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INFLUENCE OF TEMPERATURE ON COMPLEMENT-DEPENDENT IMMUNE DAMAGE TO LIPOSOMES

CARL R. ALVING, KATHARINE A. URBAN and ROBERTA L. RICHARDS

Department of Membrane Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20012 (U.S.A.)

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Summary

Maximal release of trapped liposomal glucose, in the presence of saturating amounts of liposomal antigen (galactocerebroside), antiserum (anti-galactocerebroside), and complement, was dependent on temperature. At lower temperatures (20—25° C), maximal glucose release was inversely related to liposomal phospholipid fatty acyl chain length (dimyristoyl phosphatidylcholine > dipalmitoyl phosphatidylcholine > distearoyl phosphatidylcholine > sphingomyelin). At higher temperatures (32—35° C) a limiting plateau of glucose release, at approx. 60%, was reached, or approached, by all preparations. Sphingomyelin liposomes still released less glucose than those prepared from other phospholipids, even at 35° C. The titers of antiserum and complement (ABL_{50}/ml and CL_{50}/ml) were dependent on temperature, and differences based on liposomal phospholipid fatty acyl chain length were observed. Analysis of antiserum and complement-dependence on temperature, and on phospholipid type, revealed that although antibody binding to galactocerebroside undoubtedly was subject to steric hindrance due to interference by surrounding phospholipids at 20—25° C, steric hindrance did not play a major role in blocking antibody binding above 32° C.

Introduction

Despite a large number of studies that have been published in the past 11 years on complement-dependent immune reactions of liposomes [1], very little is known about the influence of temperature. The original experiments

Abbreviations: DMPC, dimyristoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine: DSPC, distearoyl phosphatidylcholine; SM, sphingomyelin.

in the field, and many subsequent experiments, were performed at room temperature (22—25° C) [2,3], but certain laboratories have reported numerous experiments at other temperatures, such as 4—6, 20, 32, 35, or 37° C. One brief report came to the conclusion that although temperature had a marked influence on permeability due to immune damage to liposomes lacking cholesterol, there was a minimal (if any) effect of temperature (from 21 to 35° C) when liposomes contained cholesterol [4]. In the present study we demonstrate that immune damage to liposomes containing cholesterol is strongly influenced by differences of temperature, and that temperature can affect: (a) complement and antibody titers; (b) the maximal ability of liposomes to release trapped marker; and (c) interactions of phospholipids and lipid antigens resulting in steric hindrance to antibody binding.

Although none of the effects that were studied herein had any apparent direct relationship to transition temperatures of phospholipids, some aspects of thermal motion of lipids in liposomes might have played a substantial role.

Materials and Methods

Lipids were purchased from the following sources: DMPC, DPPC, DSPC and cholesterol (Calbiochem, La Jolla, CA, or Sigma Chemical, St. Louis, MO); SM (Pierce Chemical Co., Rockville, IL); dicetyl phosphate (K and K Laboratories, Plainview, NY); beef galactocerebroside (Schwartz/Mann, Orangeburg, NY, or Sigma, or Supelco, Inc., Bellefonte, PA); synthetic lignoceroyl dihydrogalactocerebroside or lignoceroyl dihydrolactocerebroside (Miles Laboratories, Inc., Kankakee, IL).

Previous publications should be consulted for complete details on the following: preparation of fresh guinea-pig and human sera as complement sources; rabbit anti-galactocerebroside serum (heated at 56° C for 30 min to inactivate complement) [5]; screening of rabbit sera for naturally-occurring anti-lactocerebroside antibodies [6,7]; preparation of liposomes, measurement of trapped liposomal glucose, and complement-dependent release of trapped glucose [8,9].

Temperature studies were performed as follows. Glucose assay reagent, antiserum and liposomes were incubated in a cuvette at room temperature, and a baseline absorbance (A_{340}) was measured, as previously described [8,9]. The contents of the cuvette were transferred to a test-tube and incubated in a temperature-controlled water bath for 10 min. The reaction was started by adding complement (either guinea-pig or human serum, also preincubated at the desired temperature). After 30 min, the contents of the tube were transferred back to the cuvette, and A_{340} was measured at room temperature. The guinea-pig and human sera were indistinguishable as complement sources [5].

Results

Effect of temperature on the plateau of immune damage to liposomes

Numerous studies have shown that when liposomes containing increasing amounts of antigen are incubated with excess (saturating) antiserum and

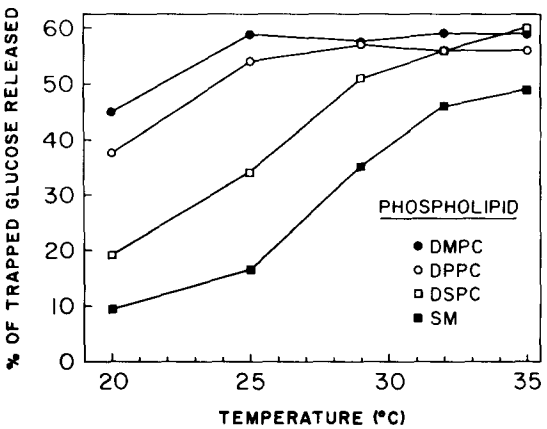

Fig. 1. Effect of temperature on maximal immune damage to liposomes. Liposomes consisted of phospholipid, cholesterol, dicetyl phosphate and galactocerebroside. Molar ratios of the lipids were 2 : 1.5 : 0.22, respectively, plus 150 μg of galactocerebroside per μmol of phospholipid. The phospholipid was 10 mM with respect to the aqueous (0.308 M glucose) swelling solution. Each cuvette contained 30 μl of antiserum and 120 μl of complement. The amounts of antigen, antiserum and complement were saturating, in that additional amounts resulted in little, if any, further glucose release. Each point is an average of two experiments.

complement, increasing amounts of trapped glucose are released. At high antigen concentration a ceiling of marker release is reached, resulting in a plateau at approx. 60% glucose release [2,8]. We found that the height of the plateau of glucose release at room temperature was inversely related to phospholipid fatty acyl chain length when DMPC, DPPC, DSPC, or SM was used to formulate the liposomes [8,10]. Fig. 1 illustrates the maximal glucose release of four liposome preparations, containing DMPC, DPPC, DSPC, or SM, at different temperatures. Each point in Fig. 1 represents the plateau for the liposome preparation at the indicated temperature. The data confirm the previously reported differences of plateaus, based on fatty acyl chain length, when the experiments were run at room temperature (20—25° C). Fig. 1 also demonstrates that such differences were greatly reduced at higher temperature, and the differences between DMPC, DPPC and DSPC were essentially eliminated at 32—35° C, although sphingomyelin liposomes still released less glucose. The upper limit of the plateau (maximal immune damage to liposomes) was not increased above approx. 60% glucose release, even at 35° C.

Effect of temperature on complement activation

Fig. 2 shows that the immunologic activity of complement (guinea-pig serum) was related to temperature. From data such as those shown in Fig. 2, a standard unit of complement, CL_{50}, can be calculated, and is defined as the amount of complement that causes half-maximal glucose release [11]. Fig. 3 demonstrates that the complement titer (CL_{50}/ml) against liposomes was influenced by temperature. Below approx. 30° C, the complement titer was not affected by liposomal phospholipid fatty acyl chain length. At 35° C the complement titer was much higher when assayed with liposomes containing

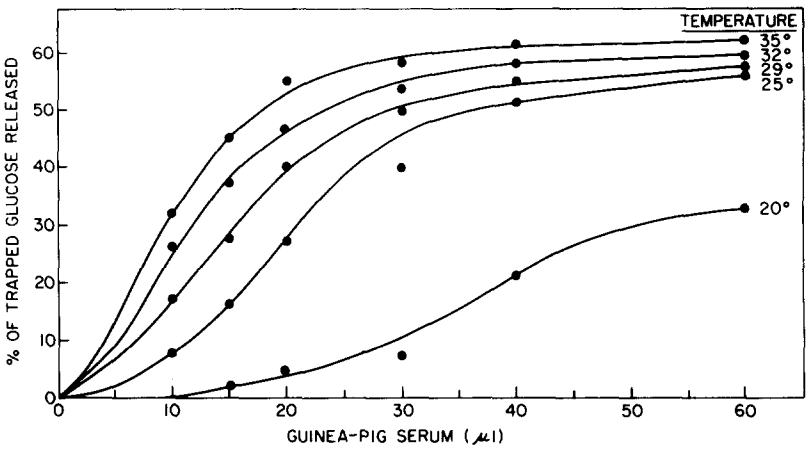

Fig. 2. Effect of temperature on complement activity. The liposomes consisted of DPPC, cholesterol, dicetyl phosphate and galactocerebroside, as described in Fig. 1. Each cuvette contained 30 μl of antiserum.

DMPC, compared with those containing DSPC. These data suggest that the differences related to phospholipid fatty acyl chain length at 20—25° C (Fig. 1) were not due to differences of complement titer, and that the merging of the plateaus (Fig. 1) at 32—35° C was not due to equalization of the complement titers.

Effect of temperature on antibody activities

At low concentrations of antiserum, the ability of the antibodies to induce complement-dependent damage to liposomes was markedly influenced by

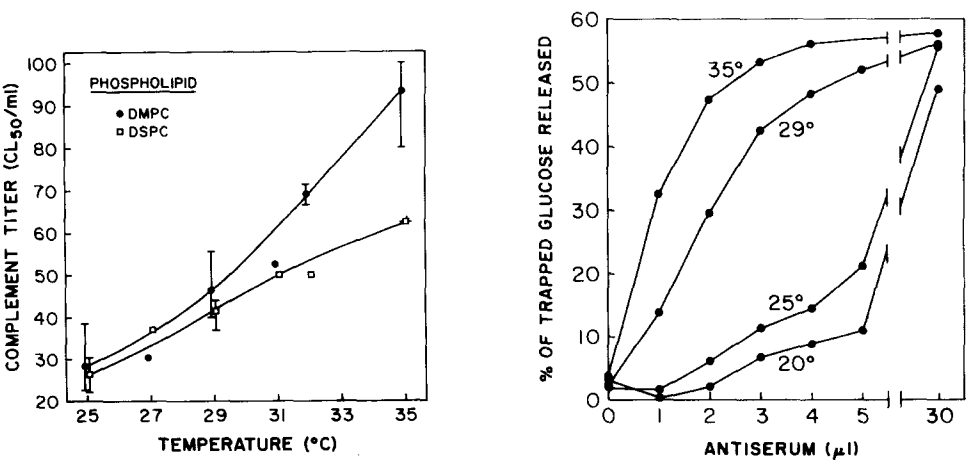

Fig. 3. Effects of temperature and phospholipid type on complement titer. Liposomes were prepared as in Fig. 1. Complement titers were calculated from experiments such as those in Fig. 2. See text for further details. Each curve is an average of three experiments and the vertical bars indicate the ranges observed. For the five points for which no range is shown, the data given are those from one of the three experiments.

Fig. 4. Effect of temperature on antiserum activity. Liposomes were prepared as described in Fig. 1 (with DMPC as the phospholipid). Each point represents an average of five experiments.

temperature (Fig. 4). Nearly all of the temperature differences were eliminated by raising the antiserum concentration to a sufficiently high level (Fig. 4), thus suggesting that at higher temperatures antibodies may have an increased ability to bind antigen or to activate complement.

A standard unit of antibody activity (ABL_{50}) is defined as the amount of antiserum (or antibody) required to cause half-maximal glucose release [10]. The ABL_{50} can be calculated from experiments such as those illustrated in Fig. 4 [10]. Fig. 5 shows that the apparent antiserum titer (ABL_{50}/ml) was strongly increased at higher temperatures. At 25°C, the antiserum titer was higher when assayed with liposomes containing DSPC compared to those containing DPPC at 25°C (Fig. 5), thus making it unlikely that the differences between DPPC and DSPC shown at 25°C in Fig. 1 were due to differences of antibody titer.

Influence of antigen size on immune damage

The above data suggested that although increased temperature could cause increased antibody and complement titers, neither of these factors satisfactorily explained the differences based on phospholipid fatty acyl chain length at 20—25°C shown in Fig. 1 or in Refs. 8 and 10. The disappearance of such differences at higher temperature (30—35°C) (Fig. 1) may have been due to increased exposure of the galactocerebroside above the plane of the lipid bilayer, with resultant increased binding of antibodies to galactocerebroside hapten at higher temperature.

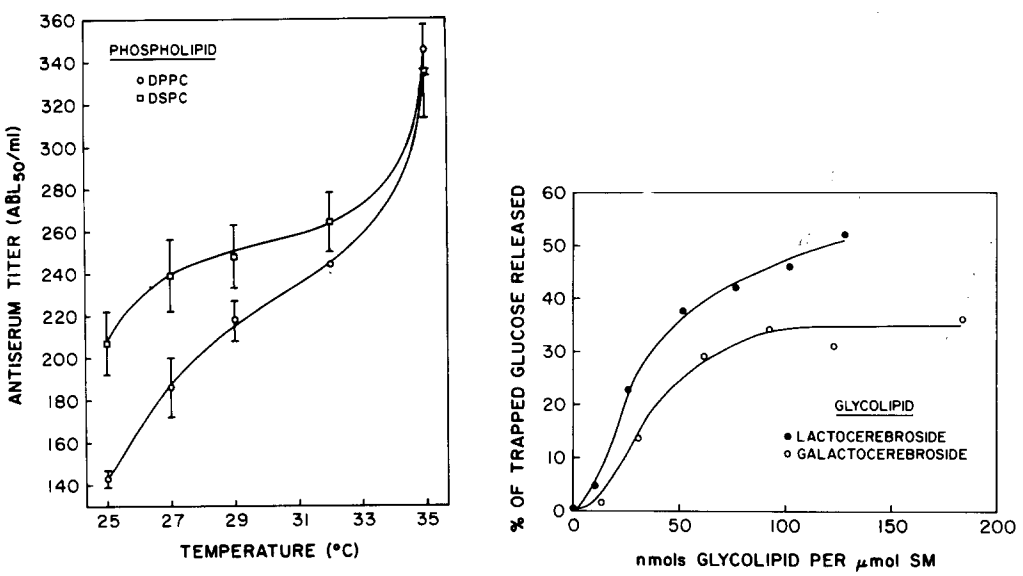

Fig. 5. Effects of temperature and phospholipid type on antiserum titer. Liposomes were prepared as in Fig. 1. Each curve is an average of two experiments and the vertical bars indicate the ranges observed.

Fig. 6. Comparison of galactocerebroside and lactocerebroside as antigens. The liposomes, prepared as described in Fig. 1, contained SM, cholesterol, dicetyl phosphate, and either lignoceroyl dihydrogalactocerebroside or lignoceroyl dihydrolactocerebroside. Each point represents a separate liposome preparation containing the indicated glycolipid, and incubated with saturating antiserum and complement (see legend to Fig. 1). The incubation temperature was 24° C.

Fig. 6 shows an experiment designed to test the concept that a longer, and thus better exposed, glycolipid would serve as a more potent antigen in liposomes. Differences were maximized by utilizing SM in the liposomes, and by running the experiment at 24° C, but qualitatively similar results were observed using liposomes containing DSPC or DPPC (for example, in Ref. 6). Saturating amounts of antiserum and complement were present in all cases. At every concentration of antigen, liposomes containing the larger antigen, lactocerebroside, released more glucose than did those containing galactocerebroside.

Discussion

This study demonstrates that temperature can have a striking influence on complement-dependent damage to liposomes. The titers of complement (Figs. 2 and 3) and antiserum (Figs. 4 and 5) were increased at higher temperature, and the effects of temperature were influenced by liposomal phospholipid composition (Figs. 1,3 and 5).

In previous reports we demonstrated (at 22—25° C) that the degree of glucose release, in the presence of saturating antiserum and complement, was inversely related to phospholipid fatty acyl chain length [8,10]. Adsorption experiments suggested that this phenomenon was due to steric hindrance of antibody binding to small lipid haptens at the membrane surface [10]. Thus, liposomes containing a short phospholipid (e.g., DMPC) and galactocerebroside as antigen, adsorbed more anti-galactocerebroside antibodies than did liposomes containing a longer phospholipid (e.g., DPPC or DSPC) [10].

Two other laboratories reported apparent confirmation of our original results, and proposed additional (or alternative) hypotheses to explain them. Brûlet and McConnell [12], using an antigen consisting of iodoacetamide spin label conjugated to dipalmitoyl phosphatidylethanolamine, showed that antibody-mediated complement fixation was inversely related to liposomal phospholipid fatty acyl chain length, but found that the effect was largely overcome by inclusion of 50 mol% cholesterol. Shin et al. [13] found that marker release due to antibody and complement, from liposomes containing Forssman antigen, was inversely related to phospholipid fatty acyl chain length. Neither of these two studies truly confirmed our original observations because: (a) high concentrations of cholesterol (43 mol%) were present in all of our liposomes; and (b) we found that the phenomenon of steric interference did not occur with liposomes containing Forssman antigen when examined with DMPC, DPPC, or DSPC, presumably because Forssman antigen was sufficiently large that it projected well above the plane of the bilayer [11]. Furthermore, Shin et al. found that marker release was inversely proportional to liposomal phospholipid fatty acyl chain length even when complement was activated in the absence of antigen or antibody, in the reactive lysis system [13].

The experiments in the present paper suggest that any apparent discrepancies in subsequent data [12,13], as described above, compared to our original experiments [8,10], were due to different experimental conditions of temperature. All of our experiments [8,10] were run at 22—25° C, whilst those of Brûlet and McConnell were run at 32° C, and those of Shin et al. were run at

37° C. In Fig. 1 we found no difference between immune damage to liposomes containing DMPC, DPPC, or DSPC when tested at, or above, 32° C. This finding is consistent with the observations of Brûlet and McConnell when they used 50 mol% cholesterol [12], but it is contrary to the observations of Shin et al. [13]. In a subsequent study of comparative efficiencies of different sizes of spin labeled phospholipid antigens, Brûlet and McConnell observed steric hindrance to antibody binding at 32° C, even with 50 mol% cholesterol [14].

The findings of Shin et al. [13], and possibly some of those of Brûlet and McConnell [12], can be explained by the effects of temperature and different phospholipids on the apparent complement titer. Fig. 3 shows that from 25 to 29° C the complement titers increased at an equal rate, and the titers were essentially the same, when tested with liposomes containing either DMPC or DSPC. In contrast, at higher temperature (e.g., 35° C) the complement titer was much lower when tested with DSPC compared to DMPC. A relatively lower complement titer against liposomal membranes could explain the lower marker release at 37° C from DSPC liposomes compared to DMPC liposomes, as reported by Shin et al. [13]. The decreased complement titer might have been due to the increased penetration distance required for traversal of the membrane by a transmembrane protein pore, as suggested by Shin et al. [13], or it could have reflected decreased efficiency of complement activation, or decreased insertion of complement components, at the surface of a more rigid membrane.

The observations in Fig. 3 also might partially explain why cholesterol caused increased complement fixation by DPPC liposomes [12,14]; the cholesterol may have had a fluidizing effect on DPPC, and thereby may have increased the complement titer.

Antiserum titer also was both increased at higher temperature, and affected by phospholipid composition (Figs. 4 and 5). The antiserum titer at lower temperatures was higher with liposomes containing DSPC, compared to DPPC (Fig. 5). Because the antiserum titer was directly related to phospholipid fatty acyl chain length, differences of antiserum titer at 22—25° C could not have been responsible for the inverse relationship between chain length and glucose release shown in Fig. 1 and in Refs. 8 and 10. At higher temperature, presumably because of increased thermal motion, differences of antiserum titers that were correlated with phospholipid fatty acyl composition, were eliminated (Fig. 5).

The discussion given above, together with previous data [8,10], leads to the conclusion that, at 22—25° C, steric hindrance to antibodies can play an important role in antibody binding to small lipid antigens. Fig. 7 shows CPK * models illustrating why such steric hindrance is not surprising, and may even be expected. Although the haptenic portion of galactocerebroside (i.e., galactose) clearly would project above the polar plane of a membrane composed of DMPC, it would be almost level with a DSPC membrane, and it would be completely level with, or even 'embedded' in, the polar plane of an SM membrane (Fig. 7). Lactocerebroside, as would be predicted from the data of

* CPK, Corey-Pauling-Koltun space-filling models.

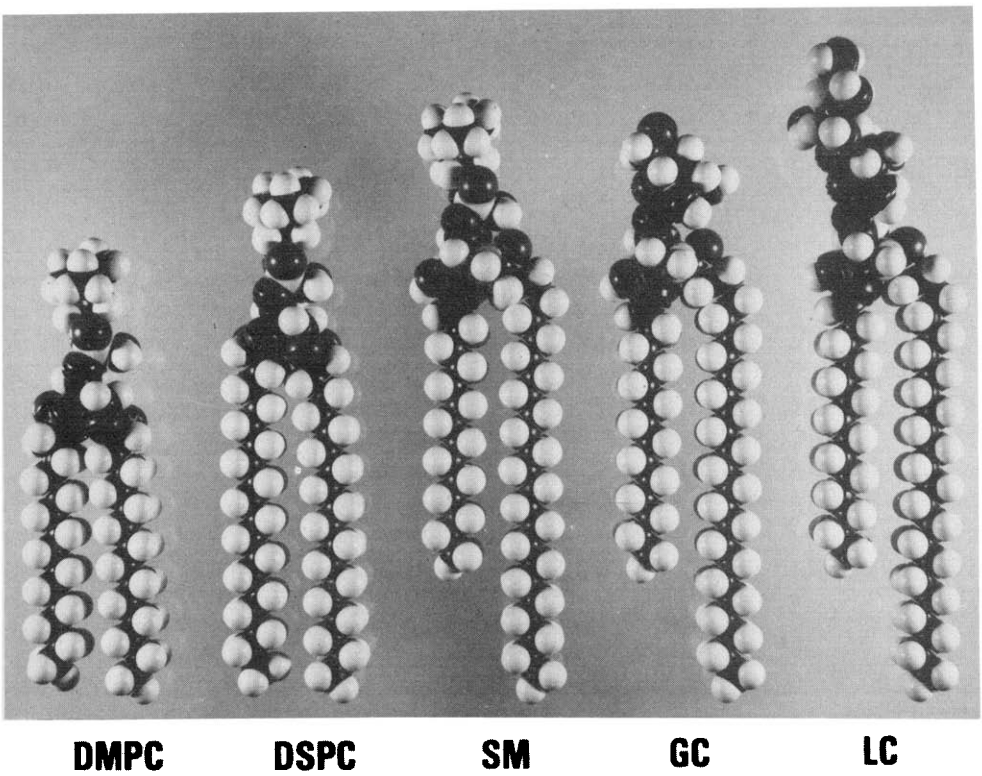

Fig. 7. CPK models of DMPC, DSPC, lignoceroyl SM, lignoceroyl galactocerebroside (GC) and lignoceroyl lactocerebroside (LC).

Fig. 6, would project much more clearly above membranes composed of DSPC or SM (Fig. 7). The data (Fig. 6) and the models (Fig. 7) support the concept, as previously suggested [6,8,10,11,14], that steric hindrance to antibody binding can be overcome by using a larger lipid antigen.

Popular schematic concepts of bilayers frequently depict the plane of the lipid membrane as flat, or comprised of smooth homogeneous pebbles (e.g., Ref. 15), whereas in fact the molecular geometry of the plane of the bilayer is heterogeneous and complex (Refs. 16 and 17, and Fig. 7). In view of the tight packing of phospholipids, and the small space available for binding of small lipid antigens to antibodies, it may even seem remarkable that, as shown in Fig. 1, molecular motion due to thermal energy can overcome many degrees of steric hindrance.

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