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Influence of glycolipids on immune reactions of phospholipid antigens in liposomes

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Complement-dependent immune damage to liposomes mediated by a murine monoclonal antibody to the liposomal bilayer was completely inhibited by ceramide tetrasaccharide (globoside) at an 8% concentration in the liposomes. Lower concentrations of globoside, or higher concentrations of ceramide tri-, di-, or monohexoside, were not inhibitory. At a globoside concentration of 5.8%, inhibition of the monoclonal antibody activity was reduced and inhibition was related to antibody concentration; but at 2% globoside, suppression of antibody activity was not observed at all. Analysis of space-filling models revealed that at 8% globoside the distance between adjacent tetrasaccharides of globoside approached the dimensions of the antigen-binding end of a 7S immunoglobulin molecule. We therefore propose that globoside, and perhaps other glycolipids, can exert steric hindrance to the binding of extracellular proteins to nonglycolipid constituents of the lipid bilayer. We conclude that microheterogeneity among polar groups of glycolipids may be a novel mechanism for allowing selective access of proteins to phospholipids on the lipid bilayer.

Introduction

Glycolipids constitute a large fraction of the total composition of the lipid bilayers of cell membranes. Most mammalian glycolipids are presumed to be present almost exclusively on the outer half of the lipid bilayer of the plasma membrane. In this location they have numerous functions or activities, including serving as cell surface receptors and as antigens [1–4].

Although neutral glycosphingolipids may account for 5–10% of the total cell lipid [1,5], they are small molecules compared to proteins and steric effects due to microheterogeneity among glycolipid molecules are rarely considered. However, it has been estimated that as many as 30 to 60% of the lipid molecules on the outer half of the plasma membrane are glycosphingolipids [1], and it has been suggested that their saccharide groups form a sort of 'sugar lawn' on the cell surface [6]. Therefore because of the sheer number of glycolipid molecules, it was considered theoretically possible that oligosaccharide groups could interfere with the interaction of soluble extracellular protein molecules with the underlying lipid bilayer on the plasma membrane of the cell.

In human erythrocytes, globoside I (a ceramide tetrahexoside) is the major glycosphingolipid and

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Abbreviations: CMH, ceramide monohexoside (*N*-lignoceroyl D,L-dihydrogalactocerebroside); CDH, ceramide dihexoside (*N*-lignoceroyl D,L-dihydrolactocerebroside); CTH, ceramide trihexoside [Gal(α1 → 4)Gal(β1 → 4)Glu-ceramide]; globoside, ceramide tetrahexoside [GalNAc(β1 → 3)Gal(α1 → 4)Gal(β1 → 4)Glu-ceramide]; Forssman glycolipid, ceramide pentahexoside [GalNAc(α1 → 3)GalNAc(β1 → 3)Gal(α1 → 4)Gal(β1 → 4)Glu-ceramide]; DMPC, DPPC, DSPC, dimyristoyl-, dipalmitoyl-, and distearoylphosphatidylcholine.

accounts for approx. 3–10% of the total lipid of the cell [1,5]. In the present study we demonstrate that globoside in the liposome bilayer, when present in the concentration range normally found in erythrocytes, strongly inhibits the activity of a monoclonal antibody that specifically recognizes the liposomal phospholipid bilayer.

Materials and Methods

Lipids

Lipids were purchased from the following sources: dimyristoyl-, dipalmitoyl-, and distearoylphosphatidylcholine, (DMPC, DPPC, and DSPC) and cholesterol (Calbiochem-Behring, La Jolla, CA); sphingomyelin (Pierce Chemical Co., Rockford, IL); dicetyl phosphate (K & K Laboratories, Plainview, NY); *N*-lignoceroyl D,L-dihydrogalactocerebroside (ceramide monohexoside, or CDH) and *N*-lignoceroyl D,L-dihydrolactocerebroside (ceramide dihexoside, or CDH) (Miles Laboratories, Inc., Kankakee, IL). Ceramide trihexoside (CTH) and ceramide tetrahexoside (globoside) were purified from human erythrocytes, and Forsmann glycolipid (ceramide pentahexoside) was extracted from sheep erythrocytes, as described previously [7].

The synthetic glycolipids (CMH and CDH) were quantified by weight. The glycolipids purified from erythrocytes were quantified on thin-layer chromatography TLC plates using a Zeiss KM3 TLC spectrophotometer [8]. After applying different concentrations of glycolipid to the TLC plate, the plate was sprayed with orcinol reagent [9], and absorbance was read at 530 nm (A_{530}). The A_{530} values were compared with standard curves consisting of A_{530} values of dried aqueous mixtures of monosaccharides in the same molar ratios found in the oligosaccharides of the glycolipids. This simplified technique required small amounts of materials and gave results that were comparable to molar values of authentic samples of the same glycolipids quantified by gas-liquid chromatography [7].

Liposomes

Multilamellar liposomes were prepared as described previously [10]. The liposomes contained phospholipid, cholesterol, and dicetyl phosphate (e.g., DMPC/CHOL/DCP) in molar ratios of 1 : 0.75 : 0.11. The concentration of glycolipid in the liposomes is expressed as %, and this term is defined as (nmol glycolipid/nmol phospholipid) $\times 100$. Glucose (0.308 M) was used as a swelling solution for dispersing the dried lipids to form liposomes, and the phospholipid was 10 mM with respect to the swelling solution. The liposomes were dialyzed for 1.5 h against 1250 vol. of 0.15 M NaCl to reduce the level of untrapped glucose.

Antibodies

A murine monoclonal IgM antibody to liposomes (DMPC/CHOL/DCP) has been described extensively in a previous publication [11]. The antibody was obtained after immunization with liposomes containing DPPC/CHOL/DCP/Lipid A, and ascites fluid was used as the antibody source [11]. Purified human monoclonal (Waldenström macroglobulin) IgM antibody to Forssman glycolipid (McG) has also been described previously [7].

Immune damage to liposomes

The assay for measuring complement-dependent immune damage to liposomes has been described in detail elsewhere [10]. In brief, an assay cuvette contained: 0.3 ml of glucose assay reagent (consisting of Tris-buffered hexokinase, glucose-6-phosphate dehydrogenase, ATP, and NADP); liposomes (3 μl); an antibody source consisting of ascites fluid containing monoclonal antibodies to liposomes [11], guinea pig serum as a complement-source (40 μl) and 0.15 M NaCl to volume, in a total volume of 0.6 ml. Glucose release was detected after 30 min at room temperature (25°C) by increased A_{340} due to reduction of the NADP. Data are expressed as % of trapped glucose released, and the total glucose trapped was determined by disrupting the liposomes with chloroform [10].

Results

Influence of different liposomal glycolipids on the activity of a monoclonal anti-liposome antibody

Liposomes containing mono-, di-, tri-, or tetrahexosyl ceramide (CMH, CDH, CTH, or globoside) were utilized as targets for complement-de-

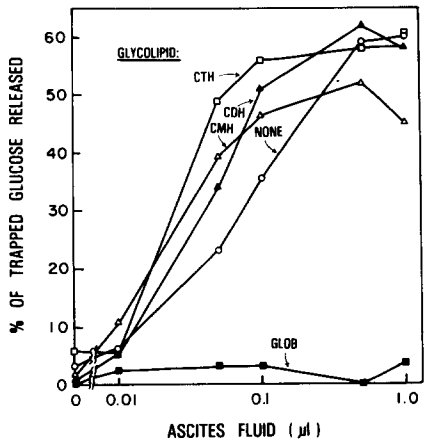

Fig. 1. Effects of glycolipids on immune reactivity of a monoclonal antibody to liposomes. The liposomes contained DMPC, cholesterol, dicetyl phosphate, and glycolipid, as indicated. The liposomal glycolipid concentration was 100 nmoles per µmole of DMPC (10%), except for CMH, which was present as 150 nmol/µmol of DMPC (15%).

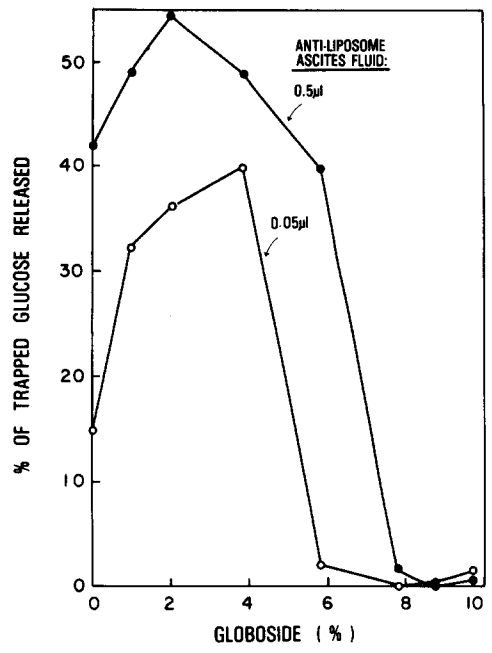

Fig. 2. Effects of globoside concentration on the activity of a monoclonal antibody to liposomes. Each point on each curve represents a different liposome preparation having the indicated amount of globoside. Each curve represents a different concentration of ascites fluid containing the monoclonal antibody.

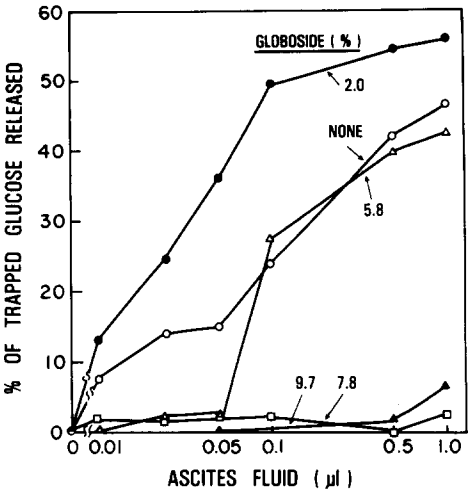

Fig. 3. Effects of antibody concentration on immune damage to liposomes containing different amounts of globoside. Each curve represents an antibody dose-response curve for liposomes containing the indicated concentration of globoside. The antibody source was ascites fluid containing monoclonal antibody to liposomes.

pendent membrane damage mediated by a monoclonal 'anti-liposome' antibody having specificity for the liposomal phospholipid bilayer. Fig. 1 shows that compared to liposomes lacking glycolipid, equivalent or increased immune damage occurred to liposomes containing CMH, CDH, or CTH. However, immune damage mediated by the anti-liposome antibody was completely inhibited when the liposomes contained globoside.

Inhibitory effect of globoside

We have previously shown that immune damage to liposomes induced by anti-liposome antibody is increased when the liposomes contain a glycolipid [11,12]. All of the glycolipids in the present study expressed this property of amplifying immune damage to liposomes. For CMH, CDH, and CTH, the increased sensitivity of liposomes is shown in Fig. 1, and for globoside it is shown in detail in Fig. 2. Between liposomal globoside concentrations of 0% and 4% increased immune damage invariably occurred (Fig. 2). However, at globoside levels above 4%, inhibition of anti-liposome antibody activity was observed, with complete inhibition occurring at globoside concentrations of 5.8 to 7.8% (Fig. 2). Separate experiments showed that the anti-liposome antibody was not nonspecifi-

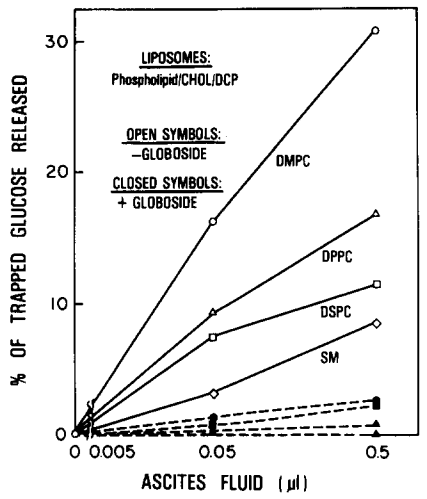

Fig. 4. Inhibitory effects of globoside on immune damage to liposomes containing several different preparations containing the indicated phospholipid. Globoside, when present in the liposomes, was in a concentration of 78 nmol/μmol of DMPC (7.8%). The antibody source was the ascites fluid containing monoclonal antibodies to liposomes. Chol, cholesterol; DCP, dicetyl phosphate; SM, sphingomyelin.

cially inhibited by *N*-acetylgalactosamine, *N*-acetylglucosamine, or galactose.

The relative inhibitory level of liposomal globoside was partly dependent on the amount of anti-liposome antibody employed (Figs. 2 and 3). At a liposomal globoside concentration of 5.8%, inhibition of anti-liposome antibody was largely overcome by increasing the amount of antibody (Figs. 2 and 3). However, at 7.8 and 9.7% globoside complete inhibition of anti-liposome antibody occurred, and inhibition was not overcome by increasing the concentration of antibody (Fig. 3).

Influence of phospholipid size on inhibition by globoside

We have previously shown that the magnitude of immune damage induced by antibodies to liposomes, when tested with liposomes containing different phospholipids, differs in the following order: immune damage to liposomes containing DMPC > DPPC > DSPC > sphingomyelin [13].

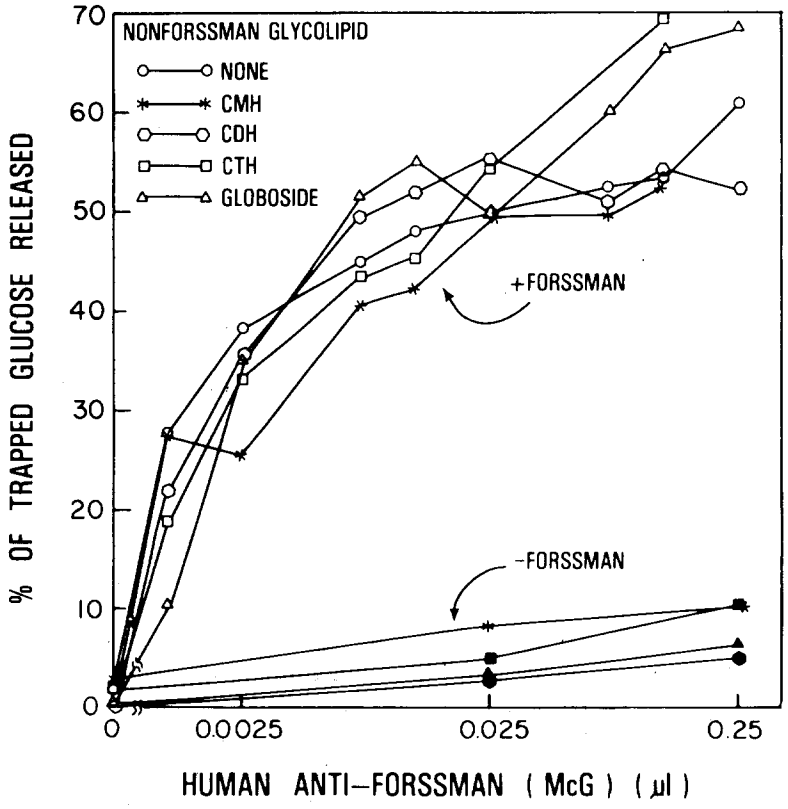

Fig. 5. Influence of glycolipid composition on immune damage to liposomes induced by a human IgM (McG) monoclonal antibody to Forssman glycolipid. The liposomes contained either Forssman glycolipid (10 nmol/μmol of DMPC, or 1%), CMH, CDH, CTH, or globoside (10%), or Forssman glycolipid (1%) combined with CMH, CDH, CTH, or globoside (10%). The antibody source consisted of 19.6 μg of purified McG protein per μl of 0.15 M NaCl [7].

Fig. 4 illustrates this observation, and also shows that the presence of globoside in each of these liposomes induces complete inhibition of immune damage by anti-liposome antibody.

Influence of globoside on monoclonal anti-Forssman antibody

Human monoclonal IgM anti-Forssman antibody (McG, a Waldenström macroglobulin) reacts with liposomal Forssman glycolipid but not with liposomal globoside (Ref. 7 and Fig. 5). As shown in Fig. 5, the simultaneous presence of globoside and Forssman glycolipid in the same liposomes did not inhibit immune damage induced by the anti-Forssman antibody.

Discussion

More than 50 different kinds of glycosphingolipids have been identified in animal organs [1]. Although numerous possible functions of glycolipids have been described, the glycolipids are usually thought of as being important elements in the 'information-controlling' machinery of the cell [1–4]. Because of the large potential amount of coded information in the oligo- and polysaccharide sequences and structures, the glycolipids are often thought of as being targets or foci for binding of extracellular molecules [1–4]. The present study suggests the possibility that microheterogeneity among glycolipid saccharide moieties also can influence the binding of extracellular proteins to nonglycolipid structures in the lipid bilayer.

In this study we demonstrate that a monoclonal antibody to liposomes lacking glycolipids was inhibited when globoside, a ceramide tetrasaccharide, was included in a concentration of more than 4% in the lipid bilayer. Similar, or higher, concentrations of CTH, CDH, or CMH did not inhibit antibody binding. Globoside did not exert a nonspecific broad-based 'immuno-inhibitory' activity since its presence did not inhibit the activity of a monoclonal antibody to Forssman glycolipid (ceramide pentahexoside) in liposomes. The monoclonal antibody to liposomes, which has been extensively described in a previous report [11], also did not bind specifically to globoside itself. If it had bound specifically to globoside then complement damage to liposomes would have been enhanced rather than suppressed by globoside.

Globoside constitutes over 65% of the glycolipid on the outer surface of human erythrocyte membranes [1,5]. The ratio of total phospholipid/globoside in the human erythrocyte is approximately 6/1 [5]. However, virtually all of the globoside is thought to be on the outer half of the lipid bilayer, and therefore the ratio of phospholipid/globoside on the surface of the erythrocyte may be closer to 3/1.

Based on the data shown in Fig. 2, the activity of the anti-liposome antibody was completely inhibited by an 8% concentration of liposomal globoside. Therefore complete inhibition of the antibody occurred at a phospholipid/globoside ratio of 12.5/1, and two globoside molecules would thus be separated by 25 phospholipid molecules. If one assumes that the globoside molecules were randomly distributed in the liposome membrane and that the average diameter of DMPC mixed with cholesterol is 8 Å [14], then the globoside molecules were separated by an average distance of approx. 200 Å. Analysis of space-filling Corey-Pauling-Koltun (CPK) models [15] reveals that the tetrasaccharide of globoside extends approx. 23 Å beyond the hydrophobic portion of the molecule. Therefore at a liposomal globoside concentration of 8% the open lipid bilayer space between two adjacent globoside tetrasaccharides at the surface of the liposome would be (200 Å-46 Å), or 154 Å. This distance is very similar to the longest dimension (142 Å) of the antigen-binding end (including two Fab regions) of an IgG molecule, as estimated from a balsa wood model based on X-ray diffraction data [16].

It should be borne in mind that the above calculation of distances between adjacent globoside oligosaccharides relies on certain assumptions, and therefore may not be perfectly precise. For example, it is conceivable that the oligosaccharide chain of globoside in liposomes may not have the same theoretical degree of freedom of motion envisioned by static Corey-Pauling-Koltun models. However, the calculation does make the point that with inhibitory concentrations of glycolipid distances between individual glycolipid molecules can approximate the size of a 7S immunoglobulin unit. The IgM (19S) antibody used in the present study

consists of a pentamer of 7S subunits, but the effective size of a 7S binding region might also be influenced by the bulkiness of the total IgM molecule. In any case the ratio of phospholipid/globoside in the erythrocyte is only 3/1 (see above) and calculations similar to those for liposomes reveal that only approx. 52 Å would separate adjacent globoside tetrasaccharides on the surface of erythrocytes. This distance between adjacent globoside molecules might be expected to be too small for penetration of an antibody molecule.

The observations and calculations described above suggest that a novel function of glycolipids might be the modulation of binding of extracellular proteins to the lipid bilayer that separates the glycolipids. In this regard, naturally-occurring antibodies to lipid bilayers have been described in normal human sera [17,18]. Binding of these antibodies to cell membranes might be inhibited by membrane glycolipids such as globoside. This could explain how these antibodies could exist in the circulation, and how they could occur without causing any apparent damage to cell membranes.

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