Influence of vesicle size on complement-dependent immune damage to liposomes

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Complement-dependent antibody-mediated damage to multilamellar lipid vesicles (MLVs) normally results in a maximum release of 50–60% of trapped aqueous marker. The most widely accepted explanation for this is that only the outermost lamellae of MLVs are attacked by complement. To test this hypothesis, complement damage to two different types of large unilamellar vesicles (LUVs), large unilamellar vesicles prepared by the reverse-phase evaporation procedure (REVs) and large unilamellar vesicles prepared by extrusion techniques (LUVETs), were determined. In the presence of excess antibody and complement the LUVs released a maximum of only approx. 25 to 40% of trapped aqueous marker, instead of close to 100% that would be expected. Since small unilamellar vesicles apparently differ from LUVs in that they can release 100% of trapped aqueous marker it appeared that the size of the vesicles was an important factor. Because of these observations the influence of MLV size on marker release was examined. Three populations of MLVs of different sizes were separated by a fluorescence activated cell sorter. Assays of the separated MLV populations showed that the degree of complement-dependent marker release was inversely related to MLV size. No detectable glucose was taken up by MLVs when glucose was present only outside the liposomes during complement lysis. Our results can all be explained by the closing, or loss, of complement channels. We conclude that complement channels are only transiently open in liposomes, and that loss of channel patency may be due to either channel closing or to loss of channels.

Introduction

When immunologically-sensitized liposomes are exposed to attack by complement, membrane

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Abbreviations: LUVs, large unilamellar vesicles; LUVETs, large unilamellar vesicles prepared by extrusion techniques; MLVs, multilamellar vesicles; REVs, large unilamellar vesicles prepared by the reverse-phase evaporation procedure; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine.

damage occurs, resulting in release of trapped aqueous marker from the liposomes (reviewed in Refs. 1–3). The rate and degree of marker release from liposomes is related to the number of immune complexes (i.e., antigen-antibody-complement lesions) formed in the liposome membrane. However, despite the presence of nonlimiting amounts of antigen, antibody, and complement, the maximum total extent of permeability from multilamellar vesicles (MLVs) is ordinarily limited to approx. 50–70% of the trapped liposomal

marker [4]. The mechanism of this plateau of marker release, which occurs even over extended periods of time, has been the subject of considerable speculation [1–3]. One major hypothesis is that only the outer few lamellae of the liposomes are attacked by complement [1]. The strongest evidence in support of this hypothesis is that small unilamellar liposomes release essentially 100% of their trapped marker [5]. In the present study this hypothesis was tested further by subjecting large unilamellar liposomes (LUVs) to complement attack. To our surprise, marker release from LUVs was less than from MLVs. By using evidence based on sorting of liposomes by size separation techniques, we have concluded in this study that the percent of trapped marker release is related to the size and aqueous volume of the liposomes. Our data can best be explained by rapid opening and closing of the complement-induced membrane lesion, resulting in transient release of trapped marker.

Materials and Methods

Lipids

Lipids were purchased from the following sources: dimyristoyl- and dipalmitoylphosphatidylcholines from Sigma Chemical Co., St. Louis, MO; cholesterol from Calbiochem-Behring, La Jolla, CA; bovine brain cerebrosides (galactosyl ceramide) from Miles Laboratories, Inc., Elkhart, IN; dicetyl phosphate from K & K Laboratories, Inc., Plainview, NY; and NBD-phosphatidylethanolamine (NBD-PE) from Pierce Chemical Co., Rockford, IL.

Liposomes

All liposomes contained dipalmitoylphosphatidylcholine or dimyristoylphosphatidylcholine, cholesterol, dicetyl phosphate and galactosyl ceramide in molar ratios of 1.0 : 0.75 : 0.11 : 0.18. The fluorescent liposomes contained dimyristoylphosphatidylcholine as phospholipid; and all other liposomes contained dipalmitoylphosphatidylcholine. The molecular weight of lignoceroyl dihydrogalactosyl ceramide ($M_r = 814$) was used in estimating the molar ratio for galactosyl ceramide. Liposomes used in glucose release assays were swollen in 0.308 M glucose; liposomes used in glucose uptake experiments were swollen in 0.308 M galactose; all other liposomes were swollen in 0.154 M NaCl.

Multilamellar liposomes (MLVs) were prepared as described previously [6]. The phospholipid concentration was 10 mM with respect to the aqueous swelling solution. Two methods were employed for preparing LUVs: large unilamellar vesicles by extrusion techniques (LUVETs) [7], and the reverse-phase evaporation procedure (REVs) [8]. The LUVETs were prepared as described previously [7] by passing 10 ml of MLVs 10 times through polycarbonate filters (100 nm pore size) at pressures greater than 600 lb/in². The LUVETs were sized by quasi-elastic light scattering using a Nicomp Model 200 Laser Particle Sizer (Nicomp Instruments, Goleta, CA) [9]. The average diameter of the LUVET preparations tested was 128 nm. The final phospholipid concentration of the LUVETs tested was 8–12 mM. All LUVETs used in these experiments were shown to be unilamellar by ³¹P-nuclear magnetic resonance analysis as described previously [7]. Reverse-phase evaporation vesicles (REVs) were prepared as described previously [8] except that at least 30 min of sonication was required to achieve a homogeneous dispersion. The REVs for these experiments were prepared using 30 μmoles of dipalmitoylphosphatidylcholine, 22.5 μmoles of cholesterol, 3.3 μmoles of dicetyl phosphate and 4.5 mg of galactosyl ceramide in 3 ml of isopropyl ether, 3 ml of chloroform and 1 ml of 0.308 M glucose. After removing untrapped glucose from the REVs by centrifugation in 0.154 M NaCl, the resulting washed REV pellet was suspended to a volume of 2 ml with 0.154 M NaCl. The phospholipid concentration of this suspension was 5.5 mM.

Complement-dependent glucose release

Complement-dependent release of trapped liposomal glucose was determined as described previously in great detail [6]. Glucose release was assayed using 500 μl of glucose assay reagent (containing hexokinase, glucose-6-phosphate dehydrogenase, ATP and NADP), sufficient 0.154 M NaCl to give a final volume of 1.0 ml, 5 μl of liposomes, 50 μl of anti-galactosyl ceramide serum, and 120 μl of human complement, unless otherwise indicated. Release of glucose was measured as in-

creased A_{340} due to reduction of NADP. Both antiserum and complement were dialyzed vs. 0.154 M NaCl to remove glucose prior to use in the glucose release assay. Rabbit anti-galactosyl ceramide serum, prepared as described previously [6,10], was heated at 56°C for 30 min to inactivate complement. Human complement [6] consisted of serum from individual humans, and was stored at −70°C. Guinea pig complement [6] consisted of serum pooled from at least 20 animals and was stored at −70°C.

Fluorescence profiles of liposomes

MLVs containing 60 ng of NBD-PE per μmol of dimyristoylphosphatidylcholine were diluted with 0.154 M NaCl and centrifuged at $12\,000 \times g$ for 10 min at 20°C. After aspiration of the supernatant, the pellet was resuspended to its original volume in 0.154 M NaCl. The washed liposomes were analyzed and sorted into three or four populations with a fluorescence activated cell-sorter [11] (see Fig. 3, below). It was necessary to centrifuge the liposomes prior to cell-sorter analysis because the large number of very small liposomes overloaded the cell-sorter before an adequate number of large liposomes could be obtained. It is likely that the removal of very small liposomes by centrifugation was responsible for the maximum release of trapped glucose from the small MLVs being only 55% (see Fig. 4, below).

Complement-dependent glucose uptake

MLVs (125 μl) swollen in 0.308 M galactose were incubated with 0.75 ml anti-galactosyl ceramide serum (giving an antiserum to liposome ratio corresponding to that for 30 μl of antiserum in Fig. 1) and either 1.125 ml of 0.308 M glucose or 1.125 ml of 0.308 M galactose for 10 min, then centrifuged 2–3 min at 27 lb/in^2 in an A-100/18 rotor in an Airfuge (Beckman Instruments, Inc., Palo Alto, CA). The resulting liposome-antibody (LA) pellets were each washed with 5 ml of 0.308 M galactose. Due to the large volumes to be centrifuged, the limited capacity of the Airfuge (approx. 1 ml per run), and the tendency of the liposomes, even with antibody bound, to float when serum was present, it took more than 2 h to obtain washed pellets. The washed liposome-antibody pellets were either suspended in 0.308 M

galactose to a volume of 0.5 ml and aliquots taken for determination of trapped glucose [6] or liposome-antibody pellets obtained in the presence of galactose were suspended to 0.25 ml with 0.308 M galactose and incubated with 2.5 ml human complement and either 3.36 ml of 0.308 M glucose or 3.36 ml of 0.308 M galactose. After 30 min, the liposomes with antibody and complement bound (LAC) were centrifuged as above, and the resulting pellets each washed with 5 ml of 0.308 M galactose. As with the LA pellets, more than 2 h of centrifuging was required to obtain washed LAC pellets. The washed LAC pellets were suspended to 0.5 ml with 0.308 M galactose and aliquots taken for determination of trapped glucose.

Results

Complement-dependent liposomal permeability changes

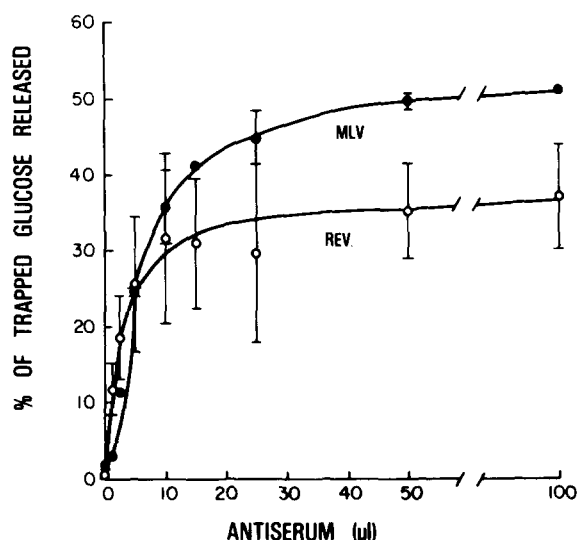As shown in Fig. 1, the maximum release of trapped glucose from REVs was approx. 40%,

Fig. 1. Effect of antibody concentration on complement damage to MLVs and REVs. Complement-dependent glucose release was measured as described in Materials and Methods, except that anti-galactosyl ceramide serum was added in the amounts indicated. Less than 5% of trapped glucose was released when heat-inactivated complement was used. Liposomes lacking antigen (galactosyl ceramide) released less than 5% of trapped glucose when 75 μl of antiserum was added. The curves are the means (±S.D.) of two or three experiments, except that the points lacking error bars were done only once.
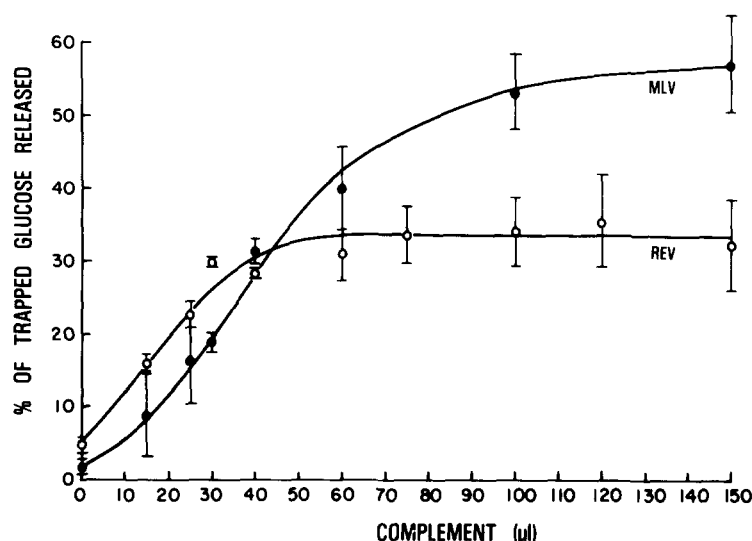
Fig. 2. Effect of complement concentration on complement damage to MLVs and REVs. Complement-dependent glucose release was measured as described in Materials and Methods, except that complement (human serum) was added in the amount indicated. Less than 5% of trapped glucose was released when heat-inactivated complement was used. Control liposomes lacking antigen released less than 4% of trapped glucose when 120 µl of complement was added. The curves are the means (±S.D.) of two or three experiments.

which is less than the 50–60% release obtained with MLVs. The results obtained with the REVs mimic the effects seen previously either with limiting antigen, antibody, or complement [4], or with vitamin A present in the liposomes [10], or with phospholipids having longer fatty acyl chains and therefore thicker bilayers [12]. In all of these cases, the net result is reduction in the number of functional channels caused by complement-containing immune complexes.

Since REVs are unilamellar (or oligolamellar) vesicles, the amount of antigen on the outer surface of the vesicles available for antibody binding may be greater for REVs than for MLVs when equivalent amounts of lipid are present. However, Fig. 1 shows that the difference in complement-dependent marker release between REVs and MLVs was not related to antibody concentration. Even when the antibody concentration was increased to a level 4-fold higher than the saturating concentration, the maximum release was greater for MLVs than for REVs. The lesser release of trapped marker from REVs also was not due to insufficient complement. Fig. 2 shows that even with almost 4-fold higher than saturating amounts of complement the plateau of marker release was lower for REVs.

In order to determine if the results with the REVs are characteristic of other large unilamellar liposomes, we also tested the immune sensitivities of LUVETs. The LUVETs, like the REVs, also gave lower maximum release of trapped glucose due to complement damage than the MLVs (data not shown).

Influence of vesicle size on complement-dependent permeability changes

The finding that the complement-dependent release of glucose from LUVs was less than from MLVs could be explained by one of the following hypotheses. (1) MLVs could be more sensitive than LUVs to immune attack; (2) either complement or antibody might be inhibited by LUVs; (3) the ratio of lipid to aqueous volume in the liposomes might affect the extent of liposomal permeability changes.

To test whether the unexpectedly low immune marker release from LUVs was due to differences in the lipid to aqueous volume ratio, MLVs of different sizes were obtained by using a fluorescence activated cell sorter. Fig. 3 shows the distribution of an MLV preparation containing a fluorescent phospholipid when four populations, based on light scattering, were counted. Most of the liposomes were small (peaks 1 and 2), based both on light scattering and relative intensity of fluorescence. This agreed with previous results obtained by Coulter counter analysis of MLVs of the same lipid composition as those used here in which more than 50% of the liposomes were found to be 1.25 µm in diameter or less [13].

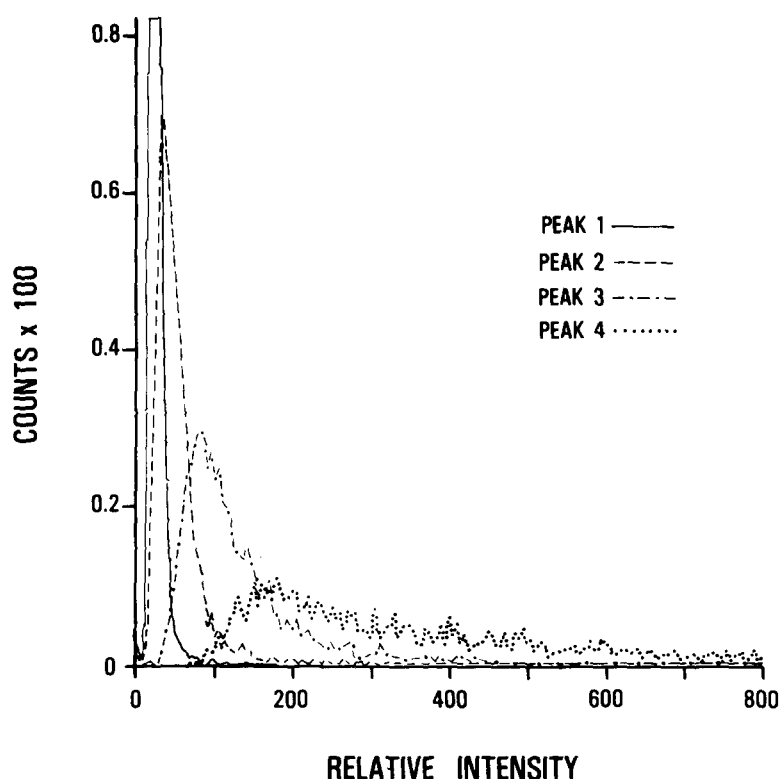The MLVs were separated, using the cell sorter,

Fig. 3. Fluorescence profiles of MLVs. The size distribution of fluorescent MLVs containing dimyristoylphosphatidylcholine as phospholipid, and prepared as described in Materials and Methods, was analyzed with a fluorescence activated cell sorter. Fluorescence histograms are shown for four populations derived from 1 preparation of MLVs, separated on the basis of light scattering. This figure is derived from one experiment, but is representative of data obtained in other experiments. The relative fluorescence intensity is depicted on the abscissa, with increasing fluorescence to the right and the number of fluorescent MLVs in each channel (counts × 100) is depicted.

into three populations which corresponded to peaks 1, 2, and (3 + 4) (Fig. 3). Each of these three populations was then tested to determine the maximum immune glucose release possible (Fig. 4). The liposomes corresponding to peak 1 of Fig. 3 were designated small MLV; those corresponding to peak 2, medium MLV; and those corresponding to peaks (3 + 4), large MLV. If MLVs were more susceptible to immune damage than REVs, all three populations of MLVs would be expected to give high levels of immune glucose release. However, as can be seen in Fig. 4, the maximum level of glucose release decreased with increasing liposomal trapping. The large MLVs released only 20% of the trapped glucose marker.

Complement-dependent uptake of glucose by MLVs

Since all our results seemed to indicate that the complement lesion in liposomes was transient, we decided to test whether, during the period of complement damage to the liposomal membrane, glucose could be taken up by the liposomes to an extent similar to that which is released from the

liposomes. MLVs containing entrapped galactose were reacted with antibody and complement in the presence of 0.17 M glucose as described in Materials and Methods. The amount of glucose taken up was only 1% of the amount of glucose calculated to be released from MLVs swollen in 0.17 M glucose.

Discussion

Several theories have been advanced to explain the limiting plateau of permeability consisting of 50–70% release of trapped marker observed with MLVs in response to attack by complement [1–3]. The most widely accepted theory is that trapped marker is released only from the outer few lamellae of the liposomes, while the aqueous volume in the inner 'core' of the liposomes contributes little, if any, to the volume of trapped marker released. The present study basically supports this hypothesis, and also reveals a simple explanation for the phenomenon. The explanation is that the complement lesion is either closed, or lost, from the
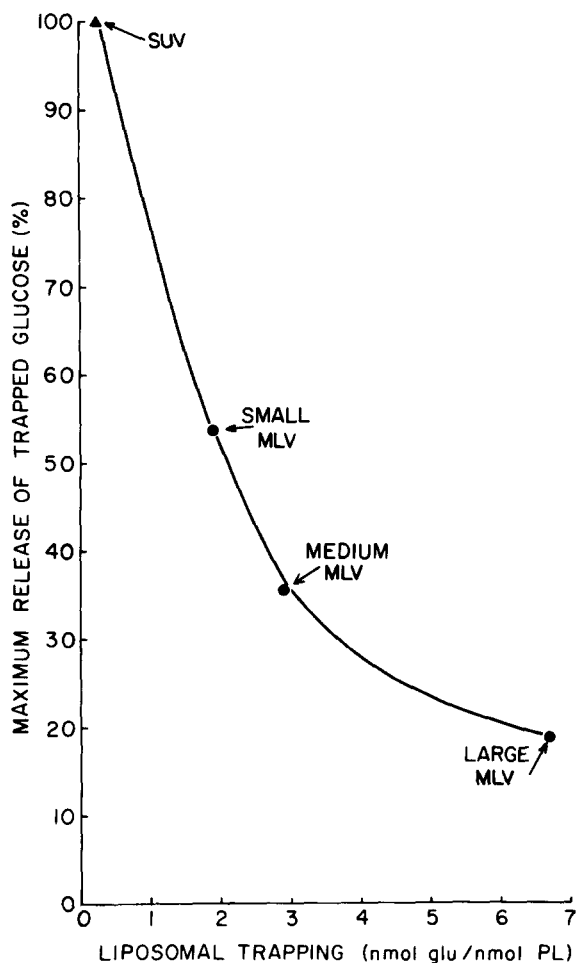
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Fig. 4. Complement damage to MLVs of different sizes. The value for SUVs was taken from [5]. MLVs (150 μl) prepared as in fig. 3 and containing trapped glucose were sorted in the fluorescence activated cell sorter into three populations, corresponding to peaks 1 (small MLV), 2 (medium MLV), and 3 + 4 (large MLV). The sorted MLVs were centrifuged 10 min at $29000 \times g$, the supernatants were removed and the pellets each were suspended in 150 μl of 0.154 M NaCl. Complement-dependent glucose release was measured on 20-μl aliquots of the sorted MLVs in the presence of saturating antibody and complement as described in Materials and Methods.

liposome membrane, shortly after formation of the transmembrane channel.

The latter conclusion is deduced mainly from data involving liposomes having different sizes and aqueous volumes. Previously it was shown that small unilamellar vesicles (SUVs) released essentially 100% of their trapped marker after comple-

ment attack [5]. We reasoned therefore that large unilamellar vesicles (LUVs) should similarly release close to 100% of marker; at least release should be greater than from MLVs. This did not occur: two types of LUVs (LUVETs and REVs) consistently released only 20–40% of trapped glucose instead of 50–60% release normally observed from MLVs. This apparently anomalous observation could be easily explained if the complement lesion were only transiently open. The percentage of trapped glucose that could diffuse out of a liposome in a given time would be lower for an LUV than for an MLV. Stated another way, diffusion of a fixed number of glucose molecules would represent a smaller percentage of the whole population of glucose molecules with LUVs than with MLVs. This is not surprising in view of the fact that much of the internal volume of MLVs is occupied by concentric lipid lamellae rather than glucose. Therefore, the aqueous glucose content for equal size particles is greater for LUVs than for MLVs.

If our hypothesis were correct, then there should be an inverse correlation between size (and therefore aqueous volume) of MLVs and the percent of trapped glucose released. When the MLVs were separated into different sizes by using a fluorescence-activated cell sorter, an inverse correlation between internal aqueous volume and percent of marker release was indeed observed (Fig. 4).

If the complement lesion were open only for a limited time, this could occur by either of two mechanisms. (1) The transmembrane channel could simply close. This seems rather unlikely because careful measurements of diffusion characteristics of complement channels in erythrocytes have revealed a long lifetime for the channels [14,15]. Furthermore, morphological observations seem to show an open tubule, even after isolation and subsequent reconstitution of the channel into fresh liposomes [16,17]. (2) The second possible mechanism, which is much more likely, is that the channel simply falls off the liposome.

The second mechanism, that the channel becomes separated from the liposome, is consistent with several other lines of evidence. It is consistent with the known ability of the complement channel to be removed from cells and to exist independently of membranes [18], and even to be isolated

intact and then incorporated into fresh liposomes [16,17]. It is also consistent with the difficulty that has been reported in observing complement lesions by electron microscopy in antigen-containing liposomes after reaction with antibody and complement and in correlating the number of channels with the degree of marker release [19,20]. Finally, it is also consistent with the fact that the membrane attack complex has a high affinity for phospholipids [21] and release of intact membrane phospholipids accompanies complement attack on liposomes [22,23].

If the complement lesion is functionally open only transiently, it should be possible for an external marker to enter the liposomes and become trapped inside when the complement lesions become functionally closed. To test this, we incubated liposomes swollen in galactose in the presence of external glucose while complement damage was occurring. However, when this experiment was performed virtually no glucose uptake was observed. There are several possible explanations for this. It may be that glucose was taken up but leaked back out during the long period of centrifugation and exposure to serum. Although the complement lesions become effectively closed so that no additional antibody and complement can diffuse into any unattacked inner lamellae, the outer bilayers may have remained fragile so that any glucose taken up during complement damage diffused out during the washing with galactose. A second possibility is that there is only one-way diffusion of saccharides across the complement lesion. However, this is unlikely since saccharides readily diffuse into C5b-9 erythrocyte ghosts [24]. Lastly, it is possible that the complement lesions, or fragments of the lesions, fall off the liposomes, again resulting in a type of fragility or leakiness, so that any glucose going into the liposomes, would diffuse out during the washing procedure. In the absence of further direct information about the physical state of bilayers attacked by complement, including the possible presence of incomplete complement channel fragments, it is impossible to determine which, if any, of the above explanations is correct. This experiment would have been most valuable if glucose uptake had been observed. However, because of uncertainty about the physical state of complement-damaged lipid bilayers the 'negative' results obtained do not lead to any definitive conclusions.

If the complement channel were released from the liposome as we propose, then its behavior would be different in liposomes than in erythrocytes. The number of complement lesions in erythrocytes has been carefully counted and it correlates well with the extent of membrane damage [25]. A major difference, of course, between cells and liposomes is the presence of intrinsic membrane proteins in cells. Interactions between the complement membrane attack complex and intrinsic membrane proteins have been observed [26]. It is possible that cross-linking of terminal complement components with membrane proteins in erythrocytes may serve to anchor the complement channel in the cell membrane. However, it has recently been reported that the complement lesion is only transiently present in nucleated cells [27,28]. Therefore it appears that the membrane environment of the complement lesion in liposomes may resemble the environment found in nucleated cells more closely than that of erythrocytes.

If the complement channel were released from liposomes, its behavior also would appear to be fundamentally different from other channels that bear a superficial morphological resemblance to complement lesions. Among these latter channels, which, in contrast to complement channels, are easily viewed by electron microscopy in liposomes, are those induced by polyene antibiotics [29] and by saponin [30]. The toxins known as lysins secreted by certain bacteria (streptolysin, tetanolysin, etc.) bind to cholesterol, and look very similar to complement channels when examined by electron microscopy [31]. However, the lysins can induce almost 100% glucose release from MLVs [32]. Our data would suggest that the mechanisms that keep all of these other channels anchored in the liposome membrane are not operative for the complement channel in liposomes.

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