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REACTIVITY OF GLYCOCONJUGATES IN MEMBRANES

I. DETERMINATION OF TRANSBILAYER DISTRIBUTION OF GANGLIOSIDES IN LIPID VESICLES BY CHEMICAL METHODS

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Two simple chemical methods are described for the determination of the transbilayer distribution of gangliosides GD_{1a} and GM_1 in phosphatidylcholine vesicles. The data presented here show an increase in the percentage of GD_{1a} exposed on the outer surface of vesicles with increasing mole fraction of GD_{1a}. The percentage of GD_{1a} exposed on 1:50 and 1:5 GD_{1a}-dipalmitoylphosphatidylcholine vesicles were found to be 67% and 83%, respectively. The same trend is seen for vesicles with dimyristoylphosphatidylcholine and distearoylphosphatidylcholine. Extrapolation of the data to infinite dilution gives 65% of GD_{1a} exposed on the surface of GD_{1a}-dipalmitoylphosphatidylcholine vesicles. The results indicate that composition-dependent changes in transbilayer distribution of GD_{1a} can only partly account for the observed increase in the reactivity of GD_{1a} in vesicles towards neuraminidase from *Clostridium perfringens* as the ratio of GD_{1a} to phosphatidylcholine increases.

Introduction

Gangliosides have been implicated as cell-surface receptors for a variety of ligands [1]. In order to understand the rate and mechanism of ganglioside-mediated phenomena at the cell-surface, model studies with vesicle-bearing gangliosides are necessary. Recently, several studies on the reactivity of the carbohydrate moiety of glycolipids including gangliosides towards lectins [2–4], cholera toxin [5], and neuraminidase [6] have been reported. All these studies have indicated a strong dependence of reactivity on the composition of the vesicle. The transbilayer distribution of glycoconjugates as observed in many mixed phospholipid systems [7,8], as well as changes in the conformation of carbohydrate head groups, may contribute to such observed differences in reactivity. This kind of information is lacking in the literature, even though several studies on liposomes bearing gangliosides have been reported recently [9–13].

In this paper an attempt has been made to correlate the initial rate of desialation of GD_{1a}-phosphatidylcholine mixed dispersions by neuraminidase from *Clostridium perfringens* with the transbilayer distribution of GD_{1a}. The difficulty in achieving this was the determination of ganglioside asymmetry, for which established methods

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Abbreviations: GM_1, galactosyl-*N*-acetylgalactosaminyl-galactosyl-(*N*-acetylneuraminyl)glucosyl ceramide; GD_{1a}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminylgalactosyl-(*N*-acetylneuraminyl)glucosyl ceramide; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; NeuAc, *N*-Acetylneuraminic acid.

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were not available. This is mainly because the widely used NMR method of determination of lipid asymmetry is of limited applicability here due to the poor resolution of resonances from ganglioside and phosphatidylcholine in ^{1}H-NMR and large amounts of material or enrichment required for ^{13}C-NMR. However, we have overcome the problem by developing two simple methods for the determination of asymmetry, which make use of neuraminidase from *Cl. perfringens* and periodate as probes. The results indicate that the increase in initial rate of desialation of GD_{1a}-phosphatidylcholine dispersions by neuraminidase from *Cl. perfringens* with increasing mole fraction of GD_{1a} can be accounted for partly by differences in transbilayer distribution of GD_{1a}.

Materials and Methods

Materials

Neuraminidase from *Cl. perfringens* (type VI), dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), distearoylphosphatidylcholine (DSPC), cholesterol, bovine serum albumin and *N*-acetylneuraminic acid (NeuAc) were obtained from Sigma, U.S.A. Sodium periodate was a product of BDH, U.K. Gangliosides were isolated from sheep brain [14]. The individual gangliosides were separated by TLC on silica gel using $CHCl_3/CH_3OH$/2.5 M ammonia (60:35:8) as the solvent. GD_{1a} bands from several plates were pooled and rechromatographed. GM_1 was prepared by the extensive hydrolysis of mixed gangliosides by neuraminidase from *Cl. perfringens* followed by TLC separation as above. NeuAc and neutral sugar contents were determined by the methods of Svennerholm [15] and Dubois et al. [16] respectively and their ratios for the GD_{1a} and GM_1 preparations were found to agree with the theoretical values.

Principle of methods used for asymmetry determination

A chemical probe for the determination of asymmetry should satisfy the following conditions. It should not diffuse into the aqueous compartment of vesicles while reacting specifically with the groups exposed on the outer surface and the vesicles should be stable as the reaction proceeds. Neuraminidase from *Cl. perfringens*, being a soluble enzyme of molecular weight 57000 [17], is expected not to penetrate the bilayer and degrade specifically ganglioside molecules on the outer surface of liposomes when added to a liposome solution. For the same reason, *Vibrio cholerae* neuraminidase can also be used for the determination of asymmetry, but complexity arises because the presence of Ca^{2+}, which is required for the activity of *V. cholerae* enzyme, may lead to processes such as vesicle aggregation and fusion. In addition, the enzyme penetrates the vesicle after 2 h of incubation so that one should be careful about the period of incubation [6]. Similarly, the periodate-resorcinol reaction of NeuAc described by Jourdian et al. [18] has been used to determine transbilayer distribution by carrying out the reaction under conditions in which the permeability of periodate through the bilayer is minimum.

Methods

Mixed vesicles containing GD_{1a} were prepared as follows. The lipids (GD_{1a}, phosphatidylcholine and cholesterol) were dissolved in $CHCl_3/Ch_3OH$ (2:1), the solvent was evaporated off using a nitrogen stream to form a thin film and kept overnight in a vacuum desiccator. This was suspended in 0.1 M sodium acetate buffer (pH 5.4) at a temperature 5–10 C deg. above the transition temperature (T_c) of the phosphatidylcholine by shaking on a vortex mixer. The suspension was then sonicated using an MSE 150 W ultrasonic disintegrator with a microtip probe (setting at Amplitude 5, Power High) for 3 min unless mentioned otherwise. During sonication, the sample was kept in a water bath of the temperature range mentioned above. The vesicles were stored at room temperature for 1 h before starting the experiment. The concentration of GD_{1a} in vesicles was determined by estimating the NeuAc content [15].

The initial rate of desialation was measured as follows: 1.333 munit (0.14 μg) neuraminidase (1 unit liberates 1 μmol NeuAc per min from sialyl lactose) dissolved in 0.1 M sodium acetate buffer (pH 5.4) along with 0.2 mg bovine serum albumin was added to vesicles containing 20 nmol GD_{1a} in a total volume of 0.2 ml. The mixture was incubated at 37°C for 15 min and the NeuAc released was estimated by Warren's procedure [19].

For the enzymatic determination of asymmetry, 16 munit (1.68 μg) of neuraminidase dissolved in acetate buffer along with 0.2 mg bovine serum albumin were added to the vesicle in a total volume of 0.2 ml. The mixture was incubated at 37°C for 5 h and the NeuAc released was estimated by Warren's procedure [19].

The periodate-resorcinol assay was carried out as follows. GD_{1a} or vesicle containing GD_{1a} in 0.3 ml of 0.033 M sodium acetate buffer (pH 5.4) were oxidized with 0.1 ml of freshly prepared 0.04 M sodium periodate in water for 25–30 min at temperatures mentioned. After oxidation, the excess periodate was removed by adding 0.05 ml of 0.16 M sodium metabisulphite and the tubes were transferred to an ice bath. To each tube 1 ml of resorcinol reagent [15] was added, shaken vigorously, and kept in ice for 5–10 min. Then the tubes were heated in a boiling water bath for 15 min and cooled in tap water for 5 min. The colour formed was extracted into a 2 ml *n*-butyl acetate/*n*-butanol (85:15, v/v) mixture and the absorbance of the organic phase at 630 nm was measured. The fraction of gangliosides exposed on the surface of vesicles (x) was calculated from the relation

$$x = \frac{A_1 - A_2}{A_3 - A_2}$$

where A_1 and A_3 are the absorbances from the vesicle and an equivalent amount of the free GD_{1a}, respectively, at room temperature and A_2 is the reading obtained from the same amount of vesicle added to the reaction mixture only after the addition of resorcinol reagent instead of at the beginning in the above procedure. (The correction with A_2 is necessary because the resorcinol reagent will disrupt the bilayer and give colour with GD_{1a} present inside the vesicle.)

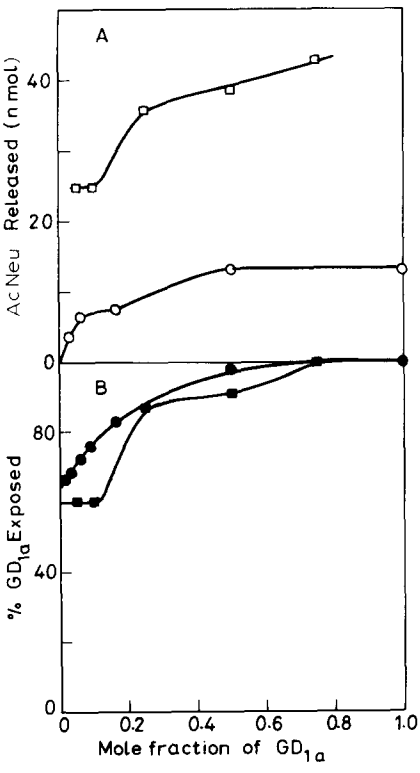
The permeability of periodate to DPPC vesicles was tested as follows. DPPC vesicles with entrapped glucose (0.5 M) was subjected to periodate oxidation and the rate was monitored by the decrease in absorbance at 223 nm [20].

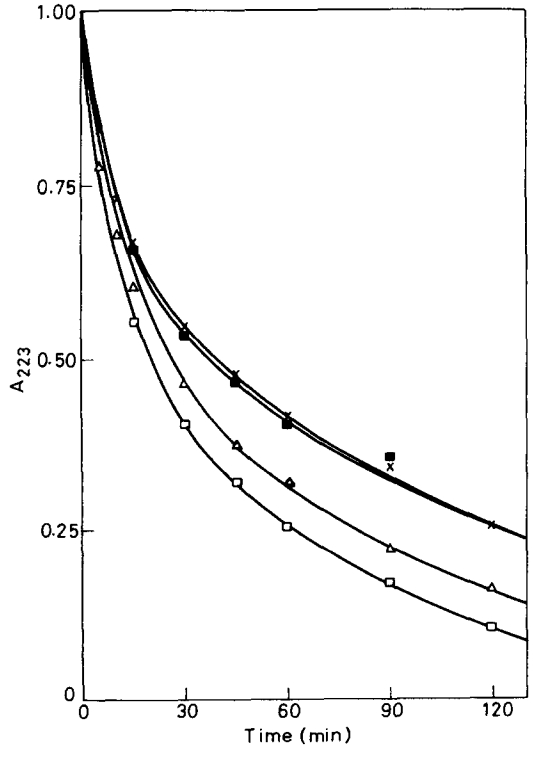
Free GD_{1a} and vesicle containing GD_{1a} at a final concentration of $4 \cdot 10^{-5}$ M were oxidized with sodium periodate at a final concentration of $1 \cdot 10^{-4}$ M in 0.01 M NaCl. The rate of oxidation was monitored by the decrease in absorbance at 223 nm [20].

Results and Discussion

Fig. 1A shows the dependence of initial rate of desialation of GD_{1a} on the composition of the vesicle at an enzyme concentration of 1.333 munit/0.2 ml incubation mixture. Similar composition-dependent differences in initial rate of periodate oxidation of GD_{1a} can be seen from Fig. 1C. In order to assess the effect of transbilayer distribution of GD_{1a} on its reactivity, a determination of asymmetry is required, for which the limiting value of desialation needs to be estimated first. With an enzyme concentration of 16 munit/0.2 ml, limiting values are attained in 1 h of incubation and remains constant even after 50 h (data not shown). Since the enzyme activity also remains unaffected upto 50 h under the present conditions of incubation, the limiting values of desialation were measured at 5 h. These values are given in Table I and correspond to the percentage of GD_{1a} exposed on the outer surface of the vesicles. The presence of remaining GD_{1a} on the inside layer of vesicles is shown by complete degradation in presence of 0.01 M Triton X-100 and also when the enzyme is entrapped inside the vesicles (Table I). The decreased level of desialation observed in this study and by earlier authors [2,5,6] is due to the inability of the enzyme to penetrate the bilayer and not due to any inaccessibility at the outer surface arising from stereochemical restraints. Hence the limiting value of desialation is a measure of the transbilayer distribution of GD_{1a}. From Fig. 2, it is evident that the distribution is not altered by further sonication beyond 3 min. Hence 3 min sonication was used for all the vesicle preparations in Table I.

The corresponding data obtained by estimating NeuAc by the periodate-resorcinol method of Jourdian et al. [18] is shown in Fig. 3 along with the accessibility of periodate towards entrapped glucose. It can be seen that within the first few minutes glucose entrapped in DPPC vesicles is not accessible to periodate oxidation, thereby suggesting that periodate cannot penetrate the bilayer in the initial stage of the reaction. The difference between curves A and B suggests that the slight





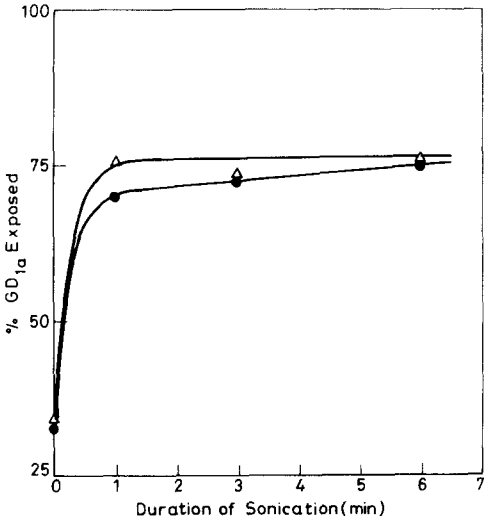


Fig. 2. Plot of percentage of GD_{1a} exposed on the 1 : 15 GD_{1a}-DPPC vesicle to neuraminidase action (●———●) and to the periodate-resorcinol reaction (△———△) as a function of the duration of sonication of the vesicle.

reduction in curve A may be due to a slight leaking of glucose. The inability of periodate to penetrate GD_{1a}-DPPC vesicle is also shown by the constant value for the percentage of GD_{1a} exposed to periodate-resorcinol reaction in Fig. 3 up to 30 min. Hence the percentage of GD_{1a} exposed to periodate-resorcinol reaction at room temperature, given in Table I, is the percentage of GD_{1a} present on the outer surface of vesicles. However, periodate penetrates vesicles at higher temperatures (in

Fig. 1. A. Initial rate of desialation (measured at 15 min) of GD_{1a}-DPPC vesicles (20 nmol GD_{1a}) by neuraminidase from *Cl. perfringens* ($E_0 = 1.333$ munit in 0.2 ml) vs. mole fraction GD_{1a} in vesicles (○———○). The initial velocity (15 min) data from Fig. 5 of Ref. 6 are replotted (□———□). For this the incubation mixture (0.25 ml) contained GD_{1a}-phosphatidylcholine vesicles (50 nmol GD_{1a}), 0.125 μmol $CaCl_2$ and 2 munit of *V. cholerae* neuraminidase in 0.04 M sodium acetate buffer, pH 5.6. B. Percentage of GD_{1a} exposed on the surface of GD_{1a}-DPPC vesicles to neuraminidase action (Table I) vs. mole fraction of GD_{1a} (●———●) and percentage of GD_{1a} exposed on the surface of GD_{1a}-phosphatidylcholine vesicles (from Ref. 6) vs. mole fraction of GD_{1a} (■———■). C. Rate curves for the periodate oxidation of GD_{1a} under different conditions in 0.01 M NaCl solution. $[GD_{1a}] = 4 \cdot 10^{-5}$ M; $[IO_4^-] = 1 \cdot 10^{-4}$ M. GD_{1a} alone (×———×); 1 : 2 GD_{1a}-DPPC vesicle (△———△); 1 : 5 GD_{1a}-DPPC vesicle (□———□) and 1 : 5 GD_{1a}-DMPC vesicle (■———■).

TABLE I

ASYMMETRY OF GANGLIOSIDES IN GANGLIOSIDE-PHOSPHATIDYLCHOLINE MIXED VESICLES

Percentage of GD_{1a} accessible to neuraminidase action is obtained by multiplying by 2 the observed percentage of AcNeu released, in order to account for the resistance of GM_1 to neuraminidase action. The enzyme was entrapped inside the vesicles by adding the enzyme along with the buffer to the thin film of lipids. The standard deviations are given for experiments with separately prepared vesicles. All the colorimetric estimations have an error of ±4%.

Composition of vesicle	% GD_{1a} accessible to neuramidase action			Percentage of ganglioside exposed to periodate-resorcinol reaction at:	
	in absence of Triton X-100	in presence of Triton X-100	when enzyme also entrapped	Room temperature	50°C
1:1 GD_{1a}-DPPC [f]	98.09			105.40	104.90
1:5 GD_{1a}-DPPC	82.78±2.75		96.72	92.77±1.35	97.66±0.22
1:10 GD_{1a}-DPPC	76.03±2.64			78.57	92.48
1:15 GD_{1a}-DPPC	72.34±2.79	100.00	99.92	73.69±1.84	98.87±4.00
1:30 GD_{1a}-DPPC	68.11±1.30	100.00	99.26	72.27	96.47
1:50 GD_{1a}-DPPC	67.29			67.86	93.83
1:10:20 GD_{1a}-cholesterol-DPPC	56.10 [e]			60.55	
1:5 GD_{1a}-DMPC	104.76			90.42 [a]	97.08
1:15 GD_{1a}-DMPC	71.23±5.35	100.00		81.02±0.53 [a]	98.49±6.81 [b]
1:5 GD_{1a}-DSPC	91.57			94.72	84.66±4.40 [c]
1:15 GD_{1a}-DSPC	83.13±1.83	96.54		94.79±1.60	81.55 [c]
1:5 GM_1-DPPC				93.03±3.27	98.80
1:15 GM_1-DPPC				81.23 [d]	

[a] The oxidation was carried out at 0°C.

[b] The same value (see footnote a) is obtained when oxidation is carried out at room temperature.

[c] The oxidation was carried out at 60°C.

[d] Calculated based on readings at room temperature and 50°C.

[e] The reported percentages of GD_{1a} exposed on GD_{1a}-cholesterol-DPPC unilamellar and multilamellar vesicles, are 65.00 and 30.00 respectively [2]. The percentage GD_{1a} expected to be exposed, based on the 30% of multilamellar liposome present in our preparation as observed by fractionation on a Sepharose 4B column, is 54.50.

[f] Dispersion.

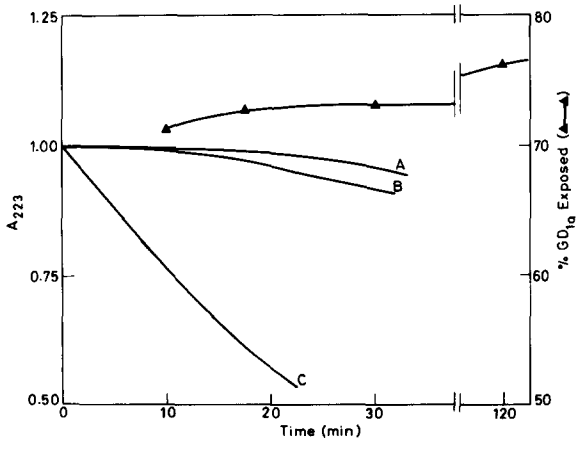


Fig. 3. (i). Rate curves for the periodate oxidation of glucose (approx. $2 \cdot 10^{-4}$ M) under different conditions with $1 \cdot 10^{-4}$ M sodium periodate. A. Glucose entrapped in DPPC vesicles 4 h after separation from free glucose using a Sephadex G-50 column. B. Glucose entrapped in DPPC vesicles 24 h after separation. C. Free glucose solution approximately equivalent to the amount trapped (from trapped volume measurements). (ii) Plot of GD_{1a} available for periodate-resorcinol reaction as a function of duration of the periodate oxidation step for 1:15 GD_{1a}-DPPC vesicle (▲——▲).

general at temperatures above the T_c of the phosphatidylcholine) and the reaction is complete (Table I). Hence for the GD_{1a}-DMPC vesicle, oxidation was carried out in an ice-bath. Thus, when the permeability of periodate is minimized, both the methods give fair agreement of values. The periodate-resorcinol method is applicable to all glycoconjugates containing NeuAc and the enzymatic method can be extended in principle, to other glycoconjugates with an appropriate enzyme.

Our values of the percentage GD_{1a} exposed on the surface of GD_{1a}-DPPC vesicles are higher than the values reported for GD_{1a}-phosphatidylcholine vesicles using *V. cholerae* neuraminidase, but agree with others if the presence of multilamellar liposomes is taken into account [2,5,6] (see Table I and Fig. 1B). We think that the variation in percentage of GD_{1a} exposed on the surface of vesicles with composition shown in Fig. 1B is a reflection of the change in transbilayer distribution of GD_{1a} with the composition. From a reciprocal plot, a limiting value of 65% of GD_{1a} exposed on a GD_{1a}-DPPC vesicle of very low GD_{1a} mole fraction can be obtained. This corresponds to almost symmetric distribution of GD_{1a} between the bilayer halves in view of the reported average outside-to-inside ratio for DPPC vesicle of 1.9 if we assume that there is not much change in the size of the vesicle with the incorporation of GD_{1a} [21]. A similar value for the transbilayer distribution of GT_1 in DPPC vesicle has been reported recently [22]. However, Fig. 1B suggests an asymmetric distribution in favour of outer layer as the mole fraction of GD_{1a} is increased.

From Fig. 1A and B, it is evident that the increase in initial rate of desialation of GD_{1a} with increasing mole fraction of GD_{1a} can be partly accounted for by the increase in effective surface concentration of GD_{1a} due to the increasing exposure of GD_{1a} with increasing mole fraction of GD_{1a}. Cestaro et al. [6] have observed a decrease in initial rate of desialation of GD_{1a}-phosphatidylcholine vesicles by neuraminidase from *V. cholerae* with increasing mole fraction of GD_{1a} at a lower concentration of the enzyme and an increasing initial rate of desialation at a higher enzyme concentration. The reason for the discrepancy is not clear. However, their results at higher enzyme concentration reproduced in Fig. 1A (taken from Fig. 5 of Ref. 6) agree with our findings. It may be emphasized here that the enzyme used in this study loses its activity in complete absence of bovine serum albumin at 37°C with a half-life of 30 min. Thus it was not possible to study desialation of gangliosides in complete absence of albumin. For the same reason Cassidy et al. [23] used bovine serum albumin in their studies. A similar situation exists also in the case of *V. cholerae* neuraminidase [24]. Recently Tettamanti and his co-workers [25] have shown that GM_1 complexes with bovine serum albumin. These complexes are presumably stabilized by hydrophobic interactions. It is likely that the reactivity of the NeuAc residue of the complex is different from that of pure ganglioside micelles. Moreover, the presence of Ca^{2+} could lead to the formation of larger aggregates, the carbohydrate moiety of which is less susceptible to reaction than that of mixed micelles, as reported [6,26]. It is not clear how the inactivation of enzyme as well as Ca^{2+}-induced formation of larger aggregates could affect the rates of desialation. In any case, the effect of asymmetry needs to be taken into account to understand the rate and mechanism of ligand-receptor interaction on the cell surface.

Finally, it may be emphasized that the enhancement in initial rate of desialation (Fig. 1A) is more than that expected from the increased exposure of GD_{1a} with increasing mole fraction of GD_{1a} up to 0.5 (Fig. 1B). This means that factors other than asymmetry, such as the valency of the ligand and change in sugar conformation, are also influencing the reactivity of gangliosides and cell-surface carbohydrates in general. Since neuraminidase from *Cl. perfringens* is a monomeric enzyme, sugar conformation seems to play a more important role in determining the reactivity of surface carbohydrates.

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