*Biochimica et Biophysica Acta*, 687 (1982) 27–34
Elsevier Biomedical Press



BBA 71096

# A NOVEL APPROACH FOR THE TOPOGRAPHICAL LOCALIZATION OF GLYCOLIPIDS ON THE CELL SURFACE

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(Received May 19th, 1981)

*Key words. Glycolipid localization, Topographical distribution, Biotin labeling, Carbohydrate moiety, (Bovine brain)*

**In this study we have developed a prototype system for distinguishing between the topographical distribution of glycolipids versus glycoproteins on the ultrastructural level. Direct modification of membrane-based sialic acids with biotin groups labels both glycolipids and glycoproteins. In this case, subsequent ultrastructural localization of biotinylated sites would not discern between these two classes of glycoconjugate in an unambiguous manner. When biotinylated cells are fixed prior to interaction with ferritin-conjugated avidin, the mean distance of marker molecules from the membrane bilayer is 8.0 nm. In contrast, if the cells are allowed to cap through the action of ferritin-avidin conjugates on unfixed cells, the average distance (13.0 nm) of the marker molecules appears even more distant from the membrane on the capped portion of the cell (uropod), whereas those on the head region are positioned in close proximity to the bilayer (3.7 nm). In order to exclusively label cell surface glycolipids on the ultrastructural level, bovine brain gangliosides were biotinylated in vitro and the haptenized gangliosides were incorporated into intact cells. In this case, marker molecules denoting the incorporated gangliosides were found in relatively close juxtaposition to the membrane surface, in a manner strikingly similar to the labeling pattern of the head region on capped cells. These results support the concept that, in the native state, the carbohydrate portion of glycolipids is positioned closer to the membrane bilayer than that of glycoproteins.**

## Introduction

Glycolipids and glycoproteins are major constituents of the cell surface membrane. Both presumably participate either alone or in concert in various biological processes necessary for normal cell function [1]. Due to the structural similarity of the carbohydrate portion, especially the terminal saccharides, on the different carrier molecules, it is difficult to differentiate between the two species of glycoconjugates with regard to the corresponding biological role(s) and respective topographical location on the cell surface. It is interesting to note that most schematic illustrations (see, for example, Refs. 2 and 3), depicting glycolipids and glycoproteins, show the former in closer juxtaposition to the membrane bilayer without any factual evidence to support this claim.

In previous studies we have shown that the chemical modification of membrane based sialic acids with various haptenic groups labels both glycolipids and glycoproteins with concomitant preservation of mitogenic activity [4–6]. Furthermore, haptenized-glycolipids can also be incorporated into cell surface membranes [7], and mitogenic signals can be transmitted intracellularly through the action of these exogenously incorporated molecules [7].

It is clear that the systematic evaluation of the location, distribution and dynamics of these two

kinds of membrane-bound glycoconjugates is a necessary prerequisite for understanding mitogenic interactions and the mechanism(s) by which mitogens induce transformation. These mitogenic systems described above provide a unique opportunity for the ultrastructural visualization of various surface constituents and, specifically, to distinguish between the topographical distribution of glycolipids and glycoproteins. The biotin molecule can be selectively implanted onto membrane-based saccharides, in particular terminal sialyl [8,9] or galactosyl [10,11] sites, for the purpose of electron microscopic analysis.

In this study we describe the modification of lymphocyte membranes with biotin hydrazide and its subsequent localization on cell surfaces via ferritin-avidin conjugates. The distribution of biotinylated compounds is compared with that of glycolipids which have been biotinylated in vitro and subsequently incorporated into the lymphocyte membrane. Our results support the intuitive consensus that the sugar residues residing on glycolipids are in closer proximity to the lipid bilayer than those of glycoproteins.

## Materials and Methods

*Chemicals and biochemicals.* Sodium periodate and potassium borohydride, analytical quality reagents, were obtained from BDH Chemicals Ltd. Highly purified, bovine brain gangliosides were obtained from K.N. Pharmaceutical Inc., Cleveland, OH. Avidin was purchased from Sigma Chem. Co., St. Louis, MO. Biotin hydrazide was synthesized as described previously [10], and ferritin-avidin conjugates were prepared by reductive alkylation [12].

*Biotinylation of gangliosides.* Biotin-modified bovine brain gangliosides were prepared via the same reaction pathway described for dinitrophenylation of gangliosides [7]: Bovine brain gangliosides (1 mg), suspended in 1 ml phosphate buffered saline, pH 7.2, were treated with sodium periodate (2 mM) for 30 min in ice. The reaction mixture was dialyzed extensively against the buffer; biotin hydrazide (10 mM) and $MnCl_2$ (1 mM) were then added. After incubation for 30 min at 37°C the modified gangliosides were dialyzed and reduced with potassium borohydride (1 mM) for 10 min at room temperature and extensively dialyzed. Alternatively, the modified gangliosides were applied to a calibrated column of Sephadex G-25 for separation of the biotinyl gangliosides (void volume fraction) from the excess of free reagents. Thin-layer chromatography was performed on precoated plates (0.2 mm layer thickness) of DC-Alufolien Kieselgel 60 (Merck, Darmstadt). Plates were developed with chloroform/methanol/water (65:25:4, v/v) and biotinyl gangliosides were visualized with dimethylaminocinnamaldehyde spray [13]. Under these conditions the migration rate of the haptenized gangliosides was not significantly altered from that of native gangliosides. In addition, biotinylated gangliosides were still capable of reacting with resorcinol [14].

*Incorporation of biotin-tagged gangliosides into thymocytes.* Lymphocytes were obtained from thymuses of hydrocortisone-treated rats as described previously [7]. Mature thymocytes ($10^8$/ml), suspended in Dulbecco's modified Eagle's medium supplemented with 0.2 mg/ml biotinylated gangliosides, were incubated for 2 h at 37°C in a humidified incubator (5% $CO_2$-air mixture). After incubation, cells were washed and resuspended in phosphate-buffered saline, pH 7.2.

*Direct biotinylation of thymocytes.* Mature rat thymocytes were biotinylated as described earlier [4]: Briefly, cells were oxidized with 1 mM periodate for 30 min in ice, centrifuged, washed, and treated with a solution containing 10 mM biotin hydrazide and 1 mM manganese chloride. The biotin-modified cells were centrifuged, washed and reduced with 1 mM borohydride for 10 min at room temperature.

*Aldehyde-fixation of cells.* Fixation of cells was accomplished by adding a given cell suspension to an equal volume of Karnovsky's fixative [15] containing 1% formaldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After 30 min at 25°C, the cells were washed three times with buffer.

*Affinity cytochemistry.* A sample (0.5 ml) of aldehyde-fixed, biotin-modified lymphocytes was conditioned with 5% bovine serum albumin (0.5 ml) for 10 min and washed twice with phosphate-buffered saline. The pellet was resuspended in a solution containing ferritin-avidin conjugates (1 mg ferritin/ml). The cell suspension was incubated at

room temperature for 30 min, washed twice and resuspended in 0.5 ml phosphate-buffered saline. An equal volume of Karnovsky's fixative was added, and the cells were then processed further for electron microscopy.

In order to study the redistribution of biotinyl residues on viable cells, a fresh, biotin-modified cell suspension was resuspended in a solution containing ferritin-avidin conjugates (1 mg/ml ferritin). The cell suspension was incubated at 37°C for varying time intervals, centrifuged, washed and fixed with aldehydes.

*Processing for electron microscopy.* Aldehyde-fixed cells were postfixed in 1% $OsO_4$ for 1 h at 4°C, washed twice with distilled water and dehydrated in graded ethanol solutions (50%, 75%, 90%, 96% and 100%). The dehydrated cells were embedded in Epon [16], and sections approx. 600 Å thick were obtained with an MT-2 Sorval microtome. The sections were mounted on naked 400-mesh copper grids and coated with carbon before viewing in a Jeol 100-B electron microscope at 80 kV.

*Calibration of ferritin-membrane interspace.* Micrographs of a given cell type were enlarged a million-fold, and portions of perpendicularly sectioned membrane were chosen, such that the two opposing electron-dense sides of the membrane bilayer were clearly distinguishable. The distance of individual particles of the ferritin core from the outer dense line of the membrane bilayer was measured with a Vernier calliper to the nearest millimeter, and, according to the final magnification, this value was directly converted to nanometers. In this manner, the ferritin-membrane interspace was determined for at least 20 labeled cells of each type. The results were graphed in histogram form and the mean and standard deviation of the ferritin-membrane interspace were computed.

## Results

In recent studies we have shown that either avidin or ferritin-avidin conjugates stimulate lymphocytes which have been modified with periodate and biotin hydrazide [4,12]. It was of further interest to determine the ultrastructural distribution of the biotin-tagged sites on the cell surface using the affinity cytochemical methods previously developed in our laboratory [8,9,17–20]. Accordingly, when biotinylated thymocytes were fixed with aldehydes prior to incubation with ferritin-conjugated avidin, the resultant cell surface was characterized by an even distribution of ferritin particles (Fig. 1). Most of the marker molecules were located at a distance ranging between 40 to 120 Å from the outer dense line of the membrane.

In order to investigate the mobility and redistribution of biotinylated sites during avidin-induced blastogenesis, viable biotin-modified, mature thymocytes were treated with ferritin-avidin conjugates prior to fixation with aldehydes. Fixation was then performed after varying time periods of incubation at 37°C. Under these conditions, it was found that after about 30 min the majority of cells exhibited capping of sialic acid sites (Fig. 2).

Distinct morphological alterations were evident in capped cells. A polar swelling of the cytoplasm into a uropod with an accumulation of intracellular organelles, e.g. mitochondria and Golgi complexes, accompanied the capping process. The nucleus displayed a typical invagination in the region facing the swollen zone.

The uropod (Fig. 2a) was characterized by a dense multilayer of ferritin particles situated relatively distant from the membrane bilayer, whereas the head region (Fig. 2b) was sparsely labeled with ferritin molecules set in close proximity to the plane of membrane. The topographical difference in the ferritin-membrane interspace between the head and uropod regions may reflect a segregation of glycoconjugate species during the capping process; that is, the residual ferritin particles in the head region could represent glycolipids which fail to redistribute to the cap, whereas the relatively distant marker molecules in the uropod could denote, for the most part, sialoglycoproteins.

In order to test this premise, we employed the following strategy: Bovine brain gangliosides were biotinylated via the same procedure used for intact thymocytes, and the modified glycolipids were subsequently reincorporated into unmodified cells. Incubation with ferritin-conjugated avidin was then performed, and these cells were fixed at time intervals in a manner similar to that described for cells which underwent direct biotinylation. Visualization of the ferritin particles attached to exogenously incorporated gangliosides (Fig. 3) revealed

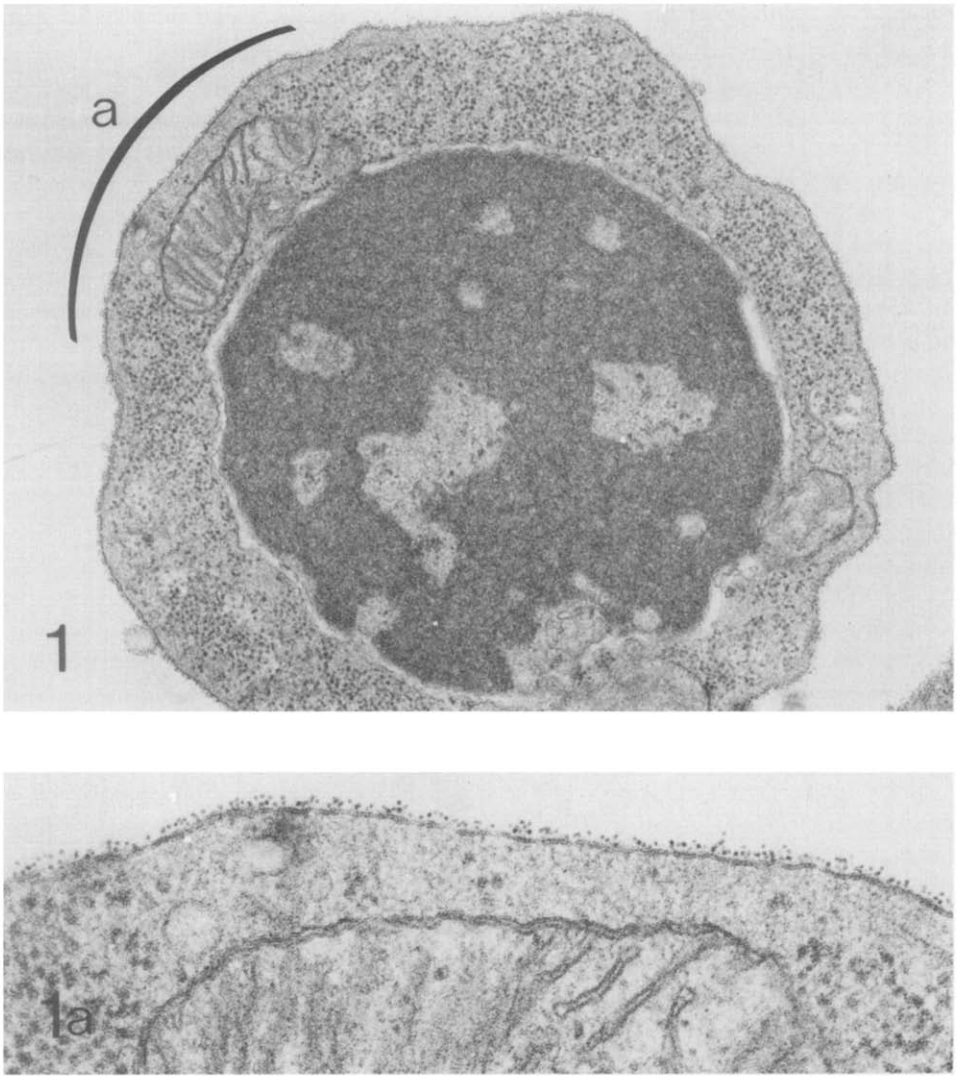


Fig 1 Mature rat thymocyte, treated successively with sodium periodate and biotin hydrazide, fixed with aldehydes and labeled with ferritin-avidin conjugates Note the even distribution pattern of ferritin particles over the entire cell surface Magnification. ×10000 (a) Higher magnification of designated area in Fig 1 Note the relatively uniform distance separating the ferritin core from the outer surface of the membrane Magnification. ×75000

an even distribution at close proximity which was similar to the labeling pattern of the head region in Fig. 2b. No capping of the cells bearing exogenously incorporated gangliosides could be detected under conditions which caused capping in directly biotinylated cells.

The distribution of ferritin particles on the above cell types or portions thereof was further evaluated by statistical analysis. The data are presented in histogram form in Fig. 4 and summarized in Table I. The results confirm the observation described above that (a) capping process is accompanied by a marked reorganization in membrane topography; (b) in the uropod region, most of the sialoglycoconjugates are positioned at extended distances from the membrane bilayer; (c) the topographical labeling pattern of the head region of capped cells is highly reminiscent of that

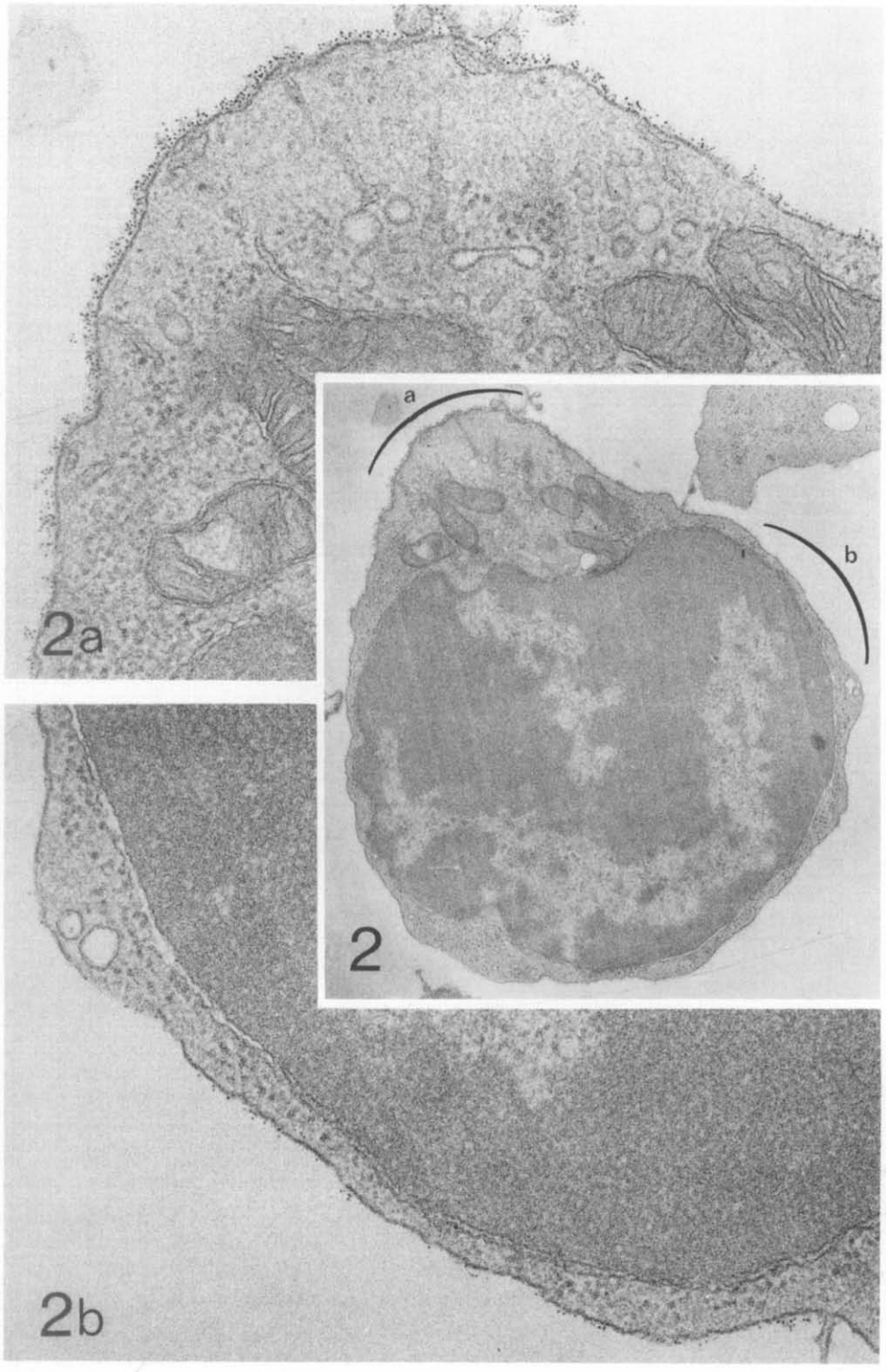


Fig 2 Mature rat thymocyte, treated successively with periodate and biotin hydrazide and labeled with ferritin-avidin conjugates Following a 30 min incubation period at 37°C, aldehyde-fixation was carried out Note the difference both in labeling density and ferritin-membrane interspace between the uropod (a) and head (b) regions (Magnification: ×5000) Inserts a and b, magnification ×60000

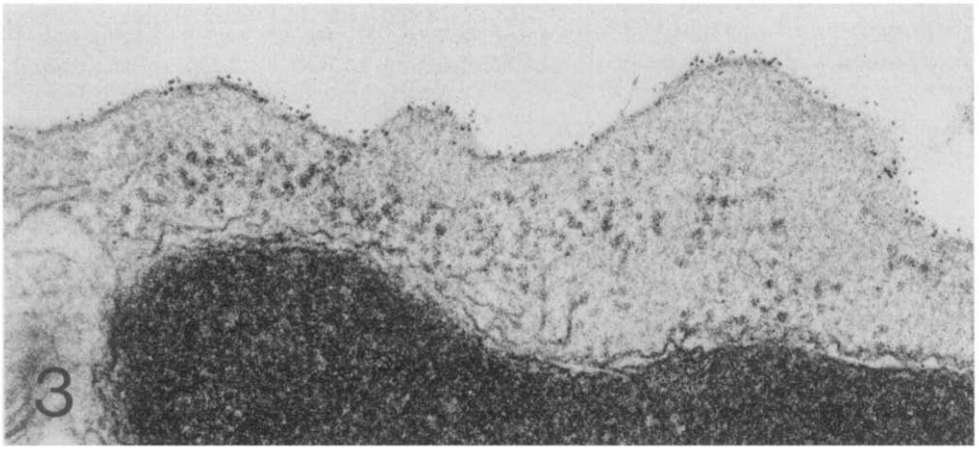


Fig. 3 Mature rat thymocyte bearing exogenously incorporated biotin-gangliosides, fixed with aldehydes and labeled with ferritin-conjugated avidin Note the relatively-close proximity of ferritin particles to the membrane surface Magnification ×60000

of cell membranes which contain exogenously incorporated gangliosides. The large standard deviations obtained may reflect an intrinsic part of membrane organization (multiple sialyl sites at varying distances from the bilayer). Alternatively, at such high magnification, the level of resolution afforded by the labeling procedure may be approaching the limits of the system.

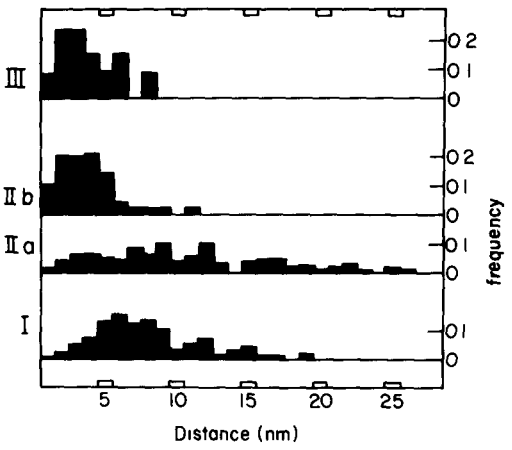


Fig 4 Histogram summarizing the distance between ferritin core particles and the outer dense line of the membrane surface of various cell types: I, aldehyde-fixed cells, II, capped cells. a, uropod region and b, head region; III, cells containing exogenously incorporated biotinyl gangliosides Ordinate frequency in percentage. Abscissa: distance (in nm) between the ferritin core and the membrane bilayer

TABLE I

AVERAGE DISTANCE OF FERRITIN-MEMBRANE INTERSPACE ON VARIOUS CELL TYPES

| Cell type | Mean ± S D (nm) |
|---|---|
| Aldehyde-fixed cells | 8 0 ± 3 5 |
| Capped cells | |
| Uropod region | 13 0 ± 11 3 |
| Head region | 3 7 ± 2 0 |
| Biotinyl ganglioside-incorporated cells | 3 8 ± 2 0 |

## Discussion

In recent studies, it was shown by various groups that glycolipids can be functionally incorporated into the surface membrane of various cell types. For example, $G_{M1}$ was incorporated in $G_{M1}$-deficient human leukemic cells, and cholera toxin was capable of causing patching and capping of the exogenously incorporated gangliosides [21]. In another study [22], the Forssman antigen, a defined glycolipid, was incorporated into various cells which lack this antigen. Subsequent monoclonal antibody-induced capping of the exogenously incorporated antigen could then be demonstrated.

These studies were performed in order to demonstrate that the incorporated gangliosides ex-

hibited biological activity as expressed by patching and capping. In a more biologically significant extension of this approach, we have incorporated haptenized gangliosides, modified via their sialyl moieties, into thymocytes membranes and have shown further that these lymphocytes, containing the exogenously incorporated molecules, can be stimulated by multivalent anti-hapten probes [7]. From all these studies, it is apparent that the gangliosides are functionally incorporated into their native states in the target cell membrane. Our use of exogenously incorporated biotinyl-gangliosides in the present study as a model system for topographical analysis of native glycolipids in the cell membrane is therefore justified.

Since the distance of the ferritin-membrane interspace in thymocytes containing exogenously incorporated gangliosides is similar to that of the head region of capped thymocytes (Table I and Fig. 4), we may therefore consider the following: (a) the carbohydrate portion of gangliosides is in close proximity to the membrane bilayer, and (b) in the capped lymphocyte, the extent of lateral mobility and consequent involvement in the capping process are apparently less for glycolipids than for glycoproteins.

It is interesting to note that in the present study some of the exogenously incorporated sites (Fig. 3) also appear in clusters or patches, albeit smaller than those described for the incorporated Forssman antigens [22]. This is not particularly surprising, since in the latter case a double-antibody (sandwich) technique was employed which would increase both the extent of crosslinking as well as the distance of marker molecules from the membrane bilayer.

We have recently shown [23] that the association of biotinylated phospholipids with the membrane surface is dependent upon the nature of the carrier lipid. In some cases biotinylated lipids may be simply adsorbed as intact liposomes onto the membrane, while other classes of biotinylated lipids are fully incorporated into the membrane, first as clusters and then as single marker sites.

The data currently available cannot unequivocally solve the problem of the distance of native gangliosides. Direct proof may result only upon interaction with electron-dense markers, e.g. ferritin-conjugated cholera toxin or interferon, which specifically and exclusively interact with native endogenous gangliosides without extraneous cross-reactivity with glycoproteins arising from overlapping carbohydrate structure. This approach would be valid provided that it is proved by independent means, e.g. isolation by affinity chromatography, that the glycolipid-specific probe fails to interact with other species of membrane glycoconjugate.

Another phenomenon worthy of comment concerns our own failure to demonstrate capping on the ultrastructural level with exogenously incorporated gangliosides, even though studies do exist (including our own) wherein parallel fluorescent affinity probes successfully induced capping detectable by fluorescent microscopy. The reason for this apparent discrepancy may be that the increased size of the ferritin-avidin conjugate (at least 10-fold) over that of native or fluorescent conjugated avidin may obstruct the mobility of the biotinyl gangliosides in the membrane. The flow of such massive complexes, which comprise large crosslinked protein conjugates implanted in the membrane by virtue of the delicate interactions of the glycolipid support, may be further impeded by the presence of endogenous membrane glycoproteins, which fail in this case to participate.

## Acknowledgements

This research was supported by a grant from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel. One of us (E.A. B.) is supported by a National Institutes of Health Public Health Service award No. F32-ES5120.

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