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BINDING OF CHOLERAGEN AND ANTI-GANGLIOSIDE ANTIBODIES TO GANGLIOSIDES INCORPORATED INTO PREFORMED LIPOSOMES

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Exogenously added gangliosides were taken up and incorporated into liposomes just as they are incorporated into cells. Ganglioside G_{M1} was rapidly taken up by liposomes containing dimyristoyl- or dipalmitoylphosphatidylcholine, cholesterol and dicetyl phosphate. When incubated with a wide range of G_{M1} concentrations for 18 h, the liposomes incorporated about 10% of the added ganglioside. The rate of G_{M1} uptake by preformed liposomes was both time- and temperature-dependent. The liposomes also incorporated other gangliosides to a similar extent. The G_{M1} taken up by preformed liposomes was predominantly located on the outer surface of the liposomes and did not appear to be internalized into the inner half of the lipid bilayer. Liposomes containing G_{M1} added after liposome formation bound as many anti-G_{M1} antibodies and as much choleragen as liposomes having G_{M1} added during the formation of the lipid bilayers. Thus, preformed liposomes sensitized by incubation with G_{M1} are a good model system for studying the interactions of antibodies and toxins with membrane-associated gangliosides.

Introduction

Cells and cell membrane preparations readily incorporate exogenously added gangliosides into the membranes. Studies with spin-labeled gangliosides suggest that the gangliosides are incorporated primarily into the plasma membranes [1]. Gangliosides that are taken up by erythrocytes are exposed on the cell surface and thus are available for binding of anti-ganglioside antibodies [2] and choleragen [3]. Gangliosides also can be taken up by cultured cells [4–8]. In cells deficient in G_{M1}, the ganglioside appears to become functionally incorporated into the plasma membrane; G_{M1}-treated cells bind more choleragen [5,7,8] and exhibit an increased responsiveness to the toxin [4,7,8].

Liposomes are a useful model membrane system for studying interactions of proteins with membrane-bound glycolipids [9–11]. Binding of choleragen to G_{M1} in liposomes appears to be the same as to G_{M1} in cells [11]. Interactions of antibodies with glycolipid antigens have been studied using liposomes [9,10]. Liposomes containing glycolipids also have been used in studies on lectin binding to glycolipids [12,13].

As observed with cells, gangliosides also can be incorporated into preformed liposomes [14–18].

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; G_{D1a}, AcNeuα2 → 3Galβ1 → 3GalNAcβ1 → 4Gal[3 ← 2αAcNeu]β1 → 4Glcβ1 → ceramide; G_{D1b}, Galβ1 → 3GalNAcβ1 → 4Gal[3 ← 2αAcNeu8 ← 2αAcNeu]β1 → 4Glcβ1 → ceramide; G_{M1}, Galβ1 → 3GalNAcβ1 → 4Gal[3 ← 2αAcNeu]β1 → 4Glcβ1 → ceramide; G_{M2}, GalNAcβ1 → 4Gal[3 ← 2αAcNeu]β1 → 4Glcβ1 → ceramide; G_{M3}, AcNeuα2 → 3Galβ1 → 4Glcβ1 → ceramide.

Preliminary reports showed that such exogenously added gangliosides are functional, as determined by binding of anti-ganglioside antibodies [15] and choleragen [15,16]. We report here a detailed study of the ability of gangliosides incorporated into preformed liposomes to function as membrane-bound receptors for binding of antibodies and choleragen.

Materials and Methods

DMPC, DPPC, cholesterol and *Vibrio cholerae* neuraminidase were purchased from Calbiochem-Behring, La Jolla, CA, or from Sigma Chemical Co., St. Louis, MO. Gangliosides were isolated and purified as described previously [19]. Choleragen was purchased from Schwarz/Mann, Spring Valley, NY.

Previous publications should be consulted for complete details on the following: preparation of liposomes, measurement of trapped liposomal glucose, complement-dependent release of trapped glucose, and preparation of fresh human serum as a complement source [20,21]; preparation of rabbit anti-mixed ganglioside serum (heated at 56°C for 30 min to inactivate complement) [21]; preparation of ^{125}I-labeled choleragen and measurement of binding of ^{125}I-labeled choleragen to liposomes [11]; and analysis of gangliosides by thin-layer chromatography [19].

The liposomes used for these studies were composed of DMPC (or DPPC), cholesterol and dicetyl phosphate, in molar ratios of 1.0 : 0.75 : 0.11. The phospholipid was 10 mM with respect to the aqueous swelling solution. Liposomes for use in the glucose release assay were swollen in 0.308 M glucose; all other liposomes were swollen in 0.154 M NaCl. The G_{M1} used for incubation with liposomes was solubilized in 0.154 M NaCl at concentrations that were always above the reported critical micelle concentration [22].

Results

Uptake of ganglioside G_{M1} by liposomes

Preformed liposomes readily incorporated ganglioside G_{M1}. The amount of [^{3}H]G_{M1} taken up by preformed liposomes after incubation with G_{M1} for 24 h at 25°C was proportional to the amount of G_{M1} added to the liposomes (Fig. 1). Over a wide range of G_{M1} concentrations, only 10% of the added G_{M1} was incorporated into the liposomes after 24 h incubation (Fig. 1). The highest level of G_{M1} incorporation corresponded

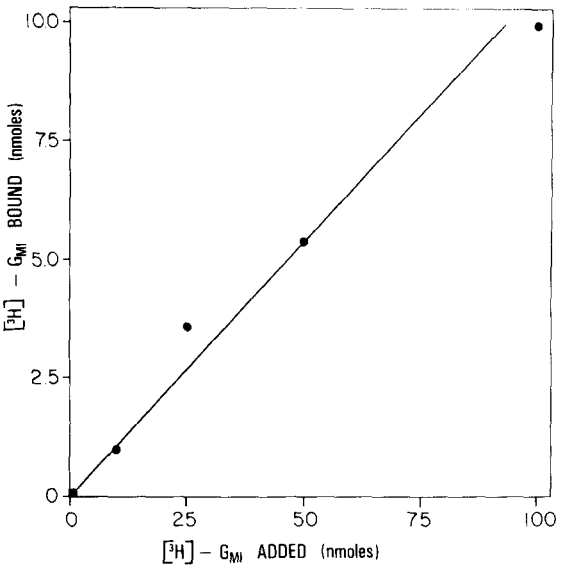

Fig. 1. Uptake of G_{M1} by preformed liposomes. Aliquots (50 μl) of liposomes were incubated with different amounts of [^{3}H]G_{M1} (1000 cpm/nmol) in 0.154 M NaCl in a total volume of 200 μl for 24 h at 25°C. The liposome/ganglioside mixtures were centrifuged at 35000 × g for 10 min. The liposome pellets obtained after removal of the supernatant were resuspended in 150 μl of 0.154 M NaCl and centrifuged again. Uptake of G_{M1} was determined by the ^{3}H recovered in the liposome pellets.

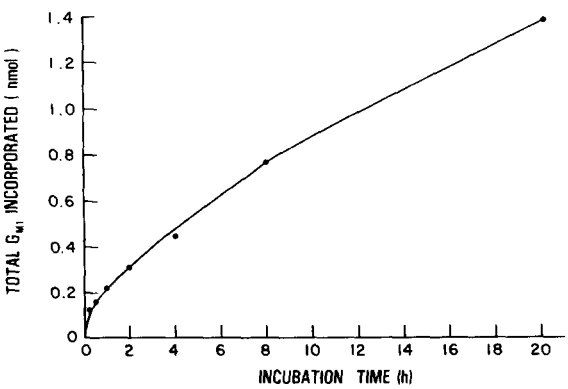

Fig. 2. Time-course of G_{M1} uptake by preformed liposomes. Aliquots of preformed liposomes (20 μl) were each incubated with 10 nmol [^{3}H]G_{M1} (20000 cpm) in 100 μl 0.154 M NaCl for various times at 25°C. Samples were centrifuged, washed and assayed for [^{3}H]G_{M1} as described in Fig. 1.

to approx. 2 mol%, when compared to the liposomal phospholipid.

The rate of G_{M1} uptake by the liposomes was initially rapid, then slowed to a smaller, but still measurable rate (Fig. 2). The continuing slow rate of uptake after 20 h of incubation suggests that uptake may be difficult to saturate. Uptake of ganglioside by preformed liposomes was temperature-dependent. Incubation of liposomes with G_{M1} at 45°C resulted in greater uptake of G_{M1} than did incubation at 25°C, particularly at higher G_{M1} concentrations (data not shown). Similar levels of uptake were observed when gangliosides other than G_{M1} were incubated with preformed liposomes (Table I). The hydrophobic portion of the ganglioside molecule appeared to be required, since uptake of the oligosaccharide portion of G_{M1} was negligible.

Binding of choleragen and antibodies by liposomal G_{M1}

The G_{M1} taken up by preformed liposomes appeared to be functionally as active as G_{M1} that was present during the formation of the liposomes. Table II shows that binding of either rabbit anti-G_{M1} serum, or choleragen plus anti-toxin, resulted in substantial complement-dependent release of trapped glucose from preformed liposomes which had been incubated with G_{M1}. This glucose release was comparable to that obtained with liposomes prepared with G_{M1} present during swelling (Ref. 21, Table 2). No significant glucose release was observed from liposomes incubated under the same conditions in the absence of G_{M1}.

Preformed liposomes, containing either DMPC or DPPC as phospholipid, were incubated with

TABLE I

UPTAKE OF VARIOUS GANGLIOSIDES BY PREFORMED LIPOSOMES

Liposomes (20-μl aliquots) were incubated with 10 nmol of the indicated gangliosides in 100 μl 0.15 M NaCl for 20 h at 25°C. Samples were centrifuged, washed and assayed as in Fig. 1. Oligo-G_{M1}, oligosaccharide portion of G_{M1}.

Ganglioside added	Ganglioside uptake (nmol)
[^{3}H]G_{M3}	1.15
[^{14}C]G_{M2}	1.24
[^{3}H]G_{M1}	0.72
[^{3}H]G_{D1a}	1.50
[^{3}H]G_{D1b}	0.95
[^{3}H]oligo-G_{M1}	0.01

TABLE II

COMPLEMENT-DEPENDENT GLUCOSE RELEASE IN THE PRESENCE OF ANTI-G_{M1} OR CHOLERAGEN PLUS ANTI-CHOLERAGEN FROM LIPOSOMES SENSITIZED WITH G_{M1}

Liposomes (100 μl) were incubated for 15 min at 25°C in the presence or absence of 125 nmol G_{M1}. The liposomes were centrifuged as described in Table I, resuspended in 100 μl 0.15 M NaCl, and dialyzed for 1 h vs. 0.15 M NaCl. Functional activity of the G_{M1} incorporated by the liposomes was assayed by complement-dependent glucose release using either horse anti-choleragen (anti-CT) serum in the presence and absence of choleragen (CT) or rabbit anti-G_{M1} serum. Complement-dependent glucose release was determined using 500 μl glucose assay reagent, sufficient 0.15 M NaCl to give a final volume of 1.0 ml, 5 μl of liposomes, either 25 μl anti-choleragen serum plus, where indicated, 12.5 μg choleragen, or 75 μl of anti-G_{M1} serum and 120 μl fresh human serum as a complement source. Control assays lacked antibody and choleragen. Glucose release with choleragen and complement, but no antibody, was approximately the same as with complement alone. Additional details concerning the glucose release procedure with choleragen and anti-choleragen can be found in Ref. 21.

Addition	% of trapped glucose released	
	$+G_{M1}$	$-G_{M1}$
Anti-G_{M1}	25.4	5.7
CT + anti-CT	56.9	2.8
Anti-CT	3.4	3.5
None (complement alone)	0.8	1.8

TABLE III

STABILITY OF CHOLERAGEN RECEPTOR ACTIVITY OF G_{M1} ADDED TO PREFORMED LIPOSOMES

Liposomes were incubated with [^{3}H]G_{M1} (100 nmol/ml of liposomes) for 2 h at 25°C and then washed as described in Fig. 1. The liposomes incorporated 5.2% of the added G_{M1}. Aliquots of the washed liposomes were incubated at the indicated temperatures for 24 h and then assayed for their ability to bind ^{125}I-labeled choleragen as described in the Materials and Methods section. The final toxin concentration was saturating at 2.5 nM (see Fig. 5).

Temperature (°C)	^{125}I-labeled choleragen bound (dpm)
4	66900 ± 2790
25	64900 ± 1150
37	71000 ± 1920

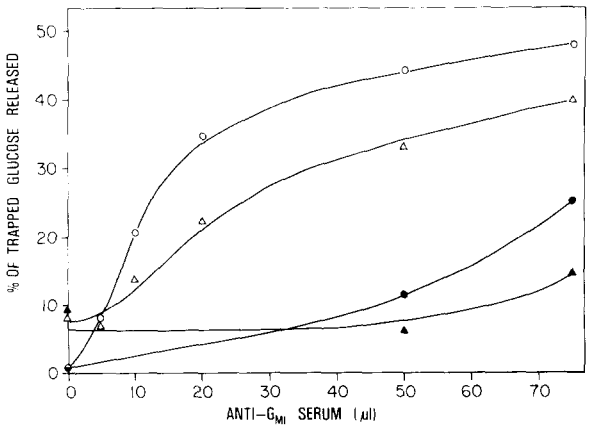

Fig. 3. Complement-dependent glucose release in the presence of anti-G_{M1} antibodies from preformed liposomes sensitized with G_{M1} by a 15 min incubation. Liposomes containing either DMPC (circles) or DPPC (triangles) as phospholipid were incubated, centrifuged and dialyzed as described in Table II. Incubation of liposomes with G_{M1} was performed at either 25 (filled symbols) or 45°C (open symbols). Complement-dependent glucose release was performed as described in Table II, except that varying amounts of anti-G_{M1} serum were used. Each curve represents a separate batch of liposomes. Liposomes incubated as above in the absence of G_{M1} gave less than 5% glucose release with the highest amount of anti-G_{M1} serum.

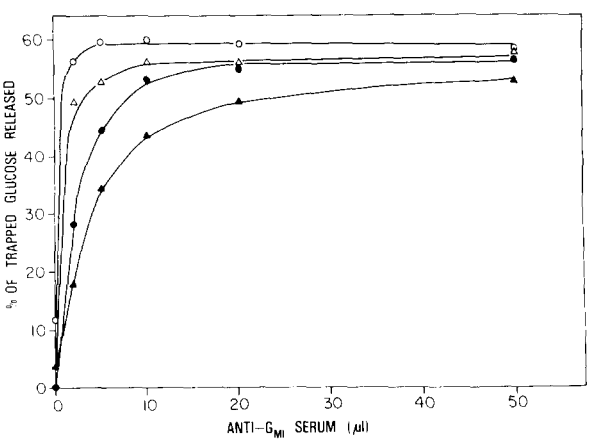

Fig. 4. Complement-dependent glucose release in the presence of anti-G_{M1} antibodies from preformed liposomes sensitized with G_{M1} by a 13 h incubation. ●, ○, DMPC; ▲, △, DPPC. This experiment was performed as in Fig. 3, except that the liposome preparations were incubated with G_{M1} for 13 h at 25 (filled symbols) or 45°C (open symbols). Liposomes incubated in the absence of G_{M1} gave less than 5% glucose release with the highest amount of anti-G_{M1} serum tested.

G_{M1} for 15 min at 25 and 45°C and then tested for their ability to bind anti-G_{M1} antibodies. As Fig. 3 shows, the antiserum titer was higher with the liposomes incubated at 45°C than with those incubated at 25°C. In addition, a higher titer was obtained when DMPC was the phospholipid.

Fig. 4 shows the results when the same experiment as in Fig. 3 was performed with liposomes incubated with G_{M1} for 13 h. The maximum glucose release obtained was higher than that obtained after a 15 min incubation, as would be expected from the data shown in Fig. 2. As with the 15 min incubation, DMPC liposomes gave higher glucose release than did DPPC liposomes.

The ability of G_{M1} taken up by preformed liposomes to bind choleragen also was tested (Fig.

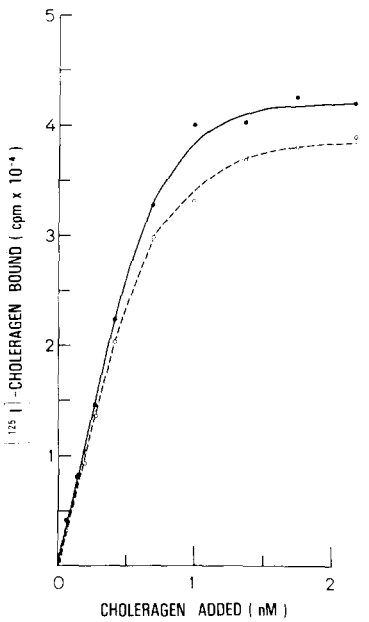

Fig. 5. Binding of ^{125}I-labeled choleragen to liposomes sensitized with G_{M1} either during (●———●) or after (○------○) formation of the liposomes. Liposomes were prepared either with 10 nmol G_{M1}/μmol DMPC present during the swelling process or were incubated after being swollen with 10 nmol G_{M1}/μmol DMPC for 22 h at 25°C. Aliquots of either liposome preparation were incubated with increasing concentrations of ^{125}I-labeled choleragen (590 cpm/fmol) in 0.2 ml of a buffer comprising 50 mM Tris-HCl (pH 7.4)/135 mM NaCl/1 mM EDTA/3 mM NaN_3/0.1% bovine serum albumin for 1 h at 25°C and filtered on 0.2 μm filters as described previously [11]. The ^{125}I-labeled choleragen bound at each point was corrected for nonspecific binding as determined by incubation of ^{125}I-labeled choleragen with liposomes lacking G_{M1}.

TABLE IV

DISTRIBUTION OF G_{D1a} INCORPORATED INTO PREFORMED LIPOSOMES

Liposomes were incubated with G_{D1a} (600 nmol/ml of liposomes) for the indicated times and temperatures and then washed as described in Fig. 1. Aliquots (50 μl) of the washed liposomes were incubated with 0.025 IU of *Vibrio cholerae* neuraminidase in 100 μl of buffer comprising 0.9% NaCl/0.1% $CaCl_2$/50 mM sodium acetate (pH 5.5) for 18 h at 37°C. Hydrolysis of G_{D1a} to G_{M1} was determined by thin-layer chromatography [19]. The data are the average of duplicate determinations, which varied less than 10%.

Time (h)	Temp. (°C)	Total G_{D1a} taken up		Neuraminidase-resistant G_{D1a}	
		pmol	%	pmol	%
3	37	1608	5.4	331	20.6
24	25	3635	12.1	610	16.8

5). Binding of choleragen either to preformed liposomes incubated with G_{M1}, or to liposomes prepared with G_{M1} present during swelling was virtually the same.

Stability of surface expression

We examined the degree to which the G_{M1} incorporated into preformed liposomes changed in its distribution in the liposomes. As seen in Table III, the ability to bind choleragen did not change when liposomes were incubated first with G_{M1} for 2 h, then in the absence of G_{M1} at higher or lower temperatures for 24 h. Similar stability of liposomal G_{M1} was observed for binding of anti-G_{M1} antibodies (data not shown).

We also determined the fraction of the G_{D1a} incorporated into preformed liposomes that was available to neuraminidase hydrolysis to G_{M1}. Table IV shows that, although the amount of G_{D1a} that was taken up increased during incubation, the percent of the total liposomal G_{D1a} that was neuraminidase-resistant did not change with time.

Discussion

Liposomes provide a useful model for studies on the interactions of various proteins, including antibodies, lectins and bacterial toxins, with membrane-bound glycolipids [11]. Both the binding and, in some cases, functional activities of proteins interacting with glycolipids can be determined by using liposomes. If the glycolipid were present during the formation of the liposomes, it would presumably be distributed throughout all the lamellae, and also would be present in both halves of the lipid bilayer [23]. In a cell membrane, glycolipids such as gangliosides are thought to be present only in the outer half of the plasma membrane bilayer [24–27]. Glycosphingolipids, including gangliosides, are known to alter the properties of lipid bilayer membranes [28,29]. It is possible, therefore, that the presence of glycolipids in both halves of the bilayer might alter the nature of interactions on the outer surface. Thus, the preparation of liposomes that have an asymmetric distribution of gangliosides might provide a better model of cell membranes than liposomes having gangliosides distributed throughout all the lamellae.

It has been shown that cells can take up exogenously added gangliosides [1,2,4–6]. These gangliosides appear to be incorporated predominantly into the outer half of the cell membrane bilayer [26], and then can bind antibody [2] or choleragen [4,5] as effectively as endogenous gangliosides. Preformed liposomes also have been shown to take up exogenously added gangliosides [14–18]. In our experiments, uptake of G_{M1} by liposomes was directly proportional to the amount of G_{M1} added to the liposomes (Fig. 1), but the maximum G_{M1} incorporation was only approx. 2 mol% when compared to the liposomal phospholipids. This is less than the maximum of 10–12 mol% reported by Felgner et al. [17], but the latter authors used unilamellar liposomes in their experiments. Unilamellar liposomes have a greater fraction of the total phospholipid on the outer surface than do multilamellar liposomes, and this may account for the greater uptake of G_{M1} by unilamellar liposomes.

In our studies, incorporation of G_{M1} was initially quite rapid, then became increasingly slow, but never stopped. The time-course of uptake reported here was similar to that reported by Kanda et al. [30] for the uptake of [^{3}H]G_{M1} by sheep erythrocytes. At the concentrations of G_{M1} used, we never were able to saturate the liposomes with G_{M1}. In contrast, Felgner et al. [17] found that even using incubation times longer than 2 days, there was a maximum amount of ganglioside which could be taken up by unilamellar liposomes composed only of DPPC. Kanda et al. [18] reported that the addition of cholesterol to DPPC liposomes increased the uptake of a spin-labeled ganglioside. All of the liposomes used for our experiments contained 43 mol% cholesterol, and this may explain why our results differ from those of Felgner et al. [17].

The differences in antibody-dependent glucose release observed between liposomes containing DMPC or DPPC were similar to those observed by us previously when galactosylceramide instead of ganglioside was the antigen [31]. These differences were not due to differences in uptake of G_{M1} by liposomes containing the two different phospholipids (data not shown), but rather were caused by differences in the degrees of complement damage that occur with liposomes containing different phospholipids [31].

As mentioned above, one reason for adding the ganglioside to preformed liposomes was to obtain liposomes with ganglioside only in the outer leaflet of the lipid bilayer. The difficulty in reaching saturation of ganglioside incorporation, however, presented the possibility that the ganglioside molecules actually were flipping to the inner half of the bilayer. It is unlikely that this occurs to any great extent, since it has been shown that the rate of flip-flop of gangliosides from the outer to the inner half of the bilayer is so slow that there is effectively no flip-flop [1,17]. We also found that the gangliosides that were taken up were stable on the outer surface of the liposomes (Tables III and IV). In addition, the bulk of the gangliosides (over 80%) were localized on the outer surface of the liposomes (Table IV). This observation with multilamellar liposomes is consistent with the study of Felgner et al. [17] who reported that all of the ganglioside taken up by unilamellar liposomes was available to neuraminidase.

Liposomes sensitized by incubation with gangliosides are a good model for cells in studies on specific interactions of proteins, such as antibodies or toxins, with membrane gangliosides. Preformed liposomes incubated with G_{M1} bind choleragen (Fig. 5) or anti-G_{M1} antibody (Table II and Ref. 21, Table 2) as effectively as liposomes prepared with G_{M1} present. Thus, the exogenously added G_{M1} is truly intercalated into the lipid bilayer and behaves functionally the same as G_{M1} incorporated during the formation of the lipid bilayer.

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