed to fluoresceinated antidinitrophenol antibody or avidin. These reagents also caused mitogenic stimulation of the thymocytes (6, 7). Others had demonstrated that cholera toxin, which binds to endogenous ganglioside GM1 (see reference 4), induced patching and capping in lymphocytes (11–13). It also had been reported that antiganglioside antibodies caused patching and capping (14).

In all of these studies, one could only assume that the observed redistribution was due to a movement of the gangliosides induced by the bound protein. As the lipid moiety of gangliosides is too short to span the bilayer, and gangliosides are presumed to be monovalent, the mechanism by which a multivalent protein causes their redistribution remains un-

1 Abbreviations used in this paper: FITC, fluorescein isothiocyanate; GM1, endogenous ganglioside; GM3, exogenous ganglioside; LY-gangliosides, Lucifer yellow CH-labeled gangliosides; Rh-gangliosides, rhodamine-labeled gangliosides; RITC, rhodamine isothiocyanate.
clear. To pursue this phenomenon further, we have prepared highly fluorescent gangliosides (15, 16) and incorporated them into lymphocytes. By inducing movement of the gangliosides with an antibody against the fluorescent group, we were able to monitor directly the redistribution of the gangliosides.

MATERIALS AND METHODS

Preparation of Fluorescent Gangliosides: Gangliosides labeled with rhodamine (15) and Lucifer yellow CH (16) were prepared as described previously. Briefly, the gangliosides were oxidized with sodium periodate, the oxidation was stopped by adding glyceral, and the gangliosides were dialyzed extensively. The oxidized gangliosides then were reacted with either isiainine rhodamine-~-alanine hydrazide (15) or Lucifer yellow CH (16) and dialyzed against distilled water. The rhodamine-labeled gangliosides (Rh-gangliosides) were reduced with KBH4 (1 mM at 25°C for 10 min). Because reduction quenched the Lucifer yellow fluorescence, the Lucifer yellow CH-labeled gangliosides (LY-gangliosides) were not reduced. The fluorescent gangliosides were separated by thin-layer chromatography on silica gel as described previously (17). They were easily detected when illuminated with a ultraviolet light and were visualized with resorcinol reagent and quantified by scanning spectrophotometry (17). In most experiments, fluorescent derivatives of bovine brain gangliosides (15, 16) were used. In some experiments, exogenous gangliosides LY-GM3, LY-GM1, and LY-GD1a were used. These were obtained by scraping the corresponding area from the chromatograms and eluting them from the silica gel with chloroform/methanol/water (10:6:3, vol/vol/vol).

Preparation of Rabbit Antibodies: Antibodies to the rhodaminyl group were raised in rabbits by immunization with rhodaminyl BSA in complete Freund's adjuvant. The antirhodamine antibodies were purified on a rhodaminyl rabbit serum albumin-Sepharose immunosorbent (18). The adsorbed antibodies were eluted with 0.1 M acetic acid at 25°C and dialyzed against Dulbecco's PBS, pH 7.4. The immunoaffinity-purified rabbit antirhodamine fraction was found to bind specifically and stoichiometrically to various rhodamine derivatives. Binding of rhodamine to the antibody was accomplished by a two-fold enhancement in rhodamine fluorescence at 590 nm. Rh-gangliosides reacted similarly with the antirhodamine antibodies. No change in fluorescence was observed when normal rabbit IgG was mixed with rhodamine derivatives.

Incorporation of Fluorescent Gangliosides into Lymphocytes: Lewis rat and B10BR mouse thymocytes were prepared as described previously (6) and suspended (10⁶ cells/ml) in Dulbecco's modified Eagle's medium containing fluorescent gangliosides (50 μg/ml). After incubating the cells for 2 h at 37°C in a humidified incubator (5% CO2/95% air), the cells were washed twice in PBS and suspended in 10⁶ cells/ml in PBS.

Antibody Treatment of Cells: The washed, fluorescently labeled cells (0.2 ml) were incubated with antirhodamine antibodies (100 μg/ml) for 30 min at 0°C, washed, and incubated at 37°C for different times. Then, they were stained with 50–100 μg/ml of fluorescein isothiocyanate (FITC)-labeled protein A (Pharmacia Fine Chemicals, Piscataway, N J) or goat anti-rabbit IgG (Miles-Yeda, Rehovoth, Israel) at 0°C for 30 min in PBS. The cells were washed above, usually fixed in 3.8% formaldehyde in PBS for 30 min, and examined with a Zeiss microscope modified for epifluorescence (Carl Zeiss Inc., New York). Photomicrographs were taken with a Pentax camera (Englewood, CO) using filters specific for either rhodamine or fluorescein fluorescence. In some experiments, the mouse thymocytes were incubated for 1 h at room temperature with anti-4-1.2 from AKR mice (New England Nuclear, Boston) diluted 1:1,000 to induce capping of this cell surface antigen. In other experiments, binding of cholera toxin to the cells was visualized using a modification of a three-layer system (19). The cells were incubated at 4°C with 10 nM cholera toxin (30 min), washed, incubated with rabbit anti-cholera toxin (1:100 for 40 min), washed, and incubated for 40 min with 50 nM rhodamine isothiocyanate (RITC)-labeled protein A or RITC-labeled goat anti-rabbit IgG (obtained from Dr. D. Segal, National Cancer Institute). The cells then were incubated at room temperature for 30–60 min to allow capping to occur.

Binding and Action of Cholera Toxin: Rat thymocytes were washed three times with DME and suspended in the same medium at 1.5–2 × 10⁶ cells/ml. The cells were incubated with and without 400 nM of the binding (B) subunit of cholera toxin (Calbiochem-Behring, LaJolla, CA) in a humidified incubator at 37°C for 30 min. The cells then were washed three times and incubated as above for 2 h in the presence or absence of 5 nM GM1. Finally, the cells were washed three times with ice-cold PBS and analyzed for binding and action of cholera toxin. Binding of 32P-cholera toxin was determined as described previously (20) except nonspecific binding was determined in the presence of 300 nM unlabeled toxin. Briefly, 10⁶ cells were incubated with 1.4 nM 32P-cholera toxin (530 cpm/μmol) in 0.2 ml of buffer (20) for 1 h at 37°C with constant shaking. The cells then were filtered and the filters were counted (20). Accumulation of cyclic AMP and activation of adenylate cyclase by cholera toxin were determined by established methods (19, 21–23). Briefly, ~10⁶ cells per ml were incubated in the appropriate medium (19) with and without 10 nM cholera toxin at 37°C with constant shaking. For cyclic AMP accumulation, the samples were boiled after 1 h and cyclic AMP was determined with a radioimmuno assay (23). For adenylate cyclase activity, the cells were collected at the indicated times and lysed (21) and the lysates assayed for cyclase activity with 50 μM GTP (22).

RESULTS

Analysis of Fluorescent Gangliosides

When the fluorescent gangliosides were separated by thin-layer chromatography and visualized under ultraviolet light, they had different mobilities than native gangliosides (Fig. 1). In that a large number of fluorescent derivatives were obtained from mixed bovine brain gangliosides (lanes 2 and 3), we compared individual species before and after modification (lanes 4–9). Whereas Rh-GM1 (lane 4) migrated more rapidly than native GM1, LY-GM1 (lanes 6 and 7) migrated more slowly. Similarly, LY-GM3 was more polar than native GM3 (lane 5). Because the rhodamine group is relatively hydrophobic and Lucifer yellow CH is both negatively charged and hydrophilic, the observed changes in mobility were predictable. The modified gangliosides still were able to react with resorcinol reagent and to be visualized on the chromatogram (lanes 8 and 9). To purify the individual fluorescent gangliosides from any unreacted ganglioside and free reagent, they were eluted from the chromatograms (lanes 3 and 7).

Incorporation of Fluorescent Gangliosides into Thymocytes

When mouse thymocytes were incubated with Rh-gangliosides, their surfaces became highly fluorescent (Fig. 2a). Based on fluorescent measurements, ~10⁴ molecules of Rh-gangliosides were taken up per cell. The gangliosides appeared to be stably incorporated inasmuch as they were not removed by extensive washing. Uptake of Rh-gangliosides by the cells was time and temperature dependent; a noticeable decrease in...
FIGURE 2 Surface fluorescence pattern of thymocytes incubated with fluorescent gangliosides. Mouse thymocytes were incubated for 2 h at 37°C with Rh-gangliosides, washed, and examined for rhodamine fluorescence before (a) or after (b) treatment with trypsin. (c-f) Cells treated with Rh-gangliosides were incubated with antirhodamine antibodies for 30 min at 37°C (c and d) or 0°C (e and f), then stained with FITC protein A at 0°C, and examined for fluorescein (c and e) and rhodamine (d and f) fluorescence. \( \times 1,000 \).

fluorescence intensity was observed when the cells were exposed to the gangliosides at 0°C. When the thymocytes were treated with trypsin (2-10 \( \mu \)g/ml for 10 min at 37°C) before or after Rh-ganglioside incorporation, the fluorescence intensity was not changed significantly (Fig. 2b). We had shown previously that trypsin treatment did not reduce the fluorescence intensity of murine thymocytes containing LY-gangliosides (16).

Redistribution of Rh-Gangliosides by Antirhodamine Antibodies

Cells containing incorporated Rh-gangliosides were incubated with antirhodamine antibodies at 0°C for 30 min, washed, and warmed at 37°C for different times. The cells then were stained at 0°C with FITC-labeled protein A or goat anti-rabbit IgG. Incubation of the antirhodamine-treated cells at 37°C led to a time-dependent reorganization of the Rh-gangliosides on the cell surface. After 10 min, patched aggregates were observed on a few cells. By 30 min, the patches had coalesced into large clusters with a caplike appearance (Fig. 2c and d). The number of cells containing caps increased with time at 37°C and the formation of caps on the uropod of the lymphocytes was pronounced. At all stages of the redistribution process, there was a coincidence of rhodamine and fluorescein fluorescence indicating coordinate movement of the Rh-gangliosides and the antirhodamine antibodies. When cells enriched with rhodaminyl or unmodified gangliosides were treated with normal rabbit IgG or antirhodamine antibodies, respectively, no fluorescein fluorescence was detected.

When the cells were maintained at 0°C, redistribution of the gangliosides was prevented and a uniform ring of rhodamine and fluorescein fluorescence was noted (Fig. 2c and f). No redistribution of Rh-gangliosides was observed in cells exposed to normal IgG followed by FITC protein A. Cells containing Rh-gangliosides also were treated with trypsin before staining with antirhodamine and FITC protein A. No significant changes in the extent of cap formation were observed. Redistribution and capping of Rh-gangliosides were induced by antirhodamine antibodies in the absence of a second antibody or FITC protein A (Fig. 3).

Coordinate Redistribution of Fluorescent Gangliosides

Mouse thymocytes were incubated with both Rh- and LY-gangliosides followed by antirhodamine antibodies. Initially, both fluorescent gangliosides remained evenly distributed on the cell surface (Fig. 4a and b). After 30 min at 37°C, the antibodies had induced extensive redistribution not only of the Rh-gangliosides but also of the LY-gangliosides into patches and caps (Fig. 4c and d). The extent of capping depended on time, temperature, and antibody concentration with up to 37% of the cells exhibiting caps. There were three
distinct populations of capped cells with a similar distribution irrespective of the extent of capping. 30% exhibited co-capping with images in both wavelengths being almost identical. In 51% of the capped cells, there was in addition to the co-cap a faint ring of Lucifer yellow fluorescence. In the remainder of the capped cells, there was no correlation between the two fluorescent gangliosides. Similar results were obtained with rat thymocytes enriched with both Rh- and LY-gangliosides (data not shown).

Antirhodamine antibodies did not induce cap formation in cells treated only with LY-gangliosides and did not react with Lucifer yellow-labeled BSA. Thus, the antirhodamine antibodies do not recognize the Lucifer yellow group. To eliminate the possibility that the observed co-capping was due to a cooperative interaction between the dyes, mouse thymocytes containing LY-gangliosides were incubated with anti-α1.2 and stained with RITC-goat anti-mouse. Although extensive capping of the α1.2 antigen (rhodamine) was observed (Fig. 4e), the distribution of the LY-gangliosides remained unaffected (Fig. 4f). Mouse thymocytes containing Rh-gangliosides were incubated with antirhodamine, fixed, and then stained with FITC concanavalin A, or anti-α1.2 followed by FITC-goat anti-mouse. In all cases, the fluorescein fluorescence appeared as a ring and did not correspond to the cap of rhodamine fluorescence. Thus, redistribution of the Rh-gangliosides by the antirhodamine antibodies does not appear to induce a corresponding redistribution of other surface markers.

Redistribution of Endogenous GM1 and Exogenous Fluorescent Gangliosides Induced by Cholera Toxin

When rat thymocytes were incubated with cholera toxin, rabbit anticholera toxin, and RITC-labeled protein A, capping was observed in agreement with previous studies (11-13). No capping was seen if the protein A (or a second antibody) was omitted; thus, cholera toxin-induced capping appeared to require extensively cross-linking as reported by others (11, 13). Cholera toxin also induced capping of exogenously inserted LY-GM1; dual fluorescence indicated that in all of the capped cells both the ganglioside and the toxin were in the same cap (Fig. 5, a and b). When cells enriched with an unrelated ganglioside, LY-GM3, were carried through the same procedure, there was a striking correspondence of patches (Fig. 5, c and d) and caps (Fig. 5, e and f) of the endogenous GM1 (stained indirectly by the three-layer immunofluorescence procedure) and the exogenous GM3 (Lucifer yellow CH fluorescence). Although only 2% of the capped cells exhibited identical co-capping, 60% of the capped cells had, in addition to a co-cap, a faint ring of fluorescence in the Lucifer yellow CH wavelength. Similar results were obtained with LY-GD1a, which also is not a receptor for cholera toxin. Thus, there appeared to be some association between the endogenous and exogenous gangliosides at the cell surface.

Evidence for Functional Insertion of Exogenous Gangliosides into Lymphocytes

It had been shown in previous studies that exposure of GM1-deficient cells to exogenous GM1 enhanced the binding and action of cholera toxin (4, 5, 10, 23). We used this technique to ascertain whether or not exogenous gangliosides were being functionally inserted into the plasma membrane of rat thymocytes. When the cells were incubated with 5 μM GM1 for 2 h, binding of 125I-cholera toxin increased around threefold (Fig. 6A), and there were small but significant increases in toxin-activated cyclic AMP production (Fig. 6B) and adenylate cyclase activity (Fig. 6C). As rat lymphocytes
have significant amounts of GM1 (24) and toxin receptors (~40,000 per cell from Fig. 6A), the small effect on toxin action by exogenous GM1 was not unexpected. To block the endogenous receptors, we incubated the cells with the B subunit of cholera toxin and then exposed the cells to GM1 (25). Now the binding of 125I-cholera toxin increased over nine-fold and cyclic AMP production and adenylate cyclase activity were enhanced substantially over that observed in cells treated with B subunit but not with GM1 (Fig. 6). Of particular interest was the reduction in the lag time for activation of the cyclase, which had been previously correlated with functional insertion of GM1 into other cells (10).

DISCUSSION

We have developed a mild procedure to introduce fluorescent groups into gangliosides (15, 16), which does not alter the ceramide moiety. Although the oligosaccharide chains have been modified, they appear to be able to interact with a number of ganglioside-binding proteins. The synthesis of dansyl- (27) and fluorescein-labeled (28) GM1 analogs, which have only one hydrocarbon chain compared with the two found in native gangliosides, have been described. Such a modification, however, may alter the anchorage and mobility of the gangliosides in the lipid bilayer.

By using our fluorescent gangliosides, we were able to directly follow the movement of gangliosides on the surface of thymocytes. Initially, the incorporated gangliosides were evenly distributed on the cell surface. When cells containing Rh-gangliosides were treated with antirhodamine antibodies, the Rh-gangliosides became redistributed into patches and caps. By using a second fluorescent indicator specific for the antibody, we were able to show that both the gangliosides and the antibodies moved in concert during the capping process. We estimated that the incorporation of Rh-gangliosides was around 10^6 molecules per thymocyte. Inasmuch as rat and mouse thymocytes have 6–11 × 10^6 molecules of gangliosides per cell (24, 29), the ganglioside content has been increased only 9–17%.

The exogenous gangliosides appeared to be inserted in the lipid bilayer and not just adsorbed to trypsin-sensitive membrane components as has been suggested in other studies (30, 31). Treatment of the lymphocytes containing Rh-gangliosides with trypsin did not prevent antirhodamine-induced capping. In contrast, cholera toxin-induced capping of a dansyl-labeled GM1 analog was reported to be inhibited when human lymphocytes were treated with trypsin (27). We also found that exogenous GM1 enhanced the responsiveness of the lymphocytes to cholera toxin. Similar results had been observed with a wide variety of other cell types (4, 5, 10, 23, 25). Tritium-labeled gangliosides taken up by cultured cells were shown to be trypsin-resistant, functional, and metabolically active (5, 10, 32, 33). More recent studies with spin-labeled gangliosides indicated that the lipid moiety was inserted into the lipid bilayer of the plasma membrane (34). Finally, fluorescent gangliosides taken up by sparse human fibroblasts were found to be completely mobile in the plane

2 As indicated in Results, cholera toxin still recognized LY-GM1. We also found that LY-GM1 was as effective as GM1 in enhancing the response of GM1-deficient rat glioma C6 cells to cholera toxin. Thus, LY-GM1 behaves as a functional receptor for the toxin. The fluorescent ganglioside derivatives also retained their ability to interact with fibronectin (26) and tetanus toxin (unpublished observations).
of the plasma membrane with a diffusion coefficient of $10^{-8}$ cm$^2$/s (35).

Several groups have described the capping of cholera toxin receptors (11–13). Because the toxin is highly specific for GM1 (4), these latter studies as well as our own indicate that the endogenous ganglioside undergoes patching and capping as detected by indirect immunofluorescence. We also were able to observe the capping of inserted LY-GM1 by cholera toxin and thus directly demonstrate the coordinate movement of the ganglioside and the toxin and conclude that GM1 itself is capped—a conclusion that was not strictly possible using the indirect methods.

Although there have been many reports that multivalent ligands induced the redistribution and capping of monovalent glycosphingolipids on the cell surface (6, 7, 11–14, 27, 36), the underlying basis for this phenomenon is unclear. In the studies involving indirect immunofluorescence, it is always possible that the ligand is cross-reacting with glycoproteins carrying similar carbohydrate determinants. Our studies using fluorescent gangliosides eliminate this possibility as the anti-ganglioside antibodies can only bind to the inserted Rh-gangliosides.

A second possibility is one of the various membrane flow models (36–39). Bretcher proposed that capping reflects the net movement of the cell membrane towards a pole that carried with it aggregates of the receptors large enough to resist counter-diffusion by Brownian motion (37). Directed movement may be induced by progressive waves on the surface of motile cells; large particles would be carried along more readily than smaller ones (38). Oliver and Berlin have suggested that membrane–microfilament interactions are important for the translocation of ligand–receptor complexes not through a direct microfilament–receptor link but rather by a mechanism which is propagated over the cell surface as a wave (39).

A third possibility is that the gangliosides are associated with transmembrane proteins which in turn interact with the cytoskeleton. Substantial amounts of gangliosides remained associated with the cytoskeleton after detergent extraction (40) and cholera toxin–GM1 complexes were resistant to detergent extraction and also remained associated with the cytoskeleton (40–42). The ability of cytoskeletal disrupting agents to inhibit cholera toxin-induced capping (11–13) and the ability to demonstrate co-capping of the toxin and $\alpha$-actinin (13) are consistent with such a mechanism.

Alternatively, gangliosides may self-associate to form microdomains in the lipid bilayer. There is evidence from electron spin resonance studies that the spin-labeled oligosaccharide chains of gangliosides exhibited a tendency toward cooperative interaction in a phospholipid bilayer (43, 44) and spin-labeled cerebrosides and ceramides inserted into cells showed spin–spin interactions indicating that they were intercalating into restricted regions of the membrane (45). Nuclear magnetic resonance studies with cerebrosides suggest that hydrogen bonding between sphingolipids occurred to a significant extent in membranes (46, 47). Differential scanning calorimetry experiments also indicated that the oligosaccharide chains of gangliosides undergo mutual cooperative interactions (48, 49). When lymphocytes containing inserted biotinylated gangliosides were fixed and stained with ferritin-conjugated aavid, clustering of the electron-dense marker along the plasma membrane was observed (50). Finally, by using the freeze-etch technique, Tillack et al. found that ganglioside and Forsman glycolipids were present in small clusters in the outer leaflet of erythrocyte membranes (51). There was no apparent relationship between the glycolipid clusters and the underlying intramembranous particles that represent transmembrane proteins. The glycolipid clusters also did not correspond to aggregates of siglalglycoproteins, which in turn did correspond closely to the intramembranous particles.

In the present study, we observed that antirhodamine antibodies induced co-capping of Rh- and LY-gangliosides inserted into lymphocytes. Similarly, cholera toxin caused the capping of LY-GM3 and LY-GD1a, which are not toxin receptors (4). The ganglioside–ganglioside interaction appears to be specific because capping of the $\theta$-1.2 antigen by a monoclonal antibody did not induce redistribution of inserted LY-gangliosides. In addition, capping of Rh-gangliosides by antirhodamine antibodies did not cause redistribution of $\theta$-1.2 antigen or concanaval A receptors. If, as our data suggest, gangliosides exist in the plasma membrane as clusters, then binding of a multivalent ligand (antibody or toxin) would result in extensive cross-linking of the clusters, which in turn could coalesce to form visible patches and caps.

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REFERENCES


$^3$ Cholera toxin was shown to bind LY-GM1 but not to other LY-gangliosides. This is consistent with all of the GM1 derivative co-capping with the toxin but not all of the GM3 and GD1a derivatives redistributing into the same cap as the toxin.


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