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Inhibitory effects of gangliosides on immune reactions of antibodies to neutral glycolipids in liposomes

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Specific immune damage to liposomes containing Forssman or globoside glycolipid was inhibited when the liposomes also contained ganglioside. The activity of a human monoclonal Waldenström macroglobulin antibody to Forssman glycolipid was inhibited by each of three gangliosides tested, G_{M3}, G_{D1a} and G_{D1b}. Inhibition of the monoclonal antibody was dependent on the amount of ganglioside in the liposomes, and was diminished by reducing the relative amount of ganglioside. Inhibition also correlated positively with the number of ganglioside sialic acid groups, with inhibition by $G_{T1b} > G_{D1a} > G_{M3}$. Naturally occurring human antibodies to globoside glycolipid were detected in 18% (9 out of 50) of normal human sera tested. Immune damage to liposomes induced by each of the three highest-reacting human anti-globoside sera was blocked by liposomal G_{M3}. We conclude that gangliosides can strongly influence immune damage to membranes induced by antibody interactions with adjacent neutral glycolipids.

Introduction

Immune reactions of antibodies against lipids are often complicated by the small sizes of many antigenic lipid molecules. Antibodies to small antigenic lipids sometimes encounter potential interference by adjacent proteins or larger lipids. For example, previous studies from this laboratory have demonstrated steric hindrance by adjacent phospholipids that inhibited the binding of antibodies to galactosyl ceramide in liposomes [1,2]. The inhibition to antibody binding was overcome by elevated temperature [3]. Conversely, we have recently demonstrated interference by a glycolipid (globoside, or ceramide tetrahexoside) that inhibited immune damage induced by a monoclonal antibody to liposomal phospholipids [4]. Inhibition by liposomal globoside occurred at a globoside epitope density such that the distance between adjacent globoside oligosaccharide groups approximated the molecular dimensions of the binding end of a 7 S immunoglobulin [4].

In the course of investigating the inhibitory effects of neutral glycolipids on the binding of antibodies to liposomal phospholipids, we inci-

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Abbreviations: globoside, ceramide tetrahexoside, GalNAc(β1 → 3)Gal(α1 → 4)Gal(β1 → 4)GlcCer; Forssman, ceramide pentahexoside, GalNAc(α1 → 3)GalNAc(β1 → 3)Gal(α1 → 4)Gal(β1 → 4)GlcCer; G_{M3}, NeuAc(α2 → 3)Gal(β1 → 4)-GlcCer; G_{D1a}, NeuAc(α2 → 3)Gal(β1 → 3)GalNAc(β1 → 4)-Gal[(3 ← 2α)NeuAc](β1 → 4)GlcCer; G_{D1b}, Gal(β1 → 3)-GalNAc(β1 → 4)Gal[(3 ← 2α)NeuAc(8 ← 2α)NeuAc](β1 → 4)GlcCer; G_{T1b}, NeuAc(α2 → 3)Gal(β1 → 3)GalNAc(β1 → 4)Gal[(3 ← 2α)NeuAc(8 ← 2α)NeuAc](β1 → 4)GlcCer; Glc, glucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; NeuAc, *N*-acetylneuraminic acid; DMPC, dimyristoylphosphatidylcholine.

dentally observed, as reported in this paper, that gangliosides caused marked inhibition of antibodies both to liposomal phospholipids and to neutral glycolipids, including globoside and Forssman glycolipid (ceramide pentahexoside), and that this occurred even though the neutral glycolipids were larger than the gangliosides. Although gangliosides themselves can serve as excellent antigens for complement-dependent immune damage to liposomes, apparently they are also potent inhibitors of immune damage induced by antibodies to adjacent neutral glycolipids.

Materials and Methods

Lipids

Lipids were purchased from the following sources: dimyristoylphosphatidylcholine (DMPC) and cholesterol (Calbiochem-Behring, La Jolla, CA); dicetyl phosphate (K & K Laboratories, Plainview, NY); *N*-lignoceroyl-DL-dihydrogalactocerebroside (ceramide monohexoside) and *N*-lignoceroyl-DL-dihydrolactocerebroside (ceramide dihexoside) (Miles Laboratories, Inc., Kankakee, IL). Ceramide tetrahexoside (globoside) and Forssman glycolipid (ceramide pentahexoside) were extracted and purified from human and sheep erythrocytes, respectively, as described previously [2].

The synthetic glycolipids (ceramide mono- and dihexoside) were quantified by weight. The glycolipids purified from erythrocytes were quantified on TLC plates using a Zeiss KM3 TLC spectrophotometer [5]. After applying different concentrations of glycolipid on the TLC plate, the plate was sprayed with orcinol reagent [6], 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 [2].

Purified gangliosides (G_{M3}, G_{D1a}, G_{T1b}) were either purchased from Supelco (Bellfonte, PA) or were kind gifts from either Drs. Peter Fishman and Roscoe Brady at the National Institutes of Health, Bethesda, MD [7], or Dr. Gino Toffano at FIDIA Research Laboratories, Abano Terme, Italy [8]. With time, G_{T1b} slowly degraded into G_{D1b}. When necessary, impurities were removed by repurification using preparative thin layer chromatography.

Preparation of liposomes. Previous publications have described extensively detailed general methodology of liposome (multilamellar vesicle) preparation [9], and preparation and immune reactivity of liposomes containing Forssman glycolipid [2], globoside glycolipid [2], or individual purified gangliosides [10]. The 'basic' liposome preparation contained dimyristoylphosphatidylcholine (DMPC), cholesterol and dicetyl phosphate in molar ratios of 1 : 0.75 : 0.11. When present, Forssman, globoside or ganglioside were also present in the molar ratios specified in the individual figures. The liposomal glycolipid concentration is often expressed as percent, and this term is defined as (nmol glycolipid/nmol phospholipid) × 100. The lipids initially in chloroform or chloroform/methanol (1 : 1) were dried together in a thin film in a pear-shaped flask using a rotary evaporator. The lipids were hydrated with 0.308 M glucose by shaking with a vortex mixer to form liposomes. The volume of glucose was selected such that the concentration of DMPC was 10 mM in the glucose. Untrapped glucose was removed by dialysis for 1.5 h against 1250 vol. 0.15 M NaCl.

Serum and antibody sources. Pooled fresh guinea pig serum was used as a complement source. Samples of 50 individual normal human sera were a kind gift from Dr. Wendell Zollinger in the Department of Bacterial Diseases at WRAIR, and consisted of preimmunization bleedings of volunteers in a vaccine testing program. The monoclonal Waldenström macroglobulin IgM(κ) antibody to Forssman has been described previously [2].

Immune damage to liposomes

The assay for measuring complement-dependent immune damage to liposomes has been described in detail elsewhere [9]. In brief, an assay cuvette contained 0.3 ml of glucose assay reagent (consisting of Tris-buffered saline, hexokinase, glucose-6-phosphate dehydrogenase, ATP and

NADP), liposomes (3 μl), an antibody source, 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 percent of trapped glucose released, and the total amount of glucose trapped was determined by disrupting the liposomes with chloroform.

Results

Inhibition of anti-Forssman activity by ganglioside G_{M3}

Complement-dependent damage to Forssman-containing liposomes mediated by monoclonal IgM anti-Forssman antibody is shown in Fig. 1. The anti-Forssman antibody was equally active at room temperature and at 35°C. However, when ganglioside G_{M3} was incorporated into the liposomes, immune damage mediated by anti-Forssman antibodies was completely inhibited (Fig. 2). The inhibitory effect of G_{M3} was dose-dependent, with an intermediate effect at a G_{M3}/Forssman ratio of 20 : 1, and complete inhibition at 30 : 1. In contrast, inhibition of anti-Forssman activity was not observed when a neutral glycolipid (ceramide mono- or dihexoside) or ceramide was included in the liposomes instead of G_{M3} (Fig. 3).

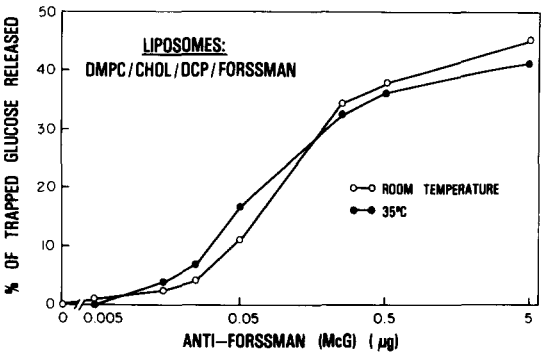

Fig. 1. Immune damage induced by a human monoclonal antibody to Forssman glycolipid. The liposomes contained a molar concentration of 0.6% Forssman glycolipid (concentration based on comparison with DMPC). Each curve represents a separate liposome preparation and was generated by eight separate assays with different quantities of purified human monoclonal anti-Forssman antibody. The differences between the curves were within the range of variability of the glucose release assay. Chol, cholesterol; DCP, dicetyl phosphate.

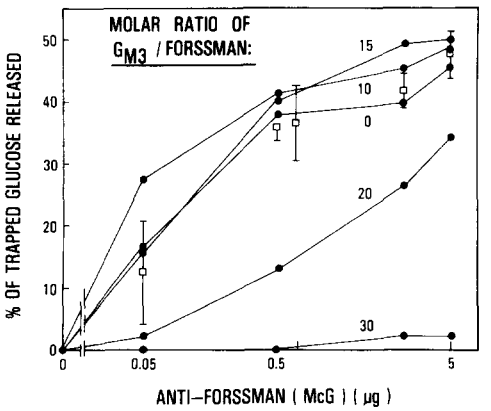

Fig. 2. Inhibition of immune damage to liposomes containing Forssman glycolipid and increasing quantities of ganglioside G_{M3}. Each line represents a separate liposome preparation containing 0.6% Forssman glycolipid and the indicated amount of ganglioside G_{M3}, and was generated by five separate assays with different quantities of purified human monoclonal anti-Forssman antibody. Most of the individual values for the curves derived from liposomes containing molar ratios of G_{M3}/Forssman (10 : 1 and 15 : 1) were within the theoretical range of variability for the glucose release assay with different liposomes. However, slight average enhancement of glucose release was often observed with liposomes containing low levels of certain glycosphingolipids (see Figs. 3 and 5a for further examples of this phenomenon, and Refs. 5 and 6 for further discussion of it). All of the values for the G_{M3}/Forssman (20 : 1 and 30 : 1) liposomes were well below the range of variability of the assay when compared with liposomes lacking G_{M3}. The open box symbols represent the means (±S.D.) of glucose release from liposomes lacking G_{M3} taken from nine experiments on different days.

Effect of temperature

Previous studies have demonstrated that incubation temperature can sometimes have a marked influence on immune reactions against glycolipids in liposomes [3]. When the size of the antigenic carbohydrate group is relatively small, steric hindrance to antibody binding can sometimes be exerted by adjacent lipid molecules at room temperature, but steric hindrance is usually overcome at higher temperatures [3]. As shown in Fig. 4, inhibition of anti-Forssman activity by G_{M3} was not reversed by running the incubation at 35°C rather than at room temperature. This observation does not eliminate the possibility that G_{M3} exerted some form of temperature-independent steric interference on antibody binding. However, any such adverse steric effect induced

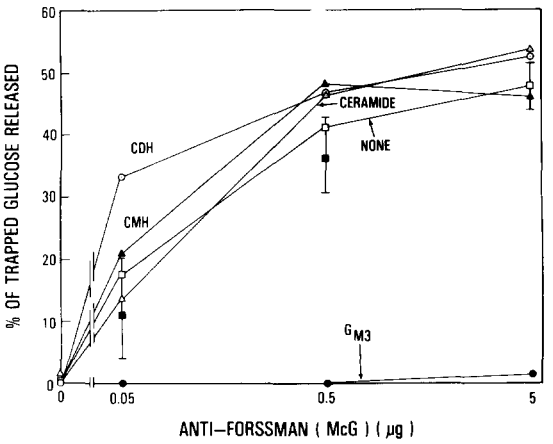

Fig. 3. Lack of inhibition by ceramide, ceramide monohexoside (CMH), or ceramide dihexoside (CDH). Each curve represents a separate liposome preparation containing Forssman glycolipid and other ceramide lipid as indicated. The curves were each generated with four separate assays with different quantities of purified human monoclonal anti-Forssman antibody. Each liposome contained 0.6% Forssman glycolipid and, where indicated, 18% ceramide, CMH, CDH, or G_{M3}. The curves for ceramide, CMH and CDH were within, or close to, the ranges of variability for the glucose release assay when compared to liposomes lacking ceramide or ceramide-linked glycolipid. However, as noted in the legend to Fig. 2, there was a consistent tendency to see slightly elevated values for those preparations that were not inhibitory to immune damage. The closed box symbols represent the means (±S.D.) of nine experiments with Forssman liposomes lacking ceramide, CMH, CDH or G_{M3}.

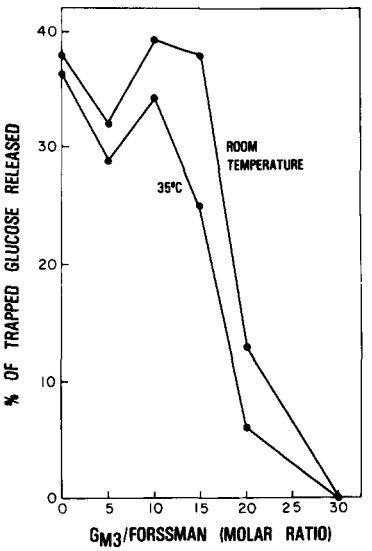

Fig. 4. Effect of G_{M3} concentration on inhibition of immune damage to liposomes containing Forssman glycolipid. Each point represents a separate liposome preparation containing one of seven indicated ratios of G_{M3}/Forssman, and the data were obtained either at room temperature (approx. 25°C) or 35°C, as shown. For each liposome preparation, the Forssman concentration was held constant at 0.6%, and the G_{M3} concentration was varied at seven different amounts as indicated. The antibody source consisted of 0.5 μg of purified monoclonal human antibody to Forssman glycolipid.

by G_{M3} was not likely to have been caused by simple bulkiness of adjacent molecules, because Forssman glycolipid is larger than G_{M3}. It is also likely that simple bulkiness would be overcome by temperature-induced increased antigenic motion as reported earlier [3].

Effects of other gangliosides

Every ganglioside tested caused inhibition of anti-Forssman antibody activity. The degree of

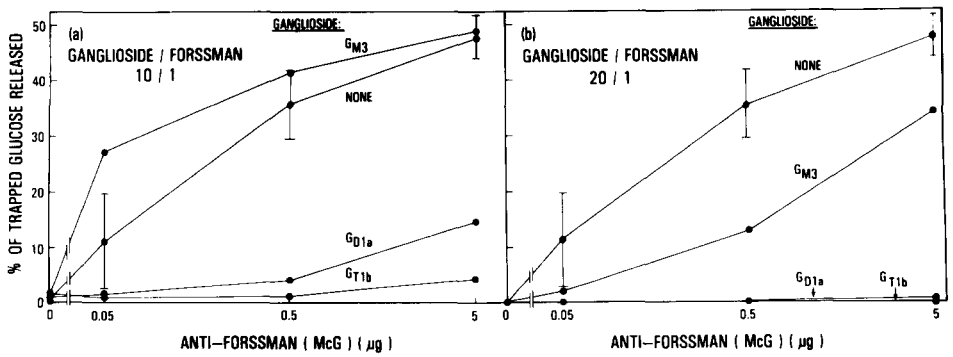

Fig. 5. Inhibition of anti-Forssman antibody by G_{M3}, G_{D1a} and G_{T1b} gangliosides. Each curve was generated by running four separate assays at 30°C, each assay using a differing amount of purified monoclonal human antibody to Forssman. For the curves lacking ganglioside each point is the mean (±S.D.) of nine separate experiments.

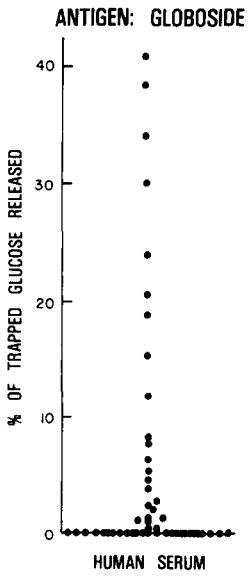

Fig. 6. Occurrence of natural antibodies to globoside among 50 normal human sera. Each point represents immune damage induced by normal human serum from liposomes containing 9.7% globoside. Each assay was run at 35°C and utilized 3 μl of liposomes, 20 μl of heated (56°C, 30 min) human serum as an antibody source, and 40 μl of fresh guinea pig serum as a complement source.

inhibition did correlate with the number of sialic acid groups present on the ganglioside. As shown in Fig. 5a, at a ganglioside/Forssman ratio of 10:1 there was no inhibition by G_{M3}, strong inhibition by G_{D1a}, and almost complete inhibition by G_{T1b}. At a ganglioside/Forssman ratio of 20:1, intermediate inhibition occurred with G_{M3}, and complete inhibition occurred with G_{D1a} and G_{T1b} (Fig. 5b).

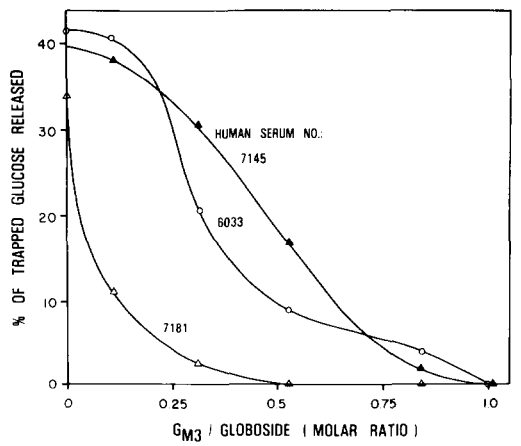

Fig. 7. Inhibition of naturally occurring human antibodies to globoside by G_{M3}. Three of the highest-reacting sera shown in Fig. 6 were tested for inhibition by G_{M3}. Each point represents one of six separate liposome preparations containing 1% globoside and a differing amount of G_{M3} as indicated. Each curve was generated using a different serum source.

Inhibition of naturally occurring human antibodies to globoside

We and others have previously demonstrated the occurrence of naturally occurring human autoantibodies to globoside [11–13]. Fig. 6 shows the results of liposomal assays performed for detecting anti-globoside antibodies among 50 individual normal human sera. Under the conditions shown, 9 out of 50 (18%) of the sera caused more that 10% release of trapped glucose. Three of the highest-reacting anti-globoside sera were tested for inhibition by ganglioside. As shown in Fig. 7, each of the three sera was completely inhibited by a low concentration of liposomal G_{M3}.

Discussion

Glycolipids constitute major fractions of the lipid bilayers of all mammalian cell membranes. It has been estimated that as many as 30–60% of the lipid molecules on the outermost lipid layer of erythrocytes consist of glycosphingolipids, of which approximately 5% are gangliosides [14]. Although gangliosides themselves are immunogenic molecules, we report in the present study that gangliosides in the lipid bilayer can have inhibitory effects on immune reactions of antibodies to neutral glycolipids.

Lipids are rather small molecules and the accessibility of the antigenic region at the surface of a lipid bilayer membrane can be highly restricted. We have previously shown that phospholipids can influence the binding of antibodies to glycolipids [1–3], and that glycolipids can influence the binding of antibodies to phospholipids [4]. It is well-known that sialylated membrane proteins can interfere with the binding of antibodies and lectins to cell surface glycolipids [15–17]. Gangliosides have also been reported to inhibit the activation of the alternative pathway of complement by liposomes [18]. However, in the present study the classical pathway of complement was activated by

a monoclonal IgM antibody to Forssman glycolipid [2].

It is clear that interference with antibody binding to lipids can occur by simple steric hindrance due to constrained availability of antigen in a small space at the membrane surface [1–3]. Inhibition caused by bulky adjacent lipids may be overcome by a higher reaction temperature [3]. Analysis of space-filling models suggested that simple blocking of antibody binding to phospholipids could occur when the average distance between adjacent glycolipid saccharide groups approached the dimensions of a 7 S immunoglobulin molecule [4]. However, it also seems likely from previous reports that a negatively charged sugar (sialic acid) can influence antibody binding to neutral glycolipids [15–17]. Inhibition of antibody binding to globoside on human erythrocytes was reversed by neuraminidase [16,17]. Antibody binding to liposomal Forssman glycolipid was inhibited by glycophorin, a sialic acid-containing glycoprotein from erythrocytes [19]. Inhibition of the antigenic activity of liposomal Forssman glycolipid by glycophorin was attributed to a 'carbohydrate-carbohydrate' interaction between the glycolipid and the sialoglycoprotein [19].

The findings in the present study are more compatible with intermolecular interactions between sialoglycolipids and neutral glycolipids (as in Ref. 19) than with simple blocking of antibody binding by adjacent bulky lipids (as in Ref. 4). The size of G_{M3} is smaller than the size of either globoside or Forssman glycolipid. If there were no interactions between adjacent ganglioside and neutral glycolipid saccharides, then the terminal sugar, which is the immunodominant group of Forssman and globoside, should be sufficiently far extended beyond the plane of the membrane to bind antibody without interference by ganglioside. Moreover, the degree of inhibition by ganglioside was correlated with the number of sialic acid groups (Fig. 5). In addition to sialic acid interactions with neutral carbohydrates, it is possible that headgroup interactions between gangliosides and phospholipids also played a role. It has been shown that the latter interactions do occur in liposomes, and that this leads to changes of membrane fluidity [20].

It should be pointed out that naturally occurring human autoantibodies to globoside were completely inhibited by small concentrations of G_{M3} (Fig. 7). Autoantibodies both to globoside and to many other lipids have widespread distributions in sera from humans and other species (Refs. 9–13, and Fig. 6). It seems likely that pathological consequences induced by such antibodies would be prevented or minimized by inhibitory effects such as those shown in Fig. 7.

In human erythrocytes, globoside serves as the P blood group substance [21]. Antibodies in the P blood group system are cold agglutinins and assays for such antibodies are not normally performed at room temperature. However, in the presence of 'warm-reacting' anti-globoside antibodies, erythrocytic globoside is a cryptic antigen [15–17]. It is likely that the crypticity of erythrocyte globoside is mainly due to overlying sialoglycoproteins rather than to gangliosides. The crypticity is reversed either by trypsin or by sialidase [16,17], and the concentration of erythrocyte gangliosides is too low to achieve the ganglioside/globoside ratios described in many of the experiments in this report [14].

In cells other than erythrocytes, gangliosides might easily achieve sufficiently high concentrations in the plasma membrane to cause inhibition of antibodies to neutral glycolipids. For example, many glycolipids have been identified as tumor-associated antigens [16,22]. It may not be coincidental that drastic increases in cellular ganglioside concentrations are often associated with tumor cells and tumorigenesis [23].

From these studies it appears that gangliosides may have a modulating influence on the binding of antibodies to neutral glycolipids. Although not specifically addressed in these experiments, it also seems reasonable to suggest that a novel and not often recognized function of gangliosides might be to regulate the binding of proteins other than antibodies to certain neutral glycolipids and phospholipids in the lipid bilayer.

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