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Liposomes as Model Membranes for Ligand–Receptor Interactions: Studies with Choleragen and Glycolipids†

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ABSTRACT: Binding of [^{125}I]choleragen to liposomes containing G_{M1} [galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide] had the characteristics previously described for the binding of the toxin to cells and membranes [P. Cuatrecasas (1973) *Biochemistry 12*, 3547]. Binding was rapid, not readily reversible, and saturable. Half-saturation occurred at 10^{-10} M choleragen, and similar concentrations of unlabeled toxin blocked the binding of [^{125}I]choleragen to the liposomes. Binding was highly specific for liposomes containing G_{M1}; only small amounts of toxin bound to liposomes containing the homologous glycolipids G_{D1b} [galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl-*N*-acetylneuraminyl)galactosylglucosylceramide], G_{A1} (galactosyl-*N*-acetylgalactosaminygalactosylglucosylceramide), and G_{M2} [*N*-acetylgalactosyl(*N*-acetylneuraminyl)galactosylglucosylceramide.] Choleragen effectively protected the G_{M1} of liposomes from external labeling by sequential treatment with galactose oxidase and NaB^3H_4; incorporation of ^{3}H into the galactose of G_{M1} was reduced by 90%. Liposomal G_{D1b}, G_{A1}, and G_{M2} were protected to a lesser extent. Binding of choleragen also reduced the labeling of the sialic acid residue of liposomal G_{M1} by $NaIO_4$ and NaB^3H_4. These results are similar to those reported for G_{M1} in intact cells [J. Moss et al. (1977) *Biochemistry 16*, 1876]. Thus, the interaction of choleragen with G_{M1} incorporated into lipid model membranes mimicked the characteristics and specificity noted with biological membranes. Liposomes appear to be useful as model membranes to explore the interaction of ligands with glycolipids incorporated into the liposomal membranes.

Liposomes (lipid model membranes) have been extremely useful in studying the interactions of antibodies with glycolipid antigens (Kinsky, 1972; Alving, 1977). Many of the properties of the antibody–antigen reaction correspond to what is observed with biological membranes. Liposomes containing various glycolipids also bind lectins (Surolia et al., 1975; Boldt et al., 1977), hormones (Maggio et al., 1977; Aloj et al., 1977; Pacuszka et al., 1978b), and toxins (Moss et al., 1976, 1977c). In none of these studies, however, has the interaction of the ligands with the liposomes been examined in detail to determine their suitability as model membranes for ligand–glycolipid interactions.

The monosialoganglioside G_{M1}[1] has been implicated as the membrane receptor for choleragen (Cuatrecasas, 1973a,b; Holmgren et al., 1973; King & van Heyningen, 1973), and its binding to cells and membranes has been well characterized (Cuatrecasas, 1973a–c). The interaction of choleragen with

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[1] Abbreviations used: 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; G_{D1a}, AcNeuα2→3Galβ1→3GalNAcβ1→4Gal[3←2αAcNeu]β1→4Glcβ1→ceramide; G_{D1b}, Galβ1→3GalNAcβ1→4Gal[3←2αAcNeu8←2αAcNeu]β1→4Glcβ1→ceramide; G_{A1}, Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→ceramide; GL-4, GalNAcβ1→4Galα1→3Galβ1→4Glcβ1→ceramide; AcNeu, *N*-acetylneuraminic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

G_{M1} in intact cells by external labeling techniques also has been described (Moss et al., 1977a). We previously reported that choleragen bound to liposomes containing G_{M1} and caused the release of trapped glucose from the liposomes (Moss et al., 1976); the B protomer (choleragenoid) was as effective as the holotoxin (Moss et al., 1977c). We now describe the characteristics and specificity of the binding of choleragen to liposomes containing G_{M1} and homologous glycolipids and its effects on the interaction of these liposomes with surface active reagents.

Experimental Procedures

Materials. Choleragen was obtained from Schwarz/Mann and was iodinated by the Chloramine-T procedure (Cuatrecasas, 1973a) to a specific radioactivity of 31–48 μCi/μg. Gangliosides were obtained as previously described (Pacuszka et al., 1978a). G_{A1} was prepared from 20 mg of bovine brain gangliosides by mild acid hydrolysis. The gangliosides in 1 mL of H_2O were applied to a Dowex-50 (H^+) column (0.5 g) which was then washed with 4 mL of H_2O. The eluate was heated at 100 °C for 1 h, cooled, and mixed with 25 mL of chloroform:methanol (2:1, v/v). The upper phase was removed; the lower phase was washed once with 10 mL of chloroform:methanol:H_2O (3:48:47, v/v) and dried under a stream of N_2. The residue was chromatographed on a small column of Unisil (200 mg) according to Yu & Ledeen (1972). G_{A1} was recovered in the first fraction and further purified by thin-layer chromatography on Silica Gel 60 coated glass plates (E. Merck, Darmstadt, G.F.R.) in chloroform:methanol:0.25% $CaCl_2$ (60:35:8, v/v). Each glycolipid appeared at least 99% pure as assessed by thin-layer chromatography.

Binding of [^{125}I]Choleragen to Liposomes. Liposomes, prepared as described previously (Moss et al., 1976, 1977c), contained dimyristoyllecithin, cholesterol, and dicetyl phosphate in molar ratios of 2:1.5:0.22 (10 nmol of phospholipid/μL of liposomes), plus glycolipids (0.1 nmol/μL of liposomes) as indicated. The liposomes, reconstituted in phosphate-buffered saline (pH 7.4), were centrifuged at 100000*g* for 10 min in a Beckman Airfuge and suspended in the same buffer; 50–65% of the added glycolipid was recovered in the liposomes compared with 98% of the cholesterol. Binding of [^{125}I]choleragen was assayed essentially as described by Cuatrecasas (1973a). Liposomes containing 1–2 pmol of glycolipid were incubated with ^{125}I-labeled toxin (50 fmol) in 100 μL of Tris-buffered saline (pH 7.4) containing 1 mM EDTA and 0.1% bovine serum albumin for 1 h at 25 °C in 12 × 75 mm polystyrene tubes. After the addition of 1.5 mL of ice-cold buffer containing 1% albumin, the samples were filtered under vacuum on 25-mm Millipore EGWP filters (0.2 μm). The filters were washed twice with the same buffer, transfered to vials containing 6 mL of Ready-Solv HP (Beckman Instruments), and counted in a Searle Mark III liquid scintillation counter. Nonspecific binding was determined by incubating the liposomes with 2×10^{-7} M unlabeled choleragen before adding the labeled toxin. About 20–25% of the iodinated choleragen did not bind to G_{M1}-liposomes or cell membranes and presumably represented damaged or denatured toxin (Cuatrecasas, 1973a). Recovery of liposomes containing [^{14}C]cholesterol on 0.2-μm filters was 87.6%. When centrifuged at 10^5*g* for 5 min, 96.5% of the labeled liposomes were recovered, whereas recoveries on 0.5- and 1.0-μm filters were only 70.3 and 47.7%, respectively. Thus, use of 0.5-μm or larger pore filters as described by Aloj et al. (1977) results in a significant loss of liposomes.

Labeling of Glycolipids Incorporated into Liposomes. Liposomes containing different glycolipids were incubated with or without choleragen (6×10^{-5} M), sedimented by centrifugation (20000*g* for 10 min), and suspended in 1 mL of saline. The liposomes were incubated with 30 units of galactose oxidase (167 units/mg of protein from Worthington Biochemical Corp.) for 2 h at 37 °C or with 5 mM $NaIO_4$ for 10 min at 25 °C. The liposomes were sedimented as above and suspended in 0.2 mL of phosphate-buffered saline (pH 7.4), and 1 mCi of NaB^3H_4 (6 Ci/mmol from Amersham Corp.) was then added to the suspension. In some experiments, the NaB^3H_4 was diluted with $NaBH_4$ prior to addition. In other experiments, the liposomes were sedimented at 100000*g* in the Beckman Airfuge, and the incubation volumes were reduced to 0.1 mL.

Isolation of Labeled Glycolipids. Five volumes of chloroform:methanol (2:1, v/v) was added to the solution of labeled liposomes, and the mixture was centrifuged. The upper phase was removed and the lower phase washed twice with theoretical upper phase. When G_{A1} (or GL-4) was present, it was recovered in the lower phase. The combined upper phases containing the gangliosides were taken to dryness under a stream of N_2; the residue was dissolved in 0.2 mL of 0.6 M NaOH in methanol plus 0.4 mL of chloroform and incubated at 37 °C for 1 h to saponify phospholipids. The solution was neutralized with 30 μL of 4 M acetic acid and desalted on a small (0.5 g) column of Sephadex G-25 superfine (Fishman et al., 1976a). The gangliosides were purified further on a small (50 mg) column of Unisil (Yu & Ledeen, 1972) and separated by thin-layer chromatography (Fishman et al., 1976a). The developed chromatograms were scanned with a Berthold radioscanner and the amount of radioactivity was calculated from the area under the peak. By using a standard solution of [^{3}H]G_{M1}, the response was proportional to the amount of radioactivity applied to the plate over a 100-fold range. The gangliosides were then visualized with resorcinol reagent and quantified by densitometry by using known amounts of ganglioside applied to the same plate as standards (Fishman et al., 1979). G_{A1} and GL-4 were detected and quantified as previously described (Simmons et al., 1975).

Hydrolysis of Labeled Glycolipids and Sugar Analysis. Labeled glycolipids were isolated from liposomes as described above and hydrolyzed in 0.5 mL of 3 M HCl at 100 °C for 2 h. As carrier, 10 nmol of galactose and *N*-acetylgalactosamine were added to each sample. The hydrolysates were dried down under vacuum and chromatographed on thin-layer Silica Gel 60 coated glass plates in the solvent system 1-propanol:H_2O (7:3, v/v). In this system, the mobilities relative to glucose were, for galactose, 0.79 and, for galactosamine (the hydrolysis product of *N*-acetylgalactosamine), 0. Areas corresponding to these sugars were scraped from the chromatogram, placed in counting vials with 1 mL of H_2O, and left overnight. Radioactivity was then measured after the addition of 6 mL of Aquasol (New England Nuclear) to each vial; counting efficiency was 9%.

Results

Binding of [^{125}I]Choleragen to Liposomes. Binding of [^{125}I]choleragen to G_{M1}-liposomes was proportional to the amount of liposomes present until most of the toxin had become bound (Figure 1A). At 5×10^{-10} M ^{125}I-labeled toxin, binding was very rapid and reached a maximum by 10 min; at 2.5×10^{-11} M toxin, binding was slower and approached a plateau by 1 h (Figure 1B). When G_{M1}-liposomes were incubated with [^{125}I]choleragen for 1 h and then diluted with 20 volumes of incubation medium, essentially none of the bound toxin dissociated from the liposomes (Figure 1C). When diluted with unlabeled toxin (10^{-7} M), 45% of the bound

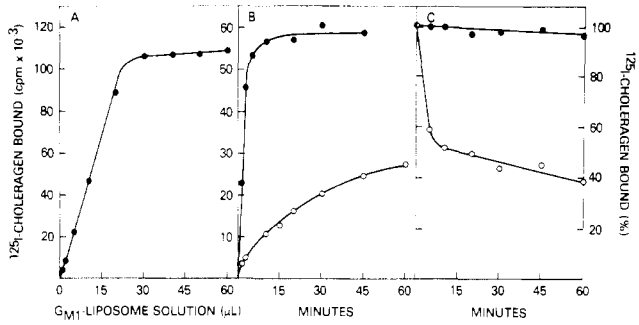

FIGURE 1: Binding of [^{125}I]choleragen to liposomes containing G_{M1}. G_{M1}-liposomes were incubated with [^{125}I]choleragen in Tris-buffered saline, pH 7.4, containing 1 mM EDTA and 0.1% bovine serum albumin at 25 °C and filtered on 0.2-μm filters as described under Methods. (A) Increasing amounts of a solution of G_{M1}-liposomes (1 pmol/10 μL) were incubated with 160000 cpm of [^{125}I]choleragen in a final volume of 0.1 mL for 1 h; values were corrected for the 960 cpm bound in the absence of liposomes. (B) G_{M1}-liposomes (10 μL) were incubated with 50 (●) or 2.5 (○) × 10^{-11} M [^{125}I]choleragen for the indicated times; values have not been corrected for nonspecific binding. (C) G_{M1}-liposomes (20 μL) were incubated with 5 × 10^{-10} M [^{125}I]choleragen for 1 h; then 2 mL of incubation medium without (●) or with (○) 10^{-7} M unlabeled choleragen was added to the samples, which were filtered at the indicated times. Values have been corrected for nonspecific binding at zero time which represented 1.4% of the total cpm bound.

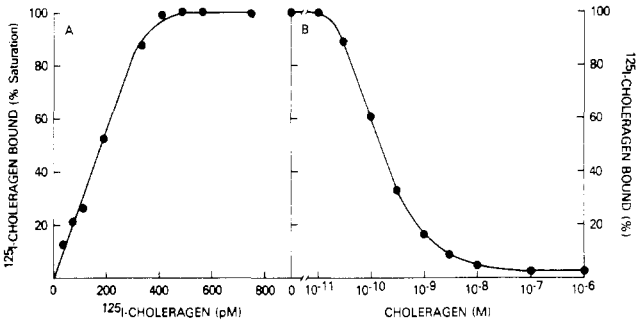

FIGURE 2: Effect of [^{125}I]choleragen and unlabeled choleragen concentrations on binding to G_{M1}-liposomes. (A) G_{M1}-liposomes were incubated with increasing concentrations of [^{125}I]choleragen for 1 h at 25 °C and filtered as described under Methods; values have been corrected for nonspecific binding which was determined in the presence of 2 × 10^{-7} M unlabeled choleragen. (B) G_{M1}-liposomes were incubated 5 min with the indicated concentration of unlabeled choleragen and then 1 h with 2.5 × 10^{-10} M [^{125}I]choleragen and filtered as described under Methods.

choleragen was rapidly displaced but the remainder only slowly dissociated from the liposomes; even after 1 h, 38% was still bound. Binding of [^{125}I]choleragen to G_{M1}-liposomes was saturable at toxin concentrations of 5 × 10^{-10} M (Figure 2A); half-saturation occurred at 1.8 × 10^{-10} M choleragen. Similar results were obtained with two different preparations of G_{M1}-liposomes and of iodinated toxin. Low concentrations of unlabeled choleragen effectively blocked the binding of ^{125}I-labeled toxin (Figure 2B); 50% inhibition occurred at 1.5 × 10^{-10} M choleragen. Similar results were obtained when the unlabeled and labeled toxin were mixed prior to the addition of the liposomes (data not shown).

The incubation conditions had little effect on the binding of [^{125}I]choleragen to G_{M1}-liposomes. There was 10% less binding at 4 °C and 6% more at 37 °C when compared with 25 °C. Binding was similar in various physiological media (phosphate-buffered saline, pH 7.4, and minimal essential medium buffered with Hepes, pH 7.4) and <20% higher in a nonphysiological medium, 25 mM Tris–acetate (pH 6.0) (Aloj et al., 1977) but containing 0.3 M sucrose to maintain the integrity of the liposomes.

Table I: Binding of [^{125}I]Choleragen to Liposomes Containing Different Glycolipids[a]

glycolipid	[^{125}I]choleragen bound (cpm): + unlabeled toxin	− unlabeled toxin	Δ	cpm bound/pmol of glycolipid[b] (as % of G_{M1})
none	2298	2326	28	
G_{A1}	1957	2863	906	0.26
G_{M2}	2287	2815	528	0.52
G_{D1b}	2411	6918	4507	5.5
G_{M1}	2284	138191	135897	100

[a] Liposomes containing the indicated glycolipid (~1 pmol) were incubated with 6.5 × 10^{-10} M [^{125}I]choleragen in 0.1 mL of incubation medium with and without 2 × 10^{-7} M unlabeled toxin for 1 h at 25 °C and filtered as described under Methods. Values are the mean of triplicate assays; 2153 cpm bound to the filters in the absence of liposomes. [b] Calculated from the actual amount of each glycolipid present in the binding assay.

Table II: Effect of Choleragen on External Labeling of G_{M1}-Liposomes by Galactose Oxidase or Sodium Periodate[a]

treatment	incorpn of ^{3}H (dpm/nmol) × 10^{-3}: − choleragen	+ choleragen	protection by choleragen (%)[b]
galactose oxidase	208	25.6	88
sodium periodate[c]	112	45.9	59
none	27.4	15.9	42

[a] Liposomes containing G_{M1} (2 nmol/15 μL) were incubated 1 h with and without choleragen (6 × 10^{-5} M), washed, incubated with galactose oxidase (30 units for 2 h), $NaIO_4$ (5 mM for 10 min), or saline, washed, and treated with 1 mCi of NaB^3H_4 (0.6 Ci/mmol) for 10 min as described under Methods. G_{M1} was isolated from the liposomes, subjected to thin-layer chromatography, analyzed for radioactivity by radioscanning, and quantified by densitometry as described under Methods. [b] Represents the reduction in ^{3}H incorporated into liposomal G_{M1} by choleragen. [c] In a second experiment with $NaIO_4$ and NaB^3H_4 (6 Ci/mmol), incorporation of ^{3}H into G_{M1} was without and with choleragen, 1.9 and 0.7 × 10^6 dpm/nmol, respectively; and the protection was 63% (average of duplicate determinations).

Binding of [^{125}I]choleragen to liposomes containing G_{M1} was highly specific (Table I). The toxin did not bind to glycolipid-free liposomes; nonspecific binding (that not inhibited by 2 × 10^{-7} M unlabeled choleragen) essentially represented the counts that bound to the filter in the absence of liposomes. There was substantially less binding to liposomes containing the homologous glycolipids G_{D1b}, G_{M2}, and G_{A1} at a concentration of [^{125}I]choleragen sufficient to saturate an equivalent amount of G_{M1}-liposomes. Even when we increased the concentration of labeled choleragen to 2 × 10^{-7} M and the amount of liposomes 100-fold, G_{M1}-liposomes bound nine times more toxin than did G_{D1b}-liposomes.[2]

Effect of Choleragen on Labeling of Liposomal Glycolipids by Galactose Oxidase and Sodium Periodate. When liposomes containing G_{M1} were sequentially treated with galactose oxidase or $NaIO_4$ and then NaB^3H_4, incorporation of ^{3}H into G_{M1} was substantially reduced by prior incubation of the liposomes with choleragen (Table II). In the absence of oxidant, there was some ^{3}H incorporation, presumably into the lipid portion of the molecule (Suzuki & Suzuki, 1972; Liao

[2] Since the choleragen concentration was saturating for G_{M1}-liposomes and, if we assume that the proportion of each ganglioside accessible to the toxin is the same, we can calculate from these data an apparent K_d of 1.5 × 10^{-6} M for choleragen binding to G_{D1b}-liposomes as described by Cuatrecasas (1973a).

Table III: Effect of Choleragen on External Labeling of Liposomal Glycolipids by Galactose Oxidase and NaB^3H_4[a]

glycolipid	nmol[b]	^{3}H incorpn [(dpm/nmol) × 10^{-3}] – choleragen	^{3}H incorpn [(dpm/nmol) × 10^{-3}] + choleragen	protection by choleragen (%)
G_{M1}	1.26	116	12.3	89
G_{D1b}	0.32	54.8	19.4	65
G_{M2}	2.09	2.4	1.6	33
G_{A1}	2.18	40.9	32.2	21

[a] Details of labeling of liposomes containing the indicated glycolipid with galactose oxidase and NaB^3H_4 and subsequent analysis of the labeled glycolipids are described in Table II and under Methods. [b] The amount of glycolipid in 15 μL of liposomes.

Table IV: Distribution of Radioactivity in Sugars Isolated from Liposomal Glycolipids Labeled with Galactose Oxidase and NaB^3H_4 in the Presence and Absence of Choleragen[a]

	^{3}H incorpn (cpm/nmol of glycolipid)			
	galactose		galactosamine[b]	
glycolipid	– choleragen	+ choleragen	– choleragen	+ choleragen
G_{M1}	8658	811	807	644
G_{D1b}	4595	1772	422	334
G_{M2}	147	98	198	151
G_{A1}	3381	2413	142	134

[a] Glycolipids isolated from the liposomes described in Table III were hydrolyzed; the hydrolysates were chromatographed on thin-layer silica gel; and the incorporation of ^{3}H into galactose and galactosamine was determined as described under Methods. When the values in cpm/nmol are corrected for counting efficienty (9%) and compared with those in Table III, most of the radioactivity introduced into the intact glycolipid was recovered in the galactose and galactosamine residues. [b] Similar results were obtained when the hydrolysates were acetylated and chromatographed. The mobility of *N*-acetylgalactosamine relative to glucose was 1.25.

et al., 1973), and this nonspecific labeling was also reduced by choleragen. Choleragen was more effective in protecting liposomal G_{M1} from galactose oxidase than from $NaIO_4$, which under these conditions oxidizes sialic acid (Van Lenten & Ashwell, 1971; Liao et al., 1973; Moss et al., 1977a; Veh et al., 1977). Choleragen did not protect liposomal G_{M2} and G_{D1b} from $NaIO_4$ oxidation; in fact, there was increased labeling of the liposomes treated with toxin (data not shown).

Exposure of liposomes containing G_{D1b}, G_{M2}, and G_{A1} to choleragen also reduced the labeling of these glycolipids by galactose oxidase and NaB^3H_4, but the relative protection by the toxin was not as great as for G_{M1}-liposomes (Table III). G_{M1} was protected the most, followed by G_{D1b}, G_{M2}, and G_{A1}; this is the same order as observed for the binding of [^{125}I]-choleragen to these glycolipids in liposomes. In the absence of toxin, incorporation of ^{3}H was greatest into G_{M1} followed by G_{D1b}, G_{A1}, and G_{M2}; this presumably represents the specificity of galactose oxidase for these glycolipids.

Distribution of Radioactivity into Individual Sugars. As galactose oxidase can oxidize both galactosyl and *N*-acetylgalactosaminyl residues of glycolipids (Bradley & Kanfer, 1964; Suzuki & Suzuki, 1972), the distribution of radioactivity into the individual sugars of these glycolipids was determined (Table IV). Most of the radioactivity incorporated into G_{M1}, G_{D1b}, and G_{A1} was associated with the galactose residue. Binding of choleragen to liposomes containing these glycolipids reduced the labeling of galactose by 90, 61, and 29%, respectively; these values are similar to the degree of protection

Table V: Effect of Choleragen on External Labeling of Liposomes Containing Mixtures of Glycolipids[a]

treatment	glycolipid	^{3}H incorpn [(dpm/nmol) × 10^{-3}] – choleragen	^{3}H incorpn [(dpm/nmol) × 10^{-3}] + choleragen
galactose oxidase	G_{M1}	262	7.6
	GL-4	150	129
sodium periodate	G_{M1}	406	61.2
	G_{M3}	308	398

[a] Liposomes (20 μL) containing 2 nmol each of G_{M1} and GL-4 or G_{M1} and G_{M3} were incubated with and without choleragen (6×10^{-6} M) in phosphate-buffered saline, pH 7.4 (final volume, 0.1 mL), for 1 h at 25 °C. Then the liposomes were incubated with galactose oxidase (15 units for 2 h at 37 °C) or $NaIO_4$ (10 mM for 10 min at 25 °C) by adding the oxidant in 50 μL of the same buffer. To the samples containing $NaIO_4$, 15 μL of 0.3 M glucose was added and all samples were centrifuged at 100,000*g* for 10 min in a Beckman Airfuge. The liposomes were washed twice with 0.16 mL of the same buffer by suspending and centrifuging as above, labeled with NaB^3H_4, and analyzed for incorporation of ^{3}H as described under Methods. The increased labeling of G_{M1} by $NaIO_4$ in this experiment compared with the one in Table II may be due to the higher concentration used (10 vs. 5 mM). The increased protection of liposomal G_{M1} by choleragen is probably due to not washing the liposomes before adding the oxidants.

Table VI: Effect of Liposomal Charge on Interaction of Choleragen and Galactose Oxidase with G_{M1}-Liposomes[a]

liposomal charge	^{3}H incorpn [(dpm/nmol) × 10^{-3}] – choleragen	^{3}H incorpn [(dpm/nmol) × 10^{-3}] + choleragen	protection by choleragen (%)
–	1180	107	91
+	417	53	87

[a] Liposomes (15 μL) containing G_{M1} (2 nmol), cholesterol, dimyristoyllecithin, and either dicetyl phosphate (negative charge) or stearylamine (positive charge) were prepared as described under Methods. The liposomes were incubated with and without choleragen and then galactose oxidase and 2 mCi of NaB^3H_4 (6 Ci/mmol) as described in Table II. Incorporation of ^{3}H into G_{M1} was determined as described under Methods.

of the intact glycolipids by choleragen (see Table III). Incorporation of ^{3}H into the galactosamine residue of these glycolipids was less than 10% of that associated with galactose and exposure of the liposomes containing these glycolipids to choleragen had little effect on labeling of galactosamine. Radioactivity was equally distributed between the galactose and galactosamine isolated from G_{M2}-liposomes and choleragen protected both residues from labeling.

Effect of Choleragen on Labeling of Liposomes Containing Mixtures of Glycolipids. Liposomes containing both G_{M1} and GL-4 were incubated with galactose oxidase in the presence and absence of choleragen and then reduced with NaB^3H_4. Choleragen effectively protected G_{M1} from being labeled, whereas GL-4 was only slightly protected (Table V). When liposomes containing both G_{M1} and G_{M3} were labeled with $NaIO_4$ and NaB^3H_4 under similar conditions, G_{M1} but not G_{M3} was protected by choleragen. The greater degree of protection observed in this experiment compared with that described in Table III was presumably due to not washing the liposomes after incubating them with choleragen.

Effects of Membrane Charge on Interaction of G_{M1}-Liposomes with Choleragen and Galactose Oxidase. G_{M1} was incorporated into liposomes containing different charges. When the liposomes were positively charged, incorporation of ^{3}H was reduced by 65% (Table VI). The relative reduction

in labeling in the presence of choleragen, however, was similar to that observed with negatively charged liposomes. Thus, a positive charge on the liposomes inhibited the oxidation of G_{M1} by galactose oxidase but did not interfere with the interaction of the ganglioside with choleragen.

Discussion

The binding of [^{125}I]choleragen to liposomes containing G_{M1}, the choleragen receptor, has many of the characteristics of toxin binding to cells and membranes. Binding to G_{M1}-liposomes was rapid as has been reported for toxin binding to fat cells, liver membranes, and fibroblasts (Cuatrecasas, 1973a; Moss et al., 1977a). Once bound to the G_{M1}-liposomes, there was no significant spontaneous dissociation of the toxin and even a large excess of unlabeled choleragen caused only a partial dissociation. Comparable results were noted with fat cells and liver membranes (Cuatrecasas, 1973b,c), intestinal membranes (Walker et al., 1974), and mouse thymocytes (Holmgren & Lonnroth, 1976). Choleragen had a very high affinity for G_{M1}-liposomes with an apparent K_d of 10^{-10} M. Similar high affinity binding has been reported for rat adipocytes (Cuatrecasas, 1973a), rat intestinal membranes (Walker et al., 1974), rat Leydig cells (Dufau et al., 1978), and mouse neuroblastoma and Friend erythroleukemic cells (P. H. Fishman and E. E. Atikkan, manuscript submitted).

The binding of choleragen to G_{M1}-liposomes was highly specific. We previously reported that choleragen bound to, and induced the release of glucose from, liposomes containing G_{M1} but not G_{D1a} (Moss et al., 1976). We now show that there is much less binding of choleragen to liposomes containing the homologous glycolipids G_{D1b}, G_{M2}, and G_{A1}. The apparent affinity of choleragen for G_{D1b}-liposomes was 10000-fold less than for G_{M1}-liposomes. Other studies have indicated that choleragen binding is highly specific for G_{M1} but choleragen will interact to a lesser extent with other glycolipids (Cuatrecasas, 1973a,b; Holmgren et al., 1973; King & van Heyningen, 1973; Staerk et al., 1974; Fishman et al., 1976b; Sattler et al., 1977).

This high degree of specificity also was shown by the ability of choleragen to protect these glycolipids incorporated into liposomes from enzymatic and chemical oxidation. G_{M1} was protected the most, followed by G_{D1b}, G_{M2}, and G_{A1}. Binding of choleragen to G_{M1}-liposomes caused a 90% reduction in the labeling of G_{M1} by galactose oxidase and NaB^3H_4. Labeling of the galactose isolated from G_{M1} was reduced to the same extent, whereas the *N*-acetylgalactosamine residue was not protected by choleragen. The effects of choleragen on the labeling of G_{D1b} and G_{A1} were also associated with reduced incorporation of ^{3}H into their galactose residues; labeling of both galactose and *N*-acetylgalactosamine was reduced by choleragen binding to G_{M2}-liposomes. In the presence of choleragen, incorporation of ^{3}H into liposomal G_{M1} by treatment with $NaIO_4$ and NaB^3H_4 was 60% less than in the absence of the toxin; labeling of liposomal G_{M2} and GD_{1b} was not reduced. Oxidation under these conditions is highly specific for sialic acid residues (Van Lenten & Ashwell, 1971; Liao et al., 1973; Moss et al., 1977a; Veh et al., 1977). By using the same labeling procedures, similar protection by choleragen of endogenous G_{M1} in human fibroblasts and of exogenous G_{M1} integrated into mouse fibroblasts has been observed (Moss et al., 1977a). Protection of the galactose and sialic acid residues of G_{M1} appeared to be a direct consequence of choleragen interacting with these residues. When other glycolipids were present in the same liposomes, they were not protected. Similar results had been obtained with intact fibroblasts; choleragen protected G_{M1} but not GL-4 from galactose oxidase and G_{M1} but not G_{M3} from $NaIO_4$ (Moss et al., 1977a).

These and other studies clearly indicate that the terminal galactose and sialic acid residues of G_{M1} are the important structural determinants for choleragen binding and, presumably, for choleragen activity on intact cells. G_{M2} and G_{D1b} were much less effective than G_{M1} in sensitizing ganglioside-deficient cells to choleragen (Fishman et al., 1976b; J. Moss et al., unpublished results). Choleragen induced the release of trapped glucose from liposomes containing G_{M1} (Moss et al., 1976, 1977c; Ohsawa et al., 1977) but not G_{D1b}, G_{M2}, or G_{A1} (unpublished observations).[3] Thus, the weak interactions of these glycolipids with choleragen appeared to be insufficient to induce perturbations in the lipid membrane, and perhaps in the choleragen structure[4] that resulted in glucose release. The specific interaction between choleragen and G_{M1} that leads to both types of perturbations may be involved in the mechanism of action of the toxin.

Variations in liposomal charge also influenced the interaction of choleragen (as well as galactose oxidase) with G_{M1}. The interaction of antibodies with glycolipid antigens incorporated into liposomes was reduced by increasing the chain length of the acyl groups in the phospholipids (Alving et al., 1974). Increased chain length reduced the binding of the lectin from *Ricinus communis* to liposomes containing galactosyl- or lactosylceramide but not G_{M1} (Surolia & Bachhawat, 1978). Further manipulations of the composition of the liposomal membrane may provide additional information on factors that can influence the binding and action of choleragen.[5] Thus, liposomes appear to be excellent model membranes for investigating the interactions of complex proteins that have glycolipids as their membrane receptors.

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[3] Choleragen-induced conductance changes in lipid bilayers containing G_{M1} but not G_{M2} (Tosteson & Tosteson, 1978).

[4] G_{M1} but not other gangliosides induced a shift in the fluorescence spectra of choleragen (Mullin et al., 1976) and its B protomer (Moss et al., 1977b). The oligosaccharide of G_{M1} induced a shift in the fluorescence and circular dichroic spectra of choleragen and its B protomer (Fishman et al., 1978). These observations are consistent with a conformational change in the choleragen molecule upon its binding to the oligosaccharide moiety of its membrane receptor G_{M1}.

[5] When G_{M1}-liposomes were prepared with sphingomyelin (average acyl chain length of 22 carbons) instead of dimyristoyllecithin and labeled by the galactose oxidase/NaB^3H_4 procedure, incorporation of ^{3}H was 944000 dpm/nmol without choleragen treatment and 209000 with choleragen treatment. Thus, the protective effect of choleragen was reduced compared with that observed with G_{M1}-liposomes containing dimyristoyllecithin (see Table VI).

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Methotrexate, a High-Affinity Pseudosubstrate of Dihydrofolate Reductase†

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ABSTRACT: Investigations have been made of the slow, tight-binding inhibition by methotrexate of the reaction catalyzed by dihydrofolate reductase from *Streptococcus faecium* A. Quantitative analysis has shown that progress curve data are in accord with a mechanism that involves the rapid formation of an enzyme–NADPH–methotrexate complex that subsequently undergoes a relatively slow, reversible isomerization reaction. From the K_i value for the dissociation of methotrexate from the E–NADPH–methotrexate complex (23 nM) and values of 5.1 and 0.013 min^{-1} for the forward and reverse rate constants of the isomerization reaction, the overall inhibition constant for methotrexate was calculated to be 58 pM. The formation of an enzyme–methotrexate complex was demonstrated by means of fluorescence quenching, and a value of 0.36 μM was determined for its dissociation constant. The same technique was used to determine dissociation constants for the reaction of methotrexate with the E–NADP and E–NADPH complexes. The results indicate that in the presence of either NADPH or NADP there is enhancement of the binding of methotrexate to the enzyme. It is proposed that methotrexate behaves as a pseudosubstrate for dihydrofolate reductase.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. The enzyme has been of considerable pharmacological interest because it is the target for a number of chemotherapeutic agents (Hitchings & Burchall, 1965) such as methotrexate, one of the first folic acid analogues to produce beneficial effects in the treatment of neoplastic disease in man (Blakley, 1969).

Because of its pharmacological importance, much effort has been directed toward an understanding of the nature of the inhibition of dihydrofolate reductase by methotrexate. Unfortunately, in earlier work on methotrexate inhibition, there was a lack of appreciation of the significance of the slow development of the inhibition and a failure to recognize that

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