Comparative Responses of Liposomes Prepared with Different Ceramide Antigens to Antibody and Complement*

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ABSTRACT: Incorporation of globoside I into lipid mixtures containing phospholipid (either lecithin, sphingomyelin, or an equimolar mixture of both) results in the generation of liposomes which release trapped glucose marker upon incubation with anti-globoside serum and unheated guinea pig serum as a source of complement. The anti-globoside serum was obtained from rabbits immunized with human erythrocytes or membranes isolated therefrom. The properties of these liposomes have been compared with analogous liposomes containing Forssman antigen which has the same tetrasaccharide unit as globoside, but differs in the configuration of the terminal glycosidic linkage. Specific antibodies are involved in the response of these liposomes because anti-Forssman (*i.e.*, rabbit anti-sheep erythrocyte) serum does not promote marker loss from globoside liposomes and, conversely, anti-globoside serum has no effect on Forssman liposomes. Using liposomes containing both globoside and Forssman antigen, evidence has been obtained which supports previous studies indicating that the extent and rate of glucose release is dependent on the number of antigen–antibody complexes formed within the liposomal bilayers. The liposomes show quantitative differences in that greater amounts of antigen and antiserum are necessary to produce half-maximal marker release from globoside, than Forssman, liposomes regardless of phospholipid composition. However, the globoside and Forssman liposomes behave qualitatively in similar fashion. In both cases, less antiserum and guinea pig serum are required to produce half-maximal glucose loss from liposomes prepared with an equimolar mixture of lecithin and sphingomyelin than either phospholipid alone, and a greater percentage of trapped marker is released from lecithin, than sphingomyelin, liposomes. These results suggest that phospholipid composition may be a significant factor in determining the sensitivity of natural membranes to immune damage.

Immunologically sensitive liposomes have also been prepared with galactocerebroside as antigen; the latter differs from both Forssman and globoside in that it contains only a single sugar residue. The galactocerebroside liposomes resemble the globoside (or Forssman) liposomes in that (a) the extent of glucose release is dependent on the amount of antigen incorporated and the concentration of anti-galactocerebroside and guinea pig serum, and (b) more marker is generally lost from lecithin, than sphingomyelin, liposomes. The anti-galactocerebroside serum was obtained from rabbits immunized with a myelin-rich beef brain fraction. An unexpected finding was that "late" anti-galactocerebroside serum (collected at the end of the immunization period) released significant quantities of glucose from lecithin, but not sphingomyelin, liposomes prepared without any antigen; this release from nonsensitized liposomes required the presence of native (*i.e.*, unheated) guinea pig serum.

Extensive studies in numerous laboratories have demonstrated the existence of a variety of cellular antigens which possess clearly distinguishable nonpolar and polar regions. Those which have been most completely characterized are the mammalian ceramide homologs where the main antigenic determinants reside in the polar regions (Rapport and Graf, 1969).

The amphipathic nature of these antigens prompted us several years ago to attempt preparation of liposomal model membranes which might be subject to immune damage. The assumption behind this approach was that proper insertion of the antigen into the lipid bilayers would leave the polar regions accessible to binding by the appropriate antibodies. The resultant antibody–antigen complex would then be able to activate the complement cascade (reviewed by Müller-Eberhard, 1968, 1969) which ultimately impairs the ability of membranes to function as restraining barriers. The validity of this approach was demonstrated in previous papers which described the preparation of liposomes that released trapped glucose marker when incubated with rabbit anti-sheep erythrocyte serum and a source of complement (Haxby *et al.*, 1968; Alving *et al.*, 1969; Kinsky *et al.*, 1969; Inoue and Kinsky, 1970). The complement requirement could be satisfied either by purified human components (Haxby *et al.*, 1969) or a variety of mammalian sera (unpublished observations), and methods which may be appropriate for the isolation of liposomes with bound complement components have been developed (Alving and Kinsky, 1971).

In these initial studies, the liposomes were generated either from a chloroform-soluble fraction derived from sheep erythrocyte membranes or from an artificial lipid mixture containing pure Forssman antigen. This was done primarily because rabbit anti-sheep erythrocyte serum with a high titer of antibodies against Forssman antigen can be easily obtained. In the present investigation, particular emphasis has been placed on liposomes containing two other ceramide antigens: globoside I (also known as cytolipin K) and galactocerebroside. These ceramides were chosen for comparative purposes because the globoside has the same tetrasaccharide unit as Forssman but with a different terminal glycosidic linkage, whereas galactocerebroside represents the simplest member of this class of compounds having only a single sugar as part of the antigenic determinant.

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Experimental Section

Except as indicated below, the sources and preparation of the various materials (lipids, cofactors and enzymes for the glucose assay reagent, rabbit anti-Forssman serum, guinea pig complement, globoside and Forssman antigen) have been described in earlier papers (Kinsky *et al.*, 1969; Inoue and Kinsky, 1970). Galactocerebroside was purchased from Pierce Chemical Co., Rockford, Ill. Prior to use, it was dissolved in a 1:1 mixture of chloroform–methanol and precipitated by the addition of ten volumes of acetone. After purification in this manner, thin-layer chromatography on silica gel plates with chloroform–methanol–water (70:30:5, v/v) as solvent revealed only two spots characteristic of galactocerebrosides containing hydroxylated and nonhydroxylated fatty acids.

Preparation of Anti-globoside Sera. This was accomplished by two procedures, using either intact human A type erythrocytes or membranes obtained from these cells for immunization. In the first method, six intravenous injections of 3.5 ml of a 10% washed erythrocyte suspension (in 0.15 M NaCl, *i.e.*, isotonic saline) were administered to each of three rabbits; the injections were equally spaced over a 3-week period. In the second procedure, washed erythrocytes were lysed by gradual dilution of packed cells with an excess of cold 0.04% acetic acid. The stroma were isolated by centrifugation at 2° (10 min, 1000*g*) and washed four times with 1 mM sodium acetate buffer (pH 5) and then three times with isotonic saline (10 min, 12,000*g*). The final stromal suspension contained 5.2 mg of protein/ml of isotonic saline and 2 ml of this suspension was administered intravenously to each of three rabbits; the animals received nine such injections at equal intervals over 3 weeks. In both of the above methods, the rabbits were killed 4 days after receiving the last injection.

Of the six sera thus obtained, all were effective in agglutinating human A erythrocytes. However, agglutination did not serve as a reliable index of anti-globoside antibodies because only three of the sera (one obtained by immunization with the intact cells and two from rabbits immunized with stroma) had a significant effect on liposomes containing globoside antigen. Most of the experiments reported in the present paper were performed with the single "intact cell" antiserum although subsequent studies have indicated that the other antisera behave in an identical manner. Prior to use, all sera (including anti-galactocerebroside serum, see below) were decomplemented by heating for 30 min at 56°, and dialyzed against Veronal-buffered saline (VBS prepared according to Mayer (1961)).

Preparation of Anti-galactocerebroside Sera. A particulate fraction from beef brain white matter was isolated by minor modification of the method outlined by Rapport and Graf (1967). The 25% sucrose homogenate was centrifuged at 2° (10 min, 800*g*) without prior layering over 30% sucrose. The resultant supernatant solution was then centrifuged (60 min, 20,000*g*) to obtain a pellet which was suspended in, and dialyzed against, cold distilled water before lyophilization. This material contained 0.2 mg of protein/mg dry weight suggesting that it is composed mainly of myelin (*cf.* Rapport *et al.*, 1964).

Rabbits were immunized by injection of 0.5 ml of antigen preparation into each footpad. The antigen preparation was made fresh each time by resuspending a sufficient amount of the lyophilized material in isotonic saline (60 mg/ml) and mixing thoroughly with an equivalent volume of complete Freund's adjuvant (purchased from Difco Laboratories, Detroit, Mich.). Injections were performed every 2 weeks for a total of 6 weeks. During the course of immunization, blood was obtained from the ear vein and the sera tested for their ability to release glucose from galactocerebroside liposomes prepared with lecithin and sphingomyelin. This screening procedure revealed a significant difference between "early" and "late" antisera as described in the Results section. For the purposes of the present investigation, early antiserum refers to that obtained by partial bleeding 10 days after the second injection; the late antiserum was obtained by killing the animals 7 days after they received the last injection.

Liposome Preparation and Glucose Release Assay. Liposomes were prepared by the same procedure previously developed for the generation of Forssman-sensitized liposomes with lipid mixtures containing a phospholipid (lecithin and/or sphingomyelin), cholesterol, and dicetyl phosphate (Kinsky *et al.*, 1969).[1] The molar ratios of these lipids, as well as the amounts of antigen added, are specified in the table and figure legends. Kinsky *et al.* (1969) also describes the spectrophotometric assay used for following loss of glucose from the liposomes, and the procedures employed in calculating the results as the per cent of trapped glucose released after 30-min incubation. In regard to the latter, it should be noted that the paper cited contains a typographical error in the description of the chloroform–Triton method by which total glucose (trapped plus untrapped) in the liposome preparations was determined; a correction has appeared (Kinsky *et al.*, 1970). The amounts of antisera and complement present during assay were adjusted to meet the requirements of the particular experiment and these, plus any specific additional details, are indicated in the appropriate legends.

Results

Properties of Globoside Liposomes. Liposomes, containing either sphingomyelin, lecithin, or a combination of the phospholipids, but no antigen, do not release significant amounts of glucose in the presence of antigloboside serum and a source of complement. Marker is lost, however, when globoside is incorporated into the lipid mixture used for generation of the liposomes (Figure 1). Release of glucose from liposomes which contain globoside is dependent on the concentration of antiserum and the source of complement (guinea pig serum). Both are required because neither guinea pig serum in the absence of antiserum (Figure 2), nor antiserum in the absence of guinea pig serum (Figure 3), has an appreciable effect. Heating of the native guinea pig serum, under conditions usually employed to inactivate hemolytic complement activity (30 min at 56°), destroys its ability to produce glucose release in the presence of antiserum.

Globoside and Forssman antigen have similar structures (GalNAc-Gal-Gal-Glc-ceramide) but differ in the nature of the terminal glycosidic linkage between *N*-acetylgalactosamine and galactose; the latter is $\alpha 1 \rightarrow 3$ in Forssman and $\beta 1 \rightarrow 3$ in the globoside. This fact enabled us to demonstrate that specific antibodies participate in the response of liposomes containing globoside (or Forssman) antigen. Table I shows that the anti-Forssman serum has no effect on globoside liposomes and, conversely, anti-globoside serum has no effect

[1] Abbreviations used are: PC, phosphatidylcholine, *i.e.*, lecithin; SM, sphingomyelin; Chol, cholesterol; DCP, dicetyl phosphate.

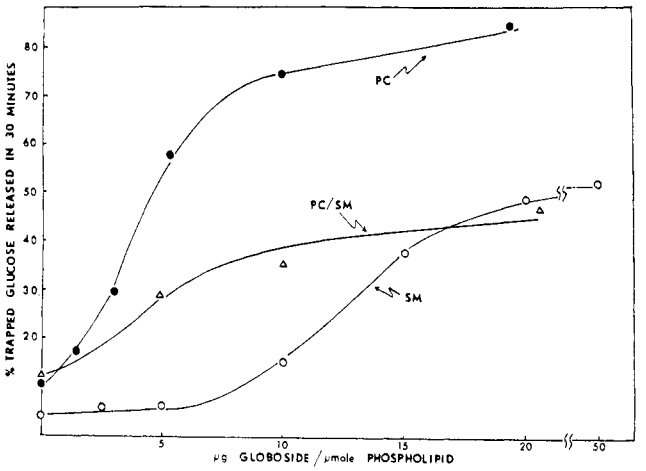

FIGURE 1: Effect of globoside on the immune sensitivity of liposomes prepared with lecithin and/or sphingomyelin. Liposomes were prepared from mixtures containing phospholipid, Chol, and DCP in molar ratios of 2:1.5:0.22, respectively, with varying amounts of globoside as indicated on the abscissa. PC (or SM) refers to liposomes containing only lecithin (or sphingomyelin) as phospholipid; PC/SM designates liposomes made with equimolar amounts of both phospholipids. Glucose release was determined in the presence of 20 μl of anti-globoside serum, plus 82.7 μl of guinea pig serum as complement.

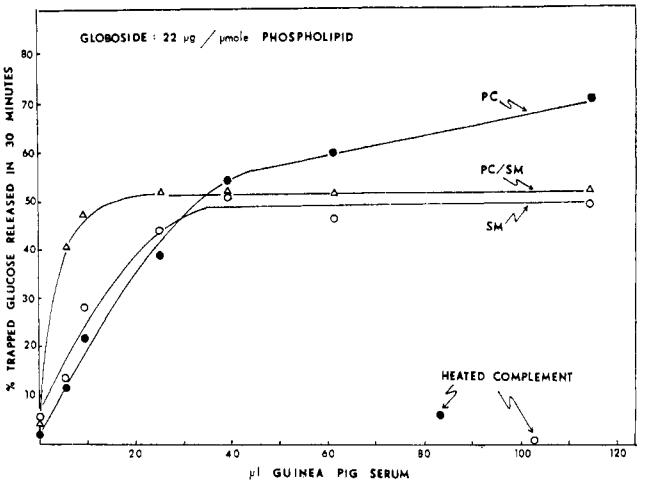

FIGURE 3: Effect of guinea pig serum concentration on globoside liposomes prepared with lecithin and/or sphingomyelin. Liposomes, containing 22 μg of globoside/μmole of phospholipid, were prepared as described in the legend to Figure 1. Glucose release was determined in the presence of 20.3 μl of anti-globoside serum, plus varying amounts of guinea pig serum as indicated on the abscissa.

on Forssman liposomes. This experiment thus supports and extends previous results indicating that serum from non-immunized rabbits does not replace antiserum in causing marker release from liposomes (Haxby *et al.*, 1968) or the binding of complement protein to liposomes (Alving and Kinsky, 1971).

Comparison of Globoside and Forssman Liposomes. The properties described in the preceding section for liposomes which contain globoside are qualitatively similar to those of liposomes which contain Forssman antigen. However, as indicated below, there are some quantitative differences, particularly with regard to antigen and antisera sensitivity.

From experiments such as described in Figure 1, it is possible to determine the amount of antigen per micromole of phospholipid which is necessary to produce half-maximal sensitization (*i.e.*, half-maximal glucose release) in the presence of saturating levels of antiserum and complement source. The results from several experiments are recorded in Table II and indicate that, with either antigen, greater amounts are required for half-maximal sensitization of liposomes made with sphingomyelin than those prepared with lecithin. However, the amounts of Forssman required to produce half-maximal glucose release from liposomes made with either lecithin or sphingomyelin are considerably smaller than the corresponding values for globoside.

From experiments such as described in Figure 2, it is

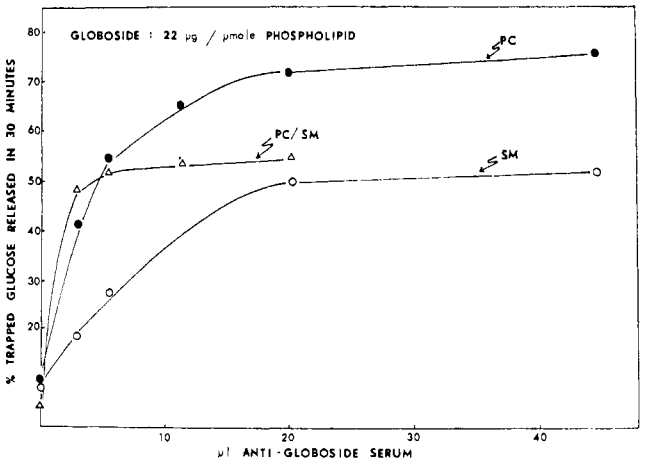

FIGURE 2: Effect of antiserum concentration on globoside liposomes prepared with lecithin and/or sphingomyelin. Liposomes, containing 22 μg of globoside/μmole of phospholipid, were prepared as described in the legend to Figure 1. Glucose release was determined in the presence of 115 μl of guinea pig serum as complement, plus varying amounts of anti-globoside serum as indicated on the abscissa.

TABLE I: Lack of Cross-Reactivity of Forssman and Globoside Liposomes Prepared with Sphingomyelin.[a]

	% Trapped Glucose Released after 30 min	
	Antigen Incorpd	
Antiserum Added	Forssman (10 μg/μmole of SM)	Globoside (22 μg/μmole of SM)
None	11.2	2.3
Anti-Forssman	57.7	1.9
Anti-globoside	14.0	53.6

[a] Liposomes were prepared from mixtures containing SM, Chol, and DCP in molar ratios of 2:1.5:0.22, respectively, with the quantities of Forssman or globoside specified in the table. Glucose release was determined in the presence of no antiserum, 20 μl of a 1:10 dilution of anti-Forssman, or 20 μl of undiluted anti-globoside, plus 82.7 μl of guinea pig serum as complement. Although not indicated in the table, it should be noted that more anti-Forssman serum (20 μl of a 1:2 dilution) also did not release any marker from the globoside liposomes.

TABLE II: Comparative Properties of Different Forssman and Globoside Liposomes.[a]

Antigen Incorpd	Liposomal Phospholipid		
	PC	PC/SM	SM
	μg of Ag/μmole of PL Required for Half-Maximal Glucose Release		
Forssman	0.8	N.D.	2.5
Globoside	4.4	5.8	13.1
	μl of AS Required for Half-Maximal Glucose Release		
Forssman	0.63	0.25	0.50
Globoside	3.2	1.6	6.2
	μl of GPS Required for Half-Maximal Glucose Release		
Forssman	12.5	2.5	5.2
Globoside	20.4	4.0	10.0

[a] Except in the case of experiments designed to determine the amount of antigen (Ag) required for half-maximal glucose release, liposomes were prepared with either 10 μg of Forssman or 20 μg of globoside/μmole of phospholipid (PL). The amounts of each antiserum (AS) required for half-maximal glucose release from the liposomes were determined in the presence of excess guinea pig serum (113 μl) as complement. Similarly, the amounts of guinea pig serum (GPS) required for half-maximal glucose release from the liposomes were determined in the presence of excess anti-Forssman (20 μl of a 1:10 dilution) or anti-globoside (20 μl of undiluted) serum. All values were calculated after correction for marker loss occurring in the absence of antigen, antiserum, or complement. See text for additional details.

TABLE III: Effect of Absorption with Erythrocytes on the Ability of Antisera to Cause Glucose Release from Forssman and Globoside Liposomes Prepared with Sphingomyelin.[a]

Antiserum Added	Erythrocytes Used for Absorption	% Trapped Glucose Released	
		Antigen Incorpd	
		Forssman (10 μg/μmole of SM)	Globoside (20 μg/μmole of SM)
None	None	16.1	4.4
Anti-Forssman	None	56.5	
Anti-Forssman	Sheep	12.6	
Anti-globoside	None		53.6
Anti-globoside	Human A		44.8

[a] Liposomes were prepared from mixtures containing SM, Chol, and DCP in molar ratios of 2:1.5:0.22, respectively, with the quantities of Forssman or globoside specified in the table. Glucose release was determined in presence of no antiserum, 20 μl of undiluted absorbed or nonabsorbed antiserum as indicated in the table, plus 113 μl of guinea pig serum as complement. Absorption was performed by incubating 1 ml each of anti-Forssman and anti-globoside serum for 60 min at 22° with 1 ml of washed packed sheep and human type A cells, respectively. After centrifugation for 10 min at 1000*g*, the sera were dialyzed overnight against cold isotonic saline before assay.

possible to determine the amount of antiserum required for half-maximal glucose release under conditions in which antigen content and the concentration of complement source are not limiting factors. Table II again illustrates the qualitative similarity of liposomes containing either Forssman or globoside because, in both cases, those which are prepared with a 1:1 equimolar mixture of lecithin and sphingomyelin are slightly more sensitive to antiserum than liposomes made with only lecithin or sphingomyelin. However, when considering liposomes with identical phospholipid composition, those which contain Forssman require significantly less antiserum for half-maximal marker release than those which contain globoside.

Previous experiments (Kinsky *et al.*, 1969) have indicated that the extent of glucose release is dependent on the number of antigen–antibody complexes which are able to initiate the complement sequence. Because formation of the latter would be favored by higher antibody concentration, the preceding observation suggests that the anti-globoside serum has a lower titer of the appropriate antibody than the anti-Forssman serum. Unfortunately, attempts made to quantitate the relative titers of anti-Forssman and anti-globoside sera by either hemagglutination tests or lysis in the presence of complement have been unsuccessful. As already noted in Experimental Section, the ability of various sera to cause glucose release from globoside liposomes was not a consistent property of sera which agglutinated human erythrocytes. Subsequent experiments showed that, compared to Forssman antigen, globoside in the native erythrocyte membrane is inaccessible to the antibody (see also Koscielak *et al.* (1968)). Thus, as indicated in Table III, absorption of the anti-Forssman serum with sheep erythrocytes completely abolished its capacity to cause glucose release from Forssman liposomes. Similar treatment of the anti-globoside serum with human A erythrocytes had, however, little effect on its ability to promote marker loss from globoside liposomes. Additional experiments (not shown) have indicated that human B and O type erythrocytes were equally as uneffective, as the A type cells, in removing anti-globoside antibodies.

From experiments such as illustrated in Figure 3, the amount of guinea pig serum (complement) necessary for half-maximal marker release was determined. The results shown in Table II indicate that both Forssman and globoside liposomes prepared with equimolar quantities of lecithin and sphingomyelin are slightly more sensitive to complement than liposomes made with either phospholipid alone. This is analogous to the situation previously described for antiserum sensitivity. It seems to us particularly significant, however, that the amounts of complement required for half-maximal glucose release are nearly identical for Forssman and globoside liposomes of similar phospholipid composition. The differences that have been observed are certainly not of the same magnitude as the differences discussed above with regard to the amounts of antigen and antisera required for half-maximal sensitization of Forssman and globoside liposomes. This finding also suggests that it is the number of antigen–antibody complexes which determines the extent of glucose release because the experiments were done under conditions expected to produce essentially the same number

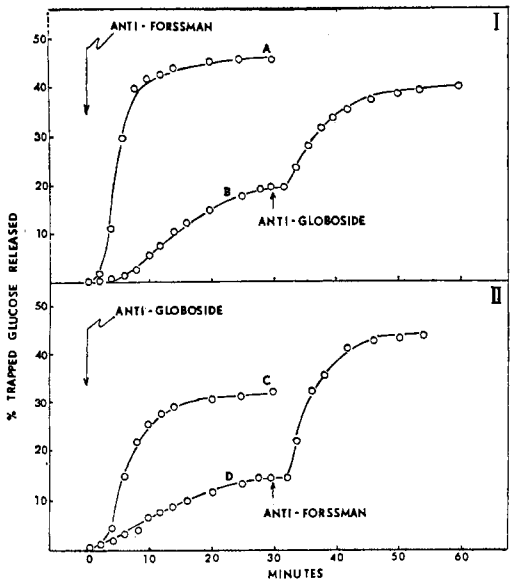

FIGURE 4: Kinetics and extent of glucose release from liposomes containing both Forssman and globoside. Liposomes were prepared from a mixture containing SM, Chol, and DCP in molar ratios of 2, 1.5, 0.22, respectively, plus 5 μg of Forssman and 10 μg of globoside per μmole of sphingomyelin. Glucose release was determined in the presence of 82.7 μl of guinea pig serum as complement. In the experiment shown in part I, the reaction was initiated by the addition of 4.0 μl (curve A) or 0.7 μl (curve B) of a 1:10 dilution of anti-Forssman serum. After 30 min, 5.3 μl of undiluted anti-globoside serum was added as indicated by the arrow (curve B). In the experiment shown in part II, the reaction was initiated by the addition of 8.2 μl (curve C) or 2.6 μl (curve D) undiluted anti-globoside serum. After 30 min, 5.3 μl of a 1:10 dilution of anti-Forssman serum was added as indicated by the arrow (curve D). See text for additional details.

of complexes within the liposomal bilayers (*i.e.*, high antigen content and excess antisera).

The lack of cross-reactivity (see Table I) between Forssman antigen and globoside antibodies, or globoside antigen and Forssman antibodies, prompted another test of this hypothesis as illustrated in Figure 4. In this experiment, marker loss from liposomes, containing *both* Forssman and globoside antigen, was determined at various times after sequential addition of the two antisera. In curve B, the reaction was initiated with a limiting quantity of anti-Forssman serum to form less than the maximum number of antigen–antibody complexes; under these conditions, glucose release was essentially completed after 30 min. At this time, addition of anti-globoside serum to produce more, and "different," antigen–antibody complexes led to a further increase in the amount of glucose released. Similar results were obtained in the converse situation (curve D) in which the reaction was begun with a limiting amount of anti-globoside serum followed, at 30 min, by the addition of anti-Forssman serum.

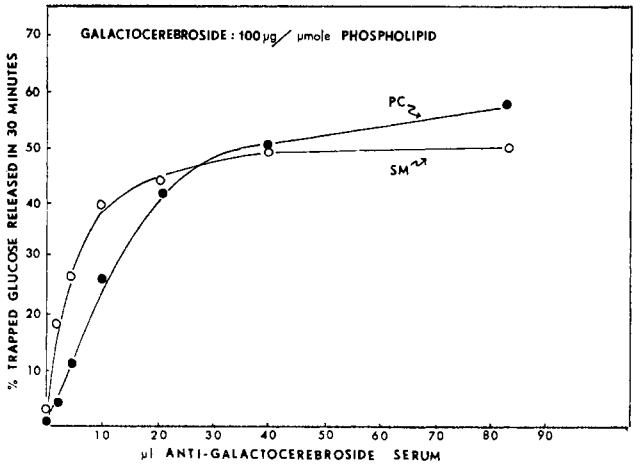

FIGURE 6: Effect of antiserum concentration on galactocerebroside liposomes prepared with lecithin or sphingomyelin. Liposomes, containing 100 μg of galactocerebroside/μmole of phospholipid, were prepared as described in the legend to Figure 5. Glucose release was determined in the presence of 113 μl of guinea pig serum as complement, plus varying amounts of early (for PC liposomes) or late (for SM liposomes) anti-galactocerebroside serum as indicated on the abscissa.

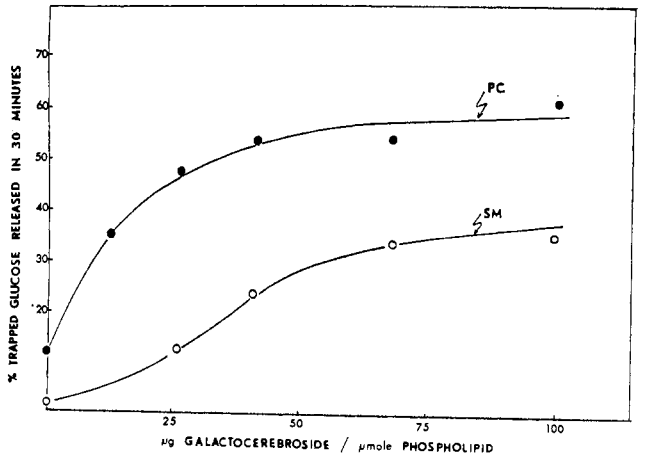

FIGURE 5: Effect of galactocerebroside on the immune sensitivity of liposomes prepared with lecithin or sphingomyelin. Liposomes were prepared from mixtures containing PC (or SM), Chol, and DCP in molar ratios of 2:1.5:0.22, respectively, with varying amounts of galactocerebroside as indicated on the abscissa. Glucose release was determined in the presence of 113 μl of guinea pig serum as complement, plus 82.7 μl of early (for PC liposomes) or late (for SM liposomes) anti-galactocerebroside serum.

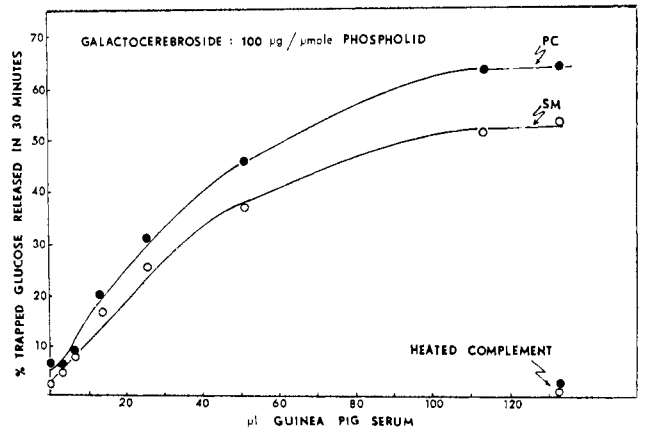

FIGURE 7: Effect of guinea pig serum concentration on galactocerebroside liposomes prepared with lecithin or sphingomyelin. Liposomes, containing 100 μg of galactocerebroside/μmole of phospholipid, were prepared as described in the legend to Figure 5. Glucose release was determined in the presence of 82.7 μl of early (for PC liposomes) or late (for SM liposomes) anti-galactocerebroside serum, plus varying amounts of guinea pig serum as indicated on the abscissa.

TABLE IV: Effect of Early and Late Anti-galactocerebroside Serum on Liposomes Prepared with and without Galactocerebroside from Lecithin or Sphingomyelin.[a]

	PC Liposomes Plus			SM Liposomes Plus		
	Galacto-cerebroside	No Antigen		Galacto-cerebroside	No Antigen	
Antiserum Source	% Glucose Released		ER[b]	% Glucose Released		*ER*[b]
Early 1	80.4	19.7		10.2	1.7	
Early 2	69.0	12.0		9.8	1.5	
Early 3	72.4	20.3		16.0	1.4	
(Av)	73.9	17.3	4.3	12.0	1.5	(8.0)
Late 1	90.5	41.0		22.4	7.6	
Late 2	91.1	36.8		26.4	7.9	
Late 3	86.0	40.8		46.2	6.6	
(Av)	89.2	39.5	2.3	31.6	7.4	4.3

[a] Liposomes were prepared from mixtures containing PC (or SM), Chol, and DCP in molar ratios of 2:1.5:0.22, respectively, without and with galactocerebroside (100 μg/μmole of phospholipid) as specified in the table. Release of glucose was determined in the presence of 82.7 μl of antiserum plus 113 μl of guinea pig serum as complement. The early and late antisera used in this experiment were obtained from three different rabbits. [b] See text for definition of enhancement ratio (*ER*) and additional details.

In curves A and C, the reaction was started with approximately six times more anti-Forssman serum and three times more anti-globoside serum than used to initiate the reactions described by curves B and D, respectively. The differences between curves A and B, and C and D, are consistent with previous results (Kinsky *et al.*, 1969) indicating that, not only the extent, but also the rate of glucose release is determined by the number of liposomal antigen–antibody complexes.

Properties of Galactocerebroside Liposomes. Liposomes which contain galactocerebroside as antigen, have been prepared with lecithin and sphingomyelin. These liposomes do not release glucose in the presence of the *appropriate* anti-galactocerebroside serum and a complement source unless antigen has been incorporated (Figure 5). The extent of glucose loss is dependent on antigen content, and also the concentration of anti-galactocerebroside serum (Figure 6) and native, *i.e.*, unheated, guinea pig serum (Figure 7), present during assay. Neither anti-galactocerebroside serum nor guinea pig serum will alone produce loss of trapped marker. It must be emphasized, however, that results such as those described in Figures 5, 6, and 7 were only obtained when the proper antisera were employed. These were the antisera which gave high enhancement ratios, previously defined (Inoue and Kinsky, 1970) as the ratio of the per cent glucose released from liposomes containing antigen/per cent glucose released from liposomes lacking antigen. As indicated below, this criterion was satisfied by early anti-galactocerebroside sera in the case of liposomes prepared with lecithin, and late anti-galactocerebroside sera in the case of liposomes made with sphingomyelin.

Properties of Anti-galactocerebroside Sera. Preparation of galactocerebroside liposomes was originally undertaken with the intention of including data (*apropos* antigen, antisera, and complement sensitivity) for comparison to the corresponding values obtained using globoside and Forssman liposomes. Extensive experiments along these lines were not done, however, because of some unexpected properties of the anti-galactocerebroside sera. These became apparent when sera were tested for their effect on galactocerebroside liposomes, prepared with either lecithin or sphingomyelin, at various times during the immunization period (see Experimental Section).

Table IV shows that sera collected 24 days after immunization had begun, which have been subsequently designated early antisera, were extremely effective in promoting glucose release from lecithin liposomes containing galactocerebroside. The average enhancement ratio, *ER*, was approximately 4 in the case of lecithin–galactocerebroside liposomes. The average enhancement ratio was 8 for sphingomyelin–galactocerebroside liposomes but the significance of this high value is probably doubtful because early antisera released surprisingly little trapped glucose from liposomes which contained the antigen (also from liposomes lacking antigen). In contrast, what we consider to be significant amounts of glucose were released from sphingomyelin–galactocerebroside liposomes when late antisera, obtained at the end of the immunization period (49 days), were employed; the average enhancement ratio in this case was approximately 4. The late anti-galactocerebroside sera also caused appreciable loss of marker from lecithin liposomes which contained antigen. However, under such circumstances, the average enhancement ratio was about half that obtained with the early antisera. As indicated in Table IV, this decline in the enhancement ratio was mainly a consequence of the marked increase in the amount of glucose released by the late antisera from lecithin liposomes which did not contain antigen. In this regard, it should be emphasized that both late anti-galactocerebroside sera and a complement source are necessary to promote loss of marker from lecithin liposomes lacking antigen. Table V shows that the late antisera cannot be replaced by normal rabbit serum and that heating of the native guinea pig serum to destroy its hemolytic complement activity has a similar effect on glucose release.

Discussion

The present investigation has shown that Forssman antigen

TABLE V: Guinea Pig Serum Requirement for Glucose Release by Late Anti-galactocerebroside Serum from Lecithin Liposomes Lacking Antigen.[a]

Additions			% Glucose Released	
Late Anti-galactocerebroside Serum	Native GPS	Heated GPS	Expt 1	Expt 2
+	+	−	67.8	41.0
+	−	+	6.1	4.2
−	+	−	7.7	N.D

[a] Liposomes were prepared from mixtures containing PC, Chol, and DCP in molar ratios of 2:1.5:0.22, respectively. Glucose release was determined in the presence of 82.7 μl of antiserum and 113 μl of guinea pig serum (GPS) as complement; expt 1 and 2 were performed with late anti-galactocerebroside serum obtained from two different rabbits. An equivalent volume of normal serum from a nonimmunized rabbit was substituted when late antiserum was omitted, and an equivalent volume of heated guinea pig serum (56° for 30 min) was used when native complement was omitted.

can be replaced by other ceramide homologs (*i.e.*, globoside and galactocerebroside) to produce liposomes which are damaged by the appropriate antibody and complement. Because such liposomes can be made from mixtures containing only lipids of known structure, they possess obvious advantages for studying the molecular basis of immune lysis. This has already been indicated by previous experiments which have employed Forssman liposomes (Kinsky *et al.*, 1969; Inoue and Kinsky, 1970). These studies have raised doubts concerning the existence of specific receptors for components of the complement sequence in natural cell membranes and the likelihood that complement-mediated lysis involves enzymatic degradation of membrane phospholipids. The fact that immunologically sensitive liposomes can be prepared with the other ceramide antigens has, therefore, some practical consequences for those interested in utilizing these model membranes because human erythrocytes are a rich source for the isolation of globoside and galactocerebroside can be obtained commercially.

During the course of this investigation, we have usually observed that, under optimal conditions (*i.e.*, high antigen content, saturating levels of antiserum and complement), liposomes prepared with lecithin release a greater percentage of their trapped marker than those made with sphingomyelin. It is likely that several factors contribute to this phenomenon. For example, liposomal bilayers prepared with sphingomyelin may be intrinsically more stable, may contain lower amounts of antigen than lecithin bilayers, and/or may contain the antigens so that they are less accessible for interaction with antibody. Indirect evidence, which indicates that sphingomyelin liposomes are more stable, has been obtained; not only do these liposomes trap a greater quantity of glucose than lecithin liposomes but also a smaller percentage of their trapped marker is lost upon treatment with Triton, standing overnight at room temperature, or hypotonic dilution with water (Kinsky *et al.*, 1969; unpublished observations). In addition to the above, the possibility that lecithin and sphingomyelin may have quantitatively and qualitatively different effects (*e.g.*, stimulatory or inhibitory) on the properties of antibodies and complement components has to be considered. Interplay of all these factors could explain why, regardless of which ceramide antigen was employed, liposomes prepared with an equimolar mixture of lecithin and sphingomyelin require less antiserum and guinea pig serum for half-maximal glucose release than those made with either phospholipid alone. In this connection, it should be noted that the greater sensitivity of the mixed phospholipid liposomes was also suggested by preliminary experiments showing that these liposomes manifest the shortest, and sphingomyelin liposomes the longest, lag phase before the onset of glucose release. The above observations suggest that phospholipid composition may be a very important factor in determining the sensitivity of natural membranes to immune damage.

Although liposomes prepared with different ceramide antigens respond qualitatively in similar fashion to antibody and complement, the significance of the quantitative differences should not be overlooked. For example, greater quantities of galactocerebroside are required for sensitization of liposomes than globoside or Forssman antigen. This could in part be due to restricted access of the antibody because liposomes prepared with galactocerebroside (possessing only a single sugar) may have most of the antigenic sites "buried" close to the liposomal bilayer. On the other hand, it seems unlikely that differences in the accessibility of antigen could explain why more globoside is required for generation of immunologically responsive liposomes than Forssman because both antigens contain four sugar residues and differ only in the nature of the terminal glycosidic link which is responsible for antibody specificity. These observations point to the desirability, in future experiments, of using purified antibodies to determine whether there is any influence of antibody class on the amount of antigen required for half-maximal glucose release. A difference might be expected in view of the evidence indicating that fewer molecules of IgM, compared to IgG, anti-Forssman antibodies are required to sensitize sheep erythrocytes to the lytic action of complement (see, *e.g.*, Borsos and Rapp, 1965).

Of particular interest is the finding that liposomes, prepared with lecithin but no antigen, release significant amounts of glucose in the presence of late anti-galactocerebroside sera and a source of native complement. In this regard, it should be noted that the phenomenon is quite reproducible. Eight out of a total of eleven rabbits immunized as described in Experimental Section have produced late antisera with this property. Experiments described in this and the previous papers (Kinsky *et al.*, 1969; Inoue and Kinsky, 1970) have demonstrated that neither anti-Forssman nor anti-globoside sera will cause appreciable marker loss in the presence of complement unless the appropriate antigen has been incorporated into the lipid mixture used for generation of the liposomes. Thus, the ability to cause significant glucose release from lecithin liposomes without any antigen seems at the moment to be a peculiar characteristic of the late anti-galactocerebroside sera.

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2′-*O*-Methyl Polynucleotides as Templates for Cell-Free Amino Acid Incorporation*

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ABSTRACT: The 2′-*O*-methyl-containing heteropolymers, poly(Cm,U) and poly(Am,C), and the three homopolymers, poly(Am), poly(Cm), and poly(Um), were tested for template activity in a cell-free amino acid incorporation system from *Escherichia coli* B. The heteropolymer, poly(Cm,U), directed the incorporation of significant levels of phenylalanine, serine, leucine, and proline, and small amounts of isoleucine and tyrosine. The total incorporation of amino acids was slightly greater with poly(Cm,U) than with poly(C,U). The heteropolymer poly(Am,C) directed the incorporation of proline, threonine, and histidine, but its template activity was lower than that of poly(A,C). Poly(Cm,U) was active as a template for a longer period of time than poly(C,U) in directing the incorporation of phenylalanine. Both poly(Am,C) and -(Cm,U) were degraded more slowly than their unmethylated analogs when incubated in reaction mixtures used for cell-free protein synthesis. Poly(Am), poly(Cm), and poly(Um) had no template activity when tested under conditions that were optimum for the template activity of the corresponding nonmethylated polymers. However, neomycin induced the template activity of the homopolymer, poly(Um), and stimulated the amino acid incorporation directed by the heteropolymers poly(Cm,U) and poly(Am,C). Thus RNA polymers only partially methylated in the 2′ position can still direct the incorporation of amino acids into protein while complete 2′-*O*-methylation renders an RNA molecule inactive as a template.

Since Nirenberg and Matthaei's discovery in 1961 of the template activity of poly(U) in a cell-free extract, RNA polymers of known nucleotide composition have been extensively used as artificial messengers to study the properties of the RNA code and the mechanism of protein synthesis. Many of these studies have examined the changes in template properties following modifications of the RNA polymer. Most of the modifications have been in the ring moiety, *e.g.*, poly(m^2A), poly(m^6A), and poly(m^3U) (McCarthy *et al.*, 1966). However, several investigations have employed polymers containing modifications in the ribose–phosphate backbone. Single-stranded DNA has been reported by McCarthy *et al.* (1966), and Morgan *et al.* (1967), to lack template activity, except in the presence of certain amino glycoside antibiotics, such as streptomycin or neomycin. In another study, Knorre *et al.* (1967), examined the effect of acetylation of the 2′-hydroxyl group in RNA. They found that neither poly(U) that was 88% acetylated in the 2′-hydroxyl position, nor poly(A) that was 98% acetylated, directed the incorporation of amino acids in a cell-free system from *Escherichia coli* B.

In contrast to 2′-*O*-acetyl ribonucleotides and deoxyribonucleotides, 2′-*O*-methyl ribonucleotides are found in RNA isolated from natural sources (Smith and Dunn, 1959; Hall, 1964; Wagner *et al.*, 1967). Nucleotides containing 2′-*O*-methylribose have been used to synthesize both 2′-*O*-methyl homopolymers (Rottman and Heinlein, 1968; Janion *et al.*, 1970), and heteropolymers containing both normal and methylated nucleotides (Rottman and Johnson, 1969). Since natural RNA species contain methyl groups on both the base and sugar moieties, the synthesis of these polymers has made it possible to ex-

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