Influence of Membrane Composition on the Interaction of a Human Monoclonal "Anti-Forssman" Immunoglobulin with Liposomes†

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ABSTRACT: This study utilizes a unique "anti-Forssman" monoclonal immunoglobulin (Waldenström macroglobulin, or WM), which was isolated from the plasma of a patient (McG), to investigate the mechanisms of immune reactions with liposomes. The purposes were to (1) examine the reactivity of the WM as an "antibody" source against Forssman in liposomes and cells, (2) determine the relative degree of specificity of the interaction with a variety of highly purified glycosphingolipids in liposomes, and (3) utilize the homogeneous immunoglobulin to study the influence of membrane composition on the antigen–antibody binding characteristics. The interactions of the macroglobulin with Forssman hapten and complement were apparently analogous to those of anti-Forssman antibodies from hemolysin (rabbit anti-sheep erythrocyte serum). Hemolysin, anti-human erythrocyte serum, and anti-galactocerebroside serum were all compared with the macroglobulin for specificity with liposomes containing one of the following glycosphingolipids: glucocerebroside, galactocerebroside, lactocerebroside (cytolipin H), globoside I, or Forssman. Each of the antisera reacted with two of the haptens, while WM reacted only with Forssman. These experiments demonstrated a marked similarity between the WM and the hemolysin anti-Forssman antibodies. The two could be distinguished, however, in the relative binding affinity to Forssman hapten. Under conditions in which the liposomal antigen concentration was limiting, the affinity of WM for Forssman, as reflected by relative glucose release from liposomes, was much less than those of hemolysin, or IgM, or IgG fractions from hemolysin. Glucose release due to WM was decreased still further in these latter experiments when a long chain phospholipid (sphingomyelin) was substituted for shorter chain phospholipids (*e.g.*, dimyristoyllecithin). By means of adsorption experiments it was demonstrated that sphingomyelin-containing liposomes bound fewer hemolytic or glucose-releasing anti-Forssman WM antibodies than did dimyristolyllecithin-containing liposomes. It was felt that the Forssman substance was probably partially "buried" in the sphingomyelin bilayer, thus accentuating the decreased affinity of the WM immunoglobulin. From this it was concluded that the liposomal phospholipid chain length may influence the binding infinity of an antibody to a membrane-associated antigen.

Recently, a human monoclonal IgM κ (Waldenström macroglobulin, or WM)[1] was discovered which agglutinated sheep erythrocytes and hemolyzed them in the presence of complement. The protein did not react with certain cells which were known to lack Forssman antigen.[2] In a brief study, complement-dependent glucose release from Forssman-containing liposomal model membranes was also observed in the presence of WM (Joseph *et al.*, 1974a). The major purpose of the present investigation was to examine in detail the properties and relative specificity of this unique monoclonal protein with regard to interactions with liposomes which were prepared from various natural or synthetic phospholipids and which contained Forssman hapten, or other haptenic glycolipids. The "antibody-like" activity of this immunoglobulin, and apparent relative affinity to Forssman hapten in liposomes, was compared with corresponding activities and apparent relative affinities of hemolysin or IgG or IgM fractions isolated from hemolysin.

The initial immunological studies of liposomal model membranes were performed with Forssman hapten (for review, see Kinsky, 1972). Anti-sheep erythrocyte serum (hemolysin) was invariably used as a source of anti-Forssman activity. Hemolysin was shown to be "specific" in that cross-reactivity did not occur with a closely related glycolipid (globoside I) (Inoue *et al.*, 1971). Although virtually all of the hemolytic antibody activity was removed by absorption with liposomes containing a mixture of sheep erythrocyte lipids (Alving *et al.*, 1969), only a fraction of the antibodies (about 65%) were removed by liposomes containing purified Forssman hapten (Kinsky *et al.*, 1969). This latter observation suggested the possibility that hemolysin was a "multispecific" antibody source and that some of the antibodies in hemolysin might have been directed against other uncharacterized glycolipid antigens of sheep erythrocytes. In the present study, the question of antibody specificity against haptens in liposomes was examined by utilizing a homologous series of purified sphingosyl glycolipids and

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[1] Abbreviations used are: WM, Waldenström macroglobulin; McG, contracted name of the patient from whose plasma the macroglobulin was isolated, and also used as a synonym for the macroglobulin; di-14:0 PC, di-16:0 PC, di-18:0 PC, synthetic lecithins consisting respectively of dimyristoyl-, dipalmitoyl-, and distearoylphosphatidylcholine; egg PC, egg lecithin; SM, sphingomyelin; DCP, dicetyl phosphate; Glu-cer and Gal-cer, gluco-, and galactocerebroside, respectively; anti-HRBC, anti-human erythrocyte serum; hemolysin, anti-sheep erythrocyte serum; GPS, guinea pig serum.

[2] W. Hammack and R. Wistar, manuscript in preparation.

several different antisera. The antisera were compared for specificity with the Waldenström macroglobulin.

The goal of many liposome experiments, to date, has been to simplify the model to a maximum extent and thereby increase the resolution of the immunological studies. Liposomes, which are artificial membranes, have an advantage as a model in that they are protein free and many of the membrane variables, particularly the antigen type and concentration and the membrane lipid composition, can be controlled. In a recent investigation (Alving *et al.*, 1974) the phospholipid composition of the liposomal membrane was found to exert an important influence on homologous immune reactions and cross-reactions. The extent of complement-dependent glucose release from liposomes in the presence of anti-galactocerebroside serum was inversely related to the fatty acyl chain lengths of synthetic phospholipids. The suggestion was made that the glycolipid antigen might have become progressively more "buried" in the phospholipid bilayer as progressively longer chain phospholipids were used, thus interfering with the antigen–antibody interaction. It was felt, therefore, that under certain conditions the relative accessibility of the antigen at the membrane might vary, and that this might influence the relative degree of immune damage. The possibility of a "steric" influence has also been introduced elsewhere, based on studies with egg lecithin and beef sphingomyelin (*cf.* Kinsky, 1972). These results raised the possibility that the relative affinity of an antibody for an antigen might vary depending on the membrane composition. This concept clearly has important implications for any study, such as the present one, in which either antibodies or antigens are being compared. Because of these "phospholipid effects," one of the additional aims of the present investigation was to provide evidence relating to the influence of phospholipid on the interactions of the Waldenström macroglobulin with Forssman hapten.

Materials and Methods

Many of the methods and sources of materials which were used have been described in previous publications, as indicated below.

Lipids. Natural and synthetic phospholipids, cholesterol, dicetyl phosphate, and beef brain galactocerebroside (*cf.* Conrad *et al.*, 1974; Alving *et al.*, 1974), synthetic *N*-lignoceroyl dihydrolactocerebroside (ceramide dihexoside, or cytolipin H), and *N*-lignoceroyl dihydroglucocerebroside were purchased from Miles Laboratories, Inc., Kankakee, Ill.

Phosphate Analysis. Phosphates were measured by the method of Gerlach and Deuticke (1963).

For enzymes and cofactors, cf. Conrad *et al.* (1974).

Preparation of Liposomes and Measurements of Trapped Glucose and Immune-Specific Glucose Release (*cf.* Kinsky *et al.*, 1969, 1970; Conrad *et al.*, 1974; Alving *et al.*, 1974). In each case the liposomes contained phospholipid, cholesterol, and dicetyl phosphate in molar ratios of 2:1.5:0.22 plus, where indicated, Forssman (or other glycolipid) in the amounts given in the appropriate figure or table. The liposomes were dispersed in sufficient 0.3 M glucose (or, where indicated, 0.15 M NaCl) such that the phospholipid was 10 mM with respect to the final suspension. The method for measuring glucose release from liposomes is briefly summarized as follows. The reaction mixture contained a final total volume of 1.0 ml in a semimicro cuvet and consisted of glucose assay reagent (500 μl), 0.15 M NaCl, liposomes, antiserum (or WM), and guinea pig serum (as a source of complement). Initially, one of the soluble immunological reactants (antibodies or complement) was omitted. The absorbance at 340 nm (A_{340}) was measured after 10 min and corrected for volume (to 1.0 ml). The reaction was then started by adding the final soluble reactant (antibodies or complement, as appropriate) and the A_{340} was measured again after 30 min at room temperature (*ca.* 22°). The A_{340} was corrected for the increased absorbance caused by addition of the final reactant, the absorbancy of which was determined separately. In contrast to certain cells, liposomes usually do not undergo light scattering changes as a result of immune damage and therefore do not need to be removed from the reaction medium. The difference between the corrected initial and final absorbancies was a value which was a direct function of the amount of glucose released. It was expressed as a per cent of the A_{340} observed due to release of all of the trapped liposomal glucose. This latter value was also measured separately after dissolving the liposomes in chloroform and Triton X-100 (Kinsky *et al.*, 1969, 1970). Further specific details are given in the appropriate figure or table legends.

Sera.[3] The fresh guinea pig serum, used as a complement source, and the rabbit antisera, were obtained and processed as previously described: GPS and rabbit anti-galactocerebroside serum (Conrad *et al.*, 1974); rabbit antisheep erythrocyte serum (hemolysin) (Haxby *et al.*, 1968); rabbit anti-human erythrocyte serum (Mayer, 1961).

Preparation of IgG and IgM Fractions from Hemolysin. A globulin fraction was obtained from 44 ml of hemolysin by precipitation with ammonium sulfate (50% of saturation). The ammonium sulfate was removed by dialysis against 0.01 M phosphate buffer (pH 7.5), and the protein was applied to a column of diethylaminoethyl cellulose (2.5 cm × 40 cm) equilibrated with the same buffer. The void volume contained only IgG when tested by immunoelectrophoresis with a polyvalent sheep antiserum to whole rabbit serum.

After removal of the IgG fraction, a linear gradient, consisting of 0.01 M phosphate buffer (pH 7.5) and 0–0.3 M NaCl (total volume 800 ml), was pumped through the column. Fractions of 20 ml each were assayed, using microtiter plates, for hemolytic activity against sheep erythrocytes. The protein corresponding to the peak of hemolytic activity was pooled, concentrated, and passed through a Sephadex G-200 column in a buffer consisting of 0.05 M Tris-HCl (pH 8.0), 0.045 M NaCl, and 0.02% NaN_3. The initial peak was pooled and concentrated. On immunoelectrophoresis it contained IgM, but no detectable IgG.

Isolation and Properties of the Waldenström Macroglobulin. This has been briefly described in a previous report (Joseph *et al.*, 1974a) and will be outlined in detail elsewhere (W. Hammack and R. Wistar, manuscript in preparation). The light chain type of WM was κ and the preparation contained <1% of contaminating "normal" IgM, as judged by the presence of λ light chains.

Absorption of WM with Liposomes. Aliquots (12.25 μg) of WM were incubated for 30 min at room temperature

[3] In conducting the research described in this report, the investigators adhered to the Guide for Laboratory Animal Facilities and Care, as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences.

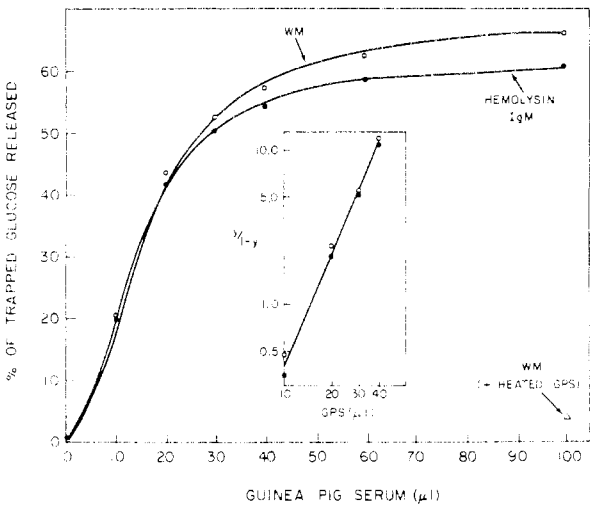

FIGURE 1: Comparison of liposomal complement titration curves obtained either with hemolysin IgM or with WM. The liposomes consisted of di-16:0 PC, cholesterol, dicetyl phosphate plus 6 nmol of Forssman/μmol of PC. In a semimicro cuvet, liposomes (4.7 μl) were preincubated for 10 min either with fresh (unheated) or heated (56°, 1 hr) GPS in the amounts shown on the abscissa, and 0.15 M NaCl in a total volume of 880 μl. After reading the initial A_{340}, the reaction was started by adding either the hemolysin IgM fraction (0.143 mg) or WM (0.490 mg). The final absorbancies were measured 30 min later. Glucose release was calculated after making the appropriate corrections (see Materials and Methods).

with appropriate quantities of liposomes (which had been dispersed in 0.15 M NaCl instead of 0.3 M glucose) (*cf.* Figures 4 and 5). Enough 0.15 M NaCl was added to make a total volume of 750 μl. The liposomes were removed by centrifugation at 15,000 rpm (27,000*g*) in 15-ml Corex tubes for 10 min at 20°; 500 μl of each supernatant was removed; 375 μl was used as an antibody source against liposomes (Figure 4) and 50 μl was used as an antibody source against erythrocytes (Figure 5).

Hemolytic Assay of Erythrocytes. The method used was that of Mayer (1961), using 10^8 sheep erythrocytes in a total reaction volume of 1.5 ml.

Isolation of Forssman and Globoside I. The preparation of erythrocyte ghosts and the initial membrane extractions were performed respectively by the methods of Dodge *et al.* (1963) and Bligh and Dyer (1959) as previously modified (Haxby *et al.*, 1968; Kinsky *et al.*, 1969). The initial extraction was performed with chloroform, methanol, and water and separated into two phases, chloroform (fraction IIa) and methanol–water (fraction IIb) (Kinsky *et al.*, 1969). Forssman and, to a lesser extent, globoside I partitioned partly into IIb in addition to IIa. The IIb was further extracted twice with equal volumes of chloroform. The chloroform extracts from IIa and IIb were pooled together, concentrated to 7 ml with a rotary evaporator, and applied to a silicic acid column (6 g, 1 cm × 10 cm). Fractions were obtained using a modification of the method of Vance and Sweeley (1967). The column was developed with 100 ml of chloroform (fraction 1, or F1) (discarded). Occasionally, relatively high concentrations of pigments were present as contaminants and, when necessary, these were greatly reduced by running 50 ml of ethyl acetate through the column (fraction discarded). The column was then developed with 500 ml of acetone–methanol (9:1) (fraction 2, or F2) to remove the glycolipids. Saponification of the F2 was sometimes used as a convenient method of removing traces of contaminating non-sphingosyl lipids (Dittmer and Wells, 1969). Some losses were frequently observed in saponification. This step was optional with Forssman because such contamination did not usually occur at the R_F on thin-layer chromatography (tlc) corresponding to Forssman. Saponification was usually needed for complete purification of globoside I, which had a higher R_F. The F2 (or saponified F2) was concentrated and streaked on precoated tlc plates (silica gel 60, EM Laboratories, Inc., Elmsford, N. Y.), using $CHCl_3/CH_3OH/H_2O$ (75:31:4.5) as the solvent system. The R_F's of Forssman and globoside I were about 0.14 and 0.22, respectively. In order to locate the appropriate glycolipid the unsprayed plate was held to a strong light and each major visible band within the R_F range of 0.1–0.4 was scraped, leaving only a small unscraped region on one side of the plate. Generally, the only detectable (or the major) glycolipid in that range was either Forssman (from sheep erythrocytes) or globoside I (from human erythrocytes). The appropriate glycolipid band was then identified by spraying the unscraped areas with 0.2% (w/v) orcinol in 75% H_2SO_4. The scraped glycolipids were eluted with 150 ml of $CHCl_3/CH_3OH$ (1:2) on a sintered glass funnel.

Compared to other methods for isolating Forssman from sheep erythrocytes (Yamakawa *et al.*, 1960; Fraser and Mallette, 1973) it was felt that this procedure was simpler and gave a higher yield. About 1.5 mg of Forssman or 12 mg of globoside was obtained from 250 ml of whole sheep or human blood, respectively.

Chromatography. The purified lipids, including all of those commercially obtained, were routinely checked for purity by tlc on precoated silica gel 60 plates. The glycolipids contained all of the expected sugars and had appropriate ratios of sugars when subjected to gas–liquid chromatography (glc) as previously described (Joseph *et al.*, 1974a). The molar amounts of Forssman and globoside I were estimated by quantitative glc analysis of the glucose content using mannitol as an internal standard (Laine *et al.*, 1972).

Protein Analysis. Proteins were estimated by the method of Lowry *et al.* (1951) using bovine γ globulin as a standard.

Results

Comparison of the Immunological Efficiencies of WM IgM and Hemolysin IgM. Liposomal complement titration curves in the presence of either WM or hemolysin IgM are presented in Figure 1. As shown in the inset, the data of both curves gave the same straight line after being transformed by the von Krogh equation and plotted on logarithmic scales (Mayer, 1961; Haxby, 1970; Knudson *et al.*, 1971). In calculating the data of the inset, the "plateau" was assumed to represent "100%" glucose release. Using the data of Figure 1, the amounts of complement required for half-maximal glucose release (*i.e.*, when $y/(1 - y) = 1.0$) were identical (14.5 μl) when measured with liposomes which had been sensitized either with hemolysin or WM.

In separate experiments (not shown) the WM was approximately equivalent to hemolysin IgM fraction in terms of sensitizing ability when compared on a weight basis. A rigorous comparison of this type would be difficult to demonstrate under these conditions, however, because of the somewhat heterogeneous composition of the hemolysin IgM fraction. Despite this, this observation would exclude the possibility that these results might have been caused by contaminating normal IgM (non-WM) which might have been isolated, along with WM, from the plasma of the patient. As noted in the Materials and Methods section, the WM

TABLE I: Specificities of the Interactions of Several Antisera and WM with Various Glycosphingolipids.[a]

Antibody Source	% of Trapped Glucose Released from Liposomes Containing One of the Following Antigens:					
	None (control)	Glu-cer	Gal-cer	Cytolipin H	Globoside I	Forssman
WM	5.8	2.6	7.5	4.6	4.3	56.0
Hemolysin	0.5	5.5	3.2	57.3	10.6	56.5
Anti-HRBC	0.5	3.6	5.9	67.6	59.0	4.0
Anti-Gal-cer	2.6	0	60.7	61.0	1.6	1.6

[a] Six liposome preparations were made which contained di-16:0 PC, cholesterol, dicetyl phosphate, and which also contained one (or none) of the glycolipids as indicated. Glucocerebroside, galactocerebroside, and cytolipin H were each in concentrations of 150 μmol of PC (approximately 184, 184, and 154 nmol/μmol of PC, respectively). Globoside I and Forssman were in concentrations of 100 and 6 nmol, respectively, per μmol of PC. The liposomes were preincubated with either WM (0.196 mg), hemolysin (5 μl), anti-HRBC serum (25 μl), or anti-galactocerebroside serum (37 μl). Glucose release was measured 30 min after adding 120 μl of fresh GPS.

had <1% contamination with normal IgM and, if this were the reactive material, it can be calculated that "normal" IgM would have more than 50 times as much anti-Forssman activity as the IgM derived from hemolysin.

Comparison of the Immunological Specificity of WM with Those of Hemolysin and Other Antisera. In order to determine the specificity of WM, six different liposome types were prepared. As shown in Table I, these liposomes each contained one (or none) of the following homologous ceramide glycolipids: glucocerebroside, galactocerebroside (both of which are ceramide monohexosides), cytolipin H (ceramide dihexoside), globoside I (ceramide tetrahexoside), or Forssman (ceramide pentahexoside). The control liposomes lacked any glycolipid hapten. Each of these liposomes was assayed for complement-dependent glucose release in the presence of either WM, hemolysin, anti-human erythrocyte (HRBC) serum, or anti-galactocerebroside serum. The anti-HRBC was directed against erythrocytes which had abnormally high concentrations of the di-, tri-, and tetrahexosyl ceramides due to a hereditary disorder (Joseph *et al.*, 1974b).

Anti-HRBC served as a source of anti-globoside antibodies (Inoue *et al.*, 1971). As shown in Table I, however, it also manifested a high degree of activity against cytolipin H. This latter activity could be attributed to the relatively high concentrations of ceramide di- and trihexosides in the immunizing cells. Each of these substances has a terminal galactose group and, based on previous results (Alving *et al.*, 1974), the antiserum might be expected to cross-react with cytolipin H. Similarly, cross-reactivity with terminal galactose groups could also explain the interaction of anti-galactocerebroside serum with cytolipin H (Table I). In view of this, the absence of cross-reactivity of anti-HRBC with galactocerebroside is unexplained. A similar specificity has been previously observed, however, in that anti-galactocerebroside serum cross-reacted with digalactosyl diglyceride but the reciprocal reactivity of anti-digalactosyl diglyceride with cerebroside did not occur (Alving *et al.*, 1974). The absence of cross-reactivity of anti-galactocerebroside serum with glucocerebroside has been reported before (Joffe *et al.*, 1963) and this is compatible with the suggestion that in some cases the terminal sugar can be an important determinant for the specificity of antibodies against a glycolipid hapten.

Globoside I and Forssman have the same terminal sugar (*N*-acetylgalactosamine) but with differing anomeric configurations, and removal of the terminal sugar of Forssman yields globoside (Siddiqui and Hakomori, 1971). As was previously found (Inoue *et al.*, 1971), reciprocal serological cross-reactivity did not occur between Forssman and globoside in liposomes (Table I). Hemolysin, which was used as the source of anti-Forssman did, however, also manifest cross-reactivity with cytolipin H (Table I).

In contrast to the antisera described above, all of which reacted with at least two glycolipids, WM reacted only with Forssman hapten. Thus, WM was able to monospecifically distinguish Forssman hapten not only from globoside I, but also from all of the other glycolipids tested (Table I). With reference to the major aims of this investigation, the data of Table I suggest that the specificity of the WM under these conditions may have been at least equivalent to that of the anti-Forssman antibodies of hemolysin.

Comparison of the Effects of Antigen Concentration on the Activities of WM and Hemolysin. The above experiments were all performed with liposomes which contained excess quantities of glycolipid haptens. Previous studies have shown that the extent of glucose release from liposomes is a function of the number of antigen-antibody complexes which are formed and which lead to membrane damage due to complement activation (*cf.* Kinsky, 1972). In the presence of excess (*i.e.*, nonlimiting) quantities of antibody and complement, the potential number of such complexes can be conveniently controlled at the membrane itself by limiting the Forssman concentration (Kinsky *et al.*, 1969; Inoue *et al.*, 1971).

In Figure 2, the relative effects of different Forssman concentrations on liposomal glucose release were compared in the presence of excess quantities of WM and hemolysin, and IgG and IgM fractions derived from hemolysin. Under these conditions, considerably less glucose release was observed from liposomes sensitized with WM than with hemolysin or either of its fractions (IgG or IgM).

The Influence of Phospholipid Composition on Glucose Release in the Presence of Either WM or Hemolysin. As mentioned above, in a study with galactocerebroside as antigen, under limiting antigen conditions glucose release was inversely related to phospholipid fatty acyl chain length (Alving *et al.*, 1974). The phospholipids used in those experiments were di-14:0 PC, di-16:0 PC, di-18:0 PC, and beef SM. The SM had the longest fatty acyl groups (on the average), more than half of which had greater than 22 carbons. The suggestion was made that this phenomenon might have been due to a steric effect at the membrane surface causing interference with antibody binding. This concept

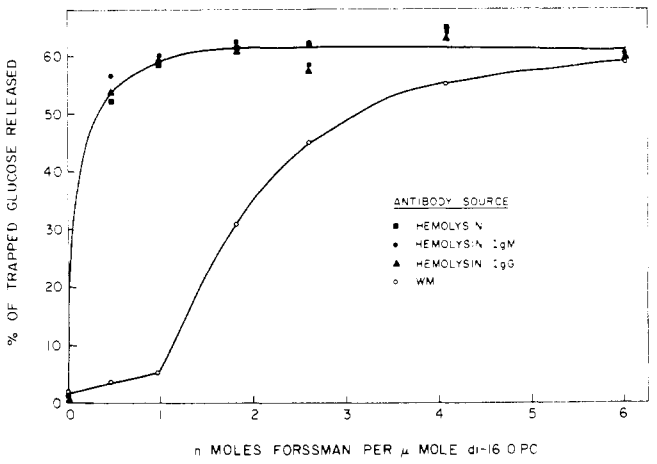

FIGURE 2: The effects of various antibodies on glucose release from liposomes containing different Forssman concentrations. Seven liposome preparations were made. In addition to di-16:0 PC, cholesterol, and dicetyl phosphate, each preparation contained a concentration of Forssman which corresponded to an amount shown on the abscissa. The procedure was the same as in Figure 1, except that the liposomes were preincubated in either hemolysin (5 μl), hemolysin IgM fraction (0.143 mg), hemolysin IgG fraction (0.610 mg), or WM (0.490 mg) and glucose release was measured 30 min after adding fresh GPS.

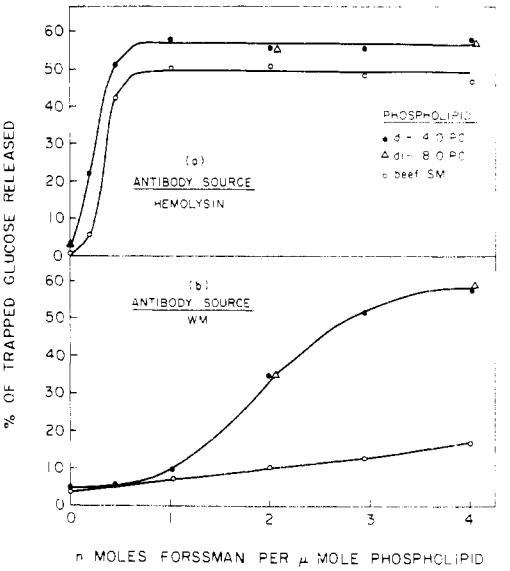

FIGURE 3: The effects of phospholipid composition on glucose release from liposomes containing varying concentrations of Forssman. The procedure was the same as in the legend of Figure 2, except that the phospholipid used to prepare the liposomes was, as indicated, either di-14:0 PC, di-18:0 PC, or beef SM. The liposomes in (a) were preincubated in 5 μl of hemolysin, and in (b) were preincubated in 0.196 mg of WM.

was supported by the observation that, under the same conditions, when a glycolipid antigen having a much larger hydrophilic group (ganglioside) was used, the resultant glucose release was affected much less by phospholipid composition, although some inhibition was observed with SM when compared with di-16:0 PC liposomes (Alving *et al.*, 1974). The overall size of Forssman is approximately equivalent to that of ganglioside, and both are ceramide haptens. It was therefore of interest to determine the influence of phospholipid composition on the interactions of hemolysin and WM with Forssman hapten.

As shown in Figure 3, the glucose release from liposomes prepared from either di-14:0 PC or di-18:0 PC was not detectably different in the presence of optimal amounts either of hemolysin (Figure 3a) or WM (Figure 3b). SM-containing liposomes, however, released less total glucose and required more Forssman for "half-maximal" glucose release with hemolysin than did liposomes containing either di-14:0 PC or di-18:0 PC (Figure 3a). This latter effect of decreased glucose release from SM liposomes compared to that observed from di-14:0 PC or di-18:0 PC liposomes was much more pronounced when WM was used as the antibody source. At the antigen concentrations shown, immunological activity of WM was barely detectable with SM-containing liposomes (Figure 3b).

Effect of Phospholipid Composition on the Binding Properties of WM. The above results are important with regard to one of the aims of this study, namely to investigate the influence of phospholipid composition on the relative degree of immunoglobulin binding. The magnitude of the differential between di-14:0 PC and SM was large in terms of glucose release from liposomes which had been sensitized with WM (Figure 3b), and because of this it was felt that similar large differences might also be detected in the degree of antibody (*i.e.*, WM) binding. This was tested as follows. Liposomes were prepared with either di-14:0 PC or beef SM, and a nonlimiting concentration of Forssman. Various quantities of these liposomes were used to "absorb" WM. In Figure 4, the absorbed WM supernatants were assayed for activity with di-14:0 PC liposomes which contained Forssman. In Figure 5, residual hemolytic activities of the supernatants were also tested with sheep erythrocytes. In both cases, all of the antibody activity was removed by absorbing with di-14:0 PC liposomes. Under the conditions shown, however, in both cases SM liposomes removed much less of the antibody activity.

Discussion

To some extent the experiments presented here are an outgrowth and further elaboration of certain earlier experiments in which Forssman hapten was utilized as an antigen in liposomes (Haxby *et al.*, 1968; Alving *et al.*, 1969; Kinsky *et al.*, 1969; Inoue *et al.*, 1971). One of the major purposes of the present investigation was to examine the immunological properties of a unique monoclonal IgM which had been shown to interact with Forssman hapten in liposomes (Joseph *et al.*, 1974a).

The results of this study support the conclusion that this protein, which was a Waldenström macroglobulin (WM or McG IgM), had a reactivity with Forssman hapten in liposomes which in certain respects was indistinguishable from those of anti-Forssman antibodies produced by immunized animals. The McG IgM was more specific in its interactions than were any of three antisera assayed. Rabbit anti-sheep erythrocyte serum (hemolysin) and anti-human erythrocyte serum served as sources of anti-Forssman and anti-globoside, respectively. Anti-galactocerebroside serum was also assayed, and this was obtained by injecting rabbits with pure galactocerebroside. Each of the three antisera reacted with at least two glycosphingolipids, while the WM protein, under the conditions shown, reacted monospecifically with Forssman hapten.

The influence of the phospholipid composition was an important consideration in this study. Previous experiments (Inoue *et al.*, 1971) demonstrated that liposomes prepared from beef SM generally released less glucose under a variety of conditions than did those prepared with egg PC. The reason for this discrepancy was not clearly delineated, but

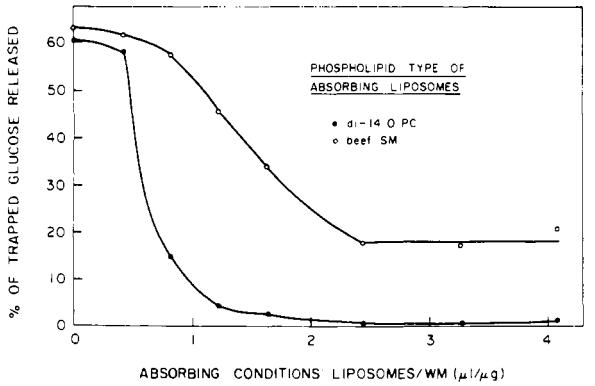

FIGURE 4: The effect of liposomal phospholipid composition on "absorption" of WM: reduction of glucose-releasing activity. WM was "absorbed" with liposomes as described in the Materials and Methods section. The absorbing liposomes contained di-14:0 PC or SM, as indicated, cholesterol, dicetyl phosphate plus 6.6 nmol of Forssman/μmol of PC. The liposomes were preincubated with the appropriate supernatants, and glucose release was measured 30 min after adding fresh GPS.

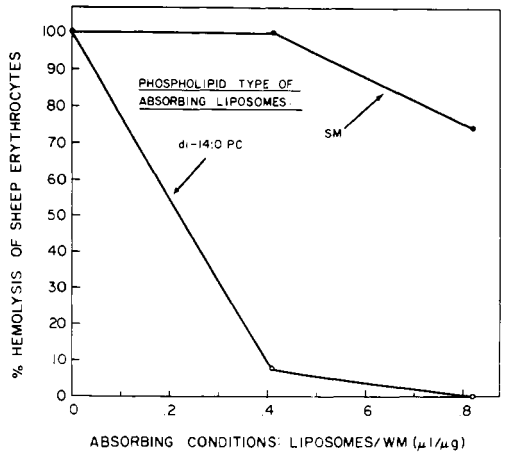

FIGURE 5: The effect of liposomal phospholipid composition on "absorption" of WM: reduction of hemolytic activity. WM was "absorbed" with liposomes, and hemolytic activity was measured as described in the Materials and Methods section. The absorbing liposomes and supernatants were the same as those used in the experiments of Figure 4.

several suggestions were made (also discussed by Kinsky, 1972): (1) SM liposomes might be more inherently "stable" than PC liposomes; (2) the antibodies might have "restricted access" to the antigen in SM-containing liposomes; or (3) PC might be "stimulatory" or SM might be "inhibitory" to antibodies and/or complement components. It was felt, initially, that the first possibility was probably correct, *viz.*, increased stability. This was based on indirect evidence that SM liposomes trapped more glucose, released a smaller quantity of glucose upon treatment with Triton X-100 or by standing overnight at room temperature, and released less glucose by "nonspecific" means during incubation in serum alone than did liposomes prepared with egg PC.

Although the relative stabilities of egg PC and beef SM might previously have played a role, as suggested above, we have eliminated this factor in our experiments by utilizing synthetic lecithins. With regard to all of the above criteria for measuring stability, we have found that liposomes prepared with either di-14:0 PC, di-16:0 PC, or di-18:0 PC were virtually indistinguishable from those made with beef SM (Conrad *et al.*, 1974; Alving *et al.*, 1974; unpublished observations). It would seem, then, that relative stability was related more to the degree of unsaturation rather than to phospholipid type. In this regard, it should be mentioned that about 74% of beef SM fatty acids were fully saturated, as opposed to only about 47% of egg PC fatty acids (Alving *et al.*, 1974).

Our previous results with synthetic PC and beef SM suggested, on the other hand, that steric factors might have been important (Alving *et al.*, 1974). As mentioned above, this possibility was inferred from the observation that, with galactocerebroside as antigen, glucose release was inversely related to phospholipid fatty acyl chain length, and this this "phospholipid effect" was much less obvious when a larger hapten (ganglioside) was substituted for cerebroside. In the experiments presented above, Forssman hapten was similar to ganglioside (and different from galactocerebroside) in that differential glucose release was not observed with di-14:0 PC, di-16:0 PC, or di-18:0 PC liposomes (Figure 3). This observation supports the hypothesis that Forssman and ganglioside both had sufficiently large oligosaccharides that they were not "buried" in the membrane surface and were accessible to antibodies when short-chain synthetic phospholipids were used in the liposomes. Thus, with short phospholipids together with Forssman hapten, steric factors may not play a significant role. It was felt, therefore, that differences of glucose release under these conditions might be a reflection of differences of antibody binding affinity. If this interpretation were correct, then the lower glucose release observed in the presence of WM (Figure 3b), compared to hemolysin (Figure 3a), from liposomes containing synthetic lecithins, may have been due to lower affinity of WM for Forssman. In this regard, it has been demonstrated that lower affinity antibodies bind to a lesser extent and release less glucose from liposomes than do higher affinity ones (Uemura and Kinsky, 1972; Six *et al.*, 1973). It should be emphasized, however, that the intrinsic affinity of the McG IgM for the Forssman oligosaccharide has not yet been determined by classical techniques. Work is now in progress comparing affinities of hemolysin and the McG IgM by means of equilibrium dialysis.

A series of experiments was performed in this study utilizing SM liposomes because of the previous reports in which the reactivity of beef SM liposomes differed under various conditions from those made with lecithin (see above). Beef SM, on the average, had longer fatty acids than did the synthetic lecithins. More than half of the SM fatty acids contained at least 22 carbons (Alving *et al.*, 1974). With SM liposomes less glucose release occurred (compared to that observed with synthetic lecithins) both with hemolysin (Figure 3a) and even more prominently with McG IgM (Figure 3b). These results raised the possibility that the binding of McG IgM to Forssman might have been diminished in the presence of SM, possibly due to steric factors in SM liposomes accentuating the lower affinity of the McG IgM. This possibility was tested by means of absorption experiments, and it was indeed found that SM liposomes bound fewer antibodies (*i.e.*, WM) than did di-14:0 PC liposomes (Figures 4 and 5).

Other techniques for studying reactions with Forssman hapten, such as hemolysis-inhibition, generally require the use of "activator" or "auxiliary" lipids (Papirmeister and Mallette, 1955; Makita *et al.*, 1966). The "auxiliary" lipids frequently have a composition (*e.g.*, lecithin, cholesterol) which is somewhat similar but not as uniform or as well characterized as that of the liposomal model membrane. In this study it may be concluded that we have simplified the

membrane-Forssman-antibody-complement interactions still further by reacting liposomes prepared from highly purified natural and synthetic lipids and Forssman hapten, with a monoclonal immunoglobulin having an "antibody-like" activity with anti-Forssman specificity. It should be reemphasized that absorption of McG IgM with purified Forssman hapten incorporated into liposomes removed all of the reactivity both to Forssman-containing liposomes (Figure 4) and to sheep erythrocytes (Figure 5). This monoclonal immunoglobulin thus had a reactivity with one of the several glycolipids present in sheep erythrocytes. Based on all of these experiments, we now feel that the IgM protein (McG) can be added to the list of monoclonal immunoglobulins (reviewed by Seligmann and Brouet, 1973) which are thought to be antibodies and for which a specific "antigen" has been described.

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