STUDIES ON THE HUMAN AND GUINEA PIG SERUM COMPLEMENT SYSTEMS WITH LIPOSOMAL MODEL MEMBRANES

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ABSTRACT

STUDIES ON THE HUMAN AND GUINEA PIG
SERUM COMPLEMENT SYSTEMS WITH
LIPOSOMAL MODEL MEMBRANES

By

Kristine Callenbach Knudson

The objective of this study was to investigate the human and guinea pig serum complement systems with liposomal model membranes. Liposomes prepared from sheep erythrocyte membranes at a concentration of 2 μmoles phospholipid/ml of galactose marker solution were used. Modifications in the procedure for preparation of liposomes, which included varying the phospholipid concentration and prolonged sonication, resulted in preparations of great stability. A simplified assay for the detection of released galactose indicative of liposome lysis was developed based on the oxidation of galactose by galactose dehydrogenase (EC 1.1.1.48) and the corresponding reduction of nicotinamide adenine dinucleotide (NAD) was monitored spectrophotometrically at 340 nm.

Liposomes were lysed by guinea pig serum (as the source of complement) in conjunction with anti-sheep cell antibody and the degree of lysis was dependent on serum concentration. Based on this dependency, the liposome system was developed into a quantitative assay for measurement of complement activity. Electron micrographs

of the serum-lysed liposomes revealed that no discrete lesions were produced during lysis, but rather the liposomes seemed to fragment.

Studies on the lysis of liposomes by fresh human serum and on the binding of functionally pure complement components indicated that at least two mechanisms were operating simultaneously to cause lysis of the liposomes. The first mechanism appeared to be the classical complement sequence, requiring all nine complement components, antibody, Ca^{++}, and Mg^{++}. The second mechanism appeared to require the late-acting components (C3-C9) and Mg^{++}, and the C3 proactivator system was hypothesized to be involved. Using specific antisera against C3 and the C3 proactivator, it was demonstrated that the C3 proactivator system participated in the lysis of liposomes. In addition, a factor, sensitive to diisopropylfluorophosphate and stable to heating at 56°C, was detected in the anti-sheep cell antiserum and this factor apparently participated in liposome lysis.

STUDIES ON THE HUMAN AND GUINEA PIG SERUM COMPLEMENT SYSTEMS WITH LIPOSOMAL MODEL MEMBRANES

By

Kristine Callenbach Knudson

A THESIS

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DEDICATION

To my husband, David, who always offered encouragement, understanding, and helpful discussions, and to my parents who instilled in me the value of a good education.

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I would like to express my sincere thanks and appreciation to Dr. David H. Bing for all his help and guidance during my graduate program. His willingness to discuss at any time my progress and problems and to offer his assistance in countless ways is greatly appreciated.

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INTRODUCTION

The complement system consists of nine components comprised of eleven proteins which interact sequentially with each other to mediate cellular injury and promote the inflammatory response. Druing the past thirty years a model system of sheep erythrocytes, rabbit anti-sheep cell antibody and serum has been used to study the consequences of the activated complement system. Using this model system, a great amount of information on the complement system was obtained. But to study the enzymatic activities of the individual complement components bound to a membrane as in the in vivo situation, an alternative model system was necessary to avoid the contamination of enzymatic reactions by the cellular contents of erythrocytes. The liposomal model membrane system appeared to be the alternative membrane system for these studies. Liposomes are vesicular lipid bilayers, prepared, in this instance, by swelling the phospholipid extract of sheep erythrocyte membranes in an aqueous galactose solution. The purpose of this study was to use the liposomal model membrane system as a tool for the study of the guinea pig and human serum complement systems.

The first component of complement (C1) binds to the cell membrane through an interaction of one of its subunits, C1q, with antibody present on the cell surface. Initial studies on the binding of C1 to liposomes indicated that the C1 was bound non-specifically in the absence of antibody, with little enhancement of binding when antibody

was present. Since previously it had been shown that fresh serum (as the source of complement) lysed liposomes, and that the second and eighth components of complement were required for lysis, the roles of other factors in the lytic mechanism were investigated.

This thesis is organized into four sections. The first is a literature review in which information on the complement system, the known alternate pathways into the complement system, and liposomal model membranes is presented. The second section consists of a published manuscript on the application of liposomes for the quantitative measurement of guinea pig complement. The third and fourth sections consist of a manuscript and a communication to be submitted for publication and concern the evidence for alternative mechanisms for lysis of liposomes and a procedure for the centrifugation of liposomes.

LITERATURE REVIEW

Part I

The Complement System

An extensive review of the complement system is beyond the scope of this survey. Several comprehensive reviews have been written (1,2,3,4,5). The following review will be concerned with the nomenclature of the complement system, highlights of the complement reaction sequence, and a brief discussion of some of the system's biological implications.

Nomenclature of Complement. The term complement is applied to a system of factors occurring in normal serum that are characteristically activated by antigen-antibody interaction and subsequently mediate a number of biologically significant consequences. Most of the information of the complement system has been derived from studies with a model system consisting of sheep erythrocytes (E) treated with rabbit antibody (A) which are then lysed upon the addition of fresh normal serum as the source of complement (C)(1,6,7). The present terminology devised for the complement system is based upon this immune lytic sequence. This terminology was agreed upon at a series of discussions arranged by the World Health Organization (8).

The nine distinct complement components which interact sequentially with each other in immune hemolysis are numbered C1-C9. The

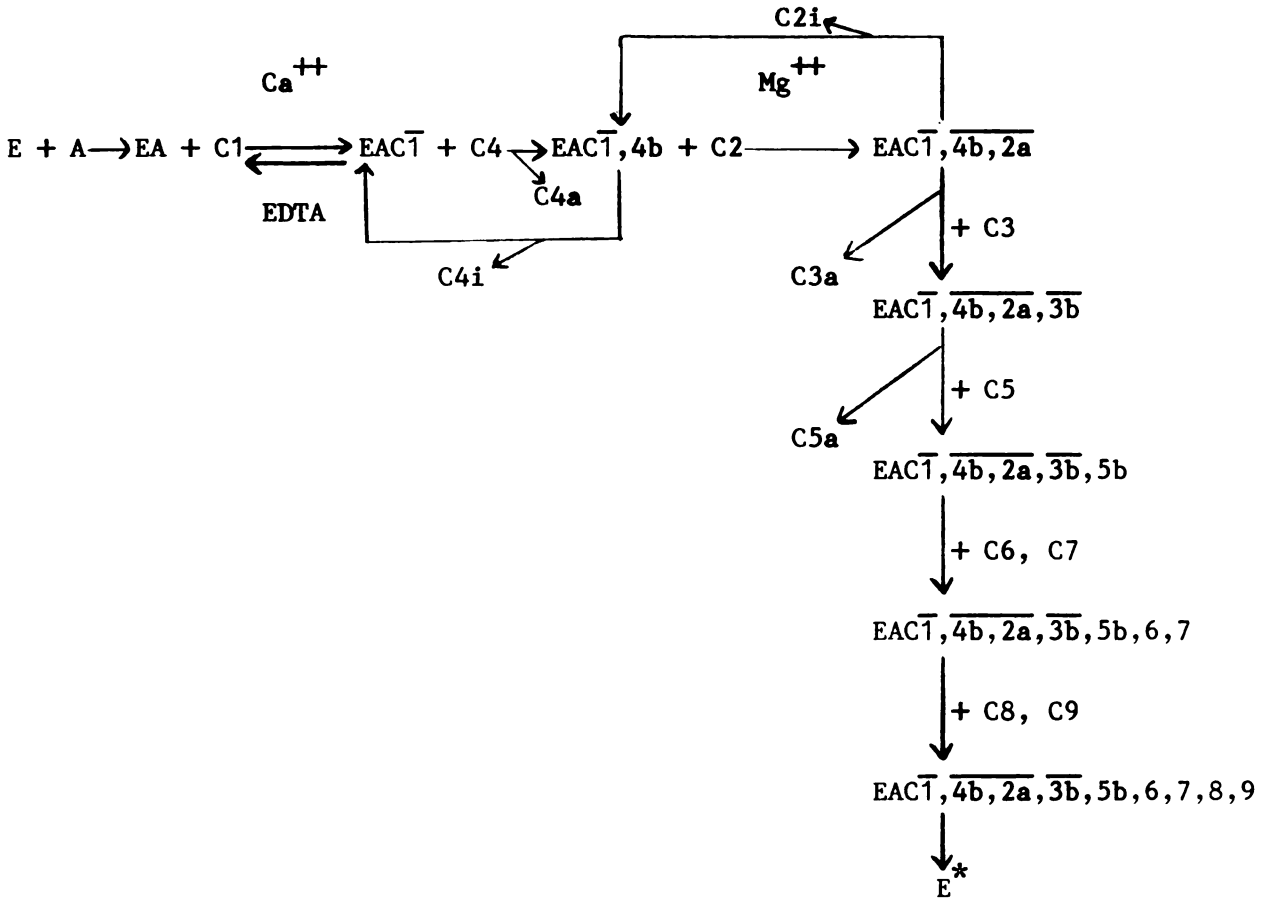
first four components are designated C1, C4, C2, and C3 because of long-standing usage, but the five remaining components are numbered sequentially (i.e., C5, C6, C7, C8, C9). Intermediate complexes are designated by EAC followed by those components which have interacted. The components enumerated after EAC denote a state of reactivity and not necessarily their physical presence (e.g., EAC1,4,2,3). Alternatively, the example may be shortened to EAC1-3. A bar or rule placed over a complement component is used to indicate that the component is in an active enzymatic or other biological state (e.g., $\overline{C1}$ or $C\overline{5,6,7}$). The loss of defined activity by a complement component is denoted by the suffix "i". Fragments which result from cleavage of complement components during the course of their reaction are suffixed sequentially with lower case letters (e.g., C3a).

The Complement Sequence. A detailed diagram of the complement sequence is shown in Figure 1. The complement system is activated upon interaction of C1 with an antigen-antibody complex. C1 is comprised of three subunits, designated C1q, C1r and C1s, which function as a unit and are bound together by Ca^{++}(9). C1q possesses the binding site of C1 for the antibody and in binding to the antibody undergoes a conformational change (10). C1r is then activated and in turn acts upon C1s converting the inactive proenzyme to its active esterase form, $\overline{C1s}$ (9,11).

$\overline{C1}$ splits C4 into two fragments, the larger of which is bound to the cell membrane to form the stable complex, EAC1,4b (12,13). C2 is also cleaved by $\overline{C1}$ and the larger fragment, C2a, forms a cell-bound complex of EAC1,4b,2a in the presence of Mg^{++} (14,15).

FIGURE 1

The Complement Sequence



The complex formed by the action of $C\overline{1}$ on C4 and C2 (EAC1,4,2) possesses a new enzymatic activity termed C3 convertase (16). This enzyme cleaves C3 into two fragments, C3a and C3b, and the larger C3b fragment is bound to the cell surface to form the intermediate EAC1,4b,2a,3b (17). This new intermediate also possesses a new enzymatic activity called C5 peptidase and acts upon the next component in the sequence, C5. Again two fragments are produced, C5a and C5b, and the larger C5b fragment binds to the complex (18,19). C6 and C7 are then bound to C5 in an unknown manner (3).

The union of C8 with the EAC1-7 cellular intermediate initiates membrane damage with C8 being present physically on the cell surface (20,21). EAC1-8 cells gradually lyse (20), but the rate of lysis is markedly enhanced by the attachment of C9 to the complex (22). A functionally impaired cell membrane exhibits ultrastructural lesions which appear as holes of about 100Å in diameter (23,24).

Biologically Active By-Products of the Complement System.

Cleavage of C3 by $C\overline{1,4,2}$ results in two fragments, C3a and C3b. As stated previously C3b is bound to the cell surface and C3a is released into solution. The C3b fragment of 180,000 molecular weight when bound to the cell appears to be the moiety responsible for immune adherence (4). Immune adherence is an agglutination phenomenon based upon the ability of antigen-antibody-complement complexes to adhere to the surface of nonsensitized particles, such as platelets, erythrocytes, and starch granules (2). Also ascribed to cell-bound C3b is phagocytosis (7,15). Conglutination is the agglutination of sensitized erythrocytes by a heat-stable and calcium-dependent factor in

bovine serum (6). Cell-bound C3 is acted upon by the conglutinogen activating factor (KAF), which has recently been shown to be identical to the C3 inactivator, to form a complex (26). This complex of bound C3 and KAF then reacts with conglutinin in the presence of Ca^{++} to cause agglutination (27).

The other fragment of C3, C3a, has a molecular weight of 6,800 and has some of the characteristics of classical anaphylatoxin (28,29). Anaphylatoxin causes smooth muscle contraction, increased vascular permeability, and release of histamine from rat peritoneal mast cells (2,4). The fragmentation of C3 by the action of cobra venom factor, plasmin, trypsin, and bacterial endotoxic lipopolysaccharide produces a C3a fragment with very similar properties to the C3a produced by the action of the C3 convertase. These other means of cleaving C3 will be discussed in a later section. Other activities ascribed to C3a are a neutrophil chemotactic factor and the degranulation of mast cells.

The cleavage of C5 also produces an anaphylatoxin, C5a, which has been shown to be distinct from C3a (29,30). This 12,000 molecular weight fragment can be formed either by the C5 peptidase ($\overline{C1,4,2,3}$) or by the action of trypsin on C5. C5 anaphylatoxin differs from C3 in its ability to release histamine from rat mast cells (4). Both anaphylatoxins are functionally similar, but subtle differences in biological and physiochemical properties distinguish them.

The complex of C5, C6 and C7 acquires neutrophil chemotactic activity following treatment with $\overline{C1,4,2,3}$ (31,32). The chemotactic factor associated with $\overline{C5,6,7}$ has been shown to activate a

proesterase in the membrane of polymorphonuclear leukocytes and this enzyme appears to be essential for the directed cell migration (33).

Part II

Alternate Pathways into the Complement System

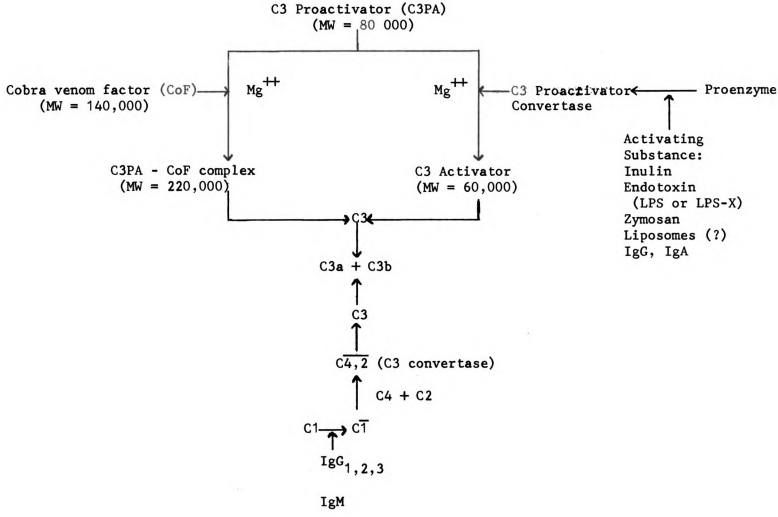
In addition to the established complement sequence of C1-C9, there now appear to be alternate mechanisms or pathways into the complement system. The complement system may be entered at points beyond the early-acting components, in particular at C3, to provide a means for complement-mediated actions independent of an immunologic event (see Figure 2). The complement proteins interact with factors unrelated to the complement system. Five of these factors and their modes of operation are discussed below.

Cobra Venom Factor. Cobra venom is known to inactivate C3 in whole serum and is also responsible for the depletion of this component in vivo (34). Addition of cobra venom to fresh human serum leads to inactivation of C3 and to physiochemical changes in the protein. The inactivation is inhibited by 0.01M EDTA and does not occur with heated serum. Treatment of isolated C3 with cobra venom does not result in inactivation of C3 (35).

The responsible protein in cobra venom, called cobra venom factor or CoF, has a molecular weight of 140,000 and a sedimentation coefficient of 7S (36,37). CoF forms a complex with a serum factor unrelated to the complement proteins (36). This serum protein, termed the C3 proactivator (C3PA), is a heat-labile pseudoglobulin with a 5S

FIGURE 2

Relationship of Alternate Pathways with the Complement Sequence



sedimentation coefficient (35). It has a molecular weight of 80,000 (37). Recent studies on this serum factor show that it possesses three different forms (38). One form (A) complexes with CoF, form B cleaves C3 in the absence of CoF, and form C is present in eluates from zymosan previously treated with serum at 17°C. It is hypothesized that the changes in the proactivator proteins are caused by an unknown enzyme in serum (38). The proactivator forms a complex with CoF which has a molecular weight of 220,000 and a S value of 9S (36,37). This complex, termed C3 activator, acts enzymatically on C3 cleaving it into two fragments, one of which has anaphylatoxin activity similar to C3a (36,37,38). The formation of the complex between CoF and C3PA is dependent on the presence of Mg^{++} with the formation being prevented by prior addition of EDTA (39) and upon the temperature (40). It does not react on cell-bound C3 and the activation of fluid-phase C3 by this complex is independent of antibody (40). In in vitro studies with guinea pig serum, CoF was found to inactivate not only C3, but also C5, C6, C7, C8 and C9 (41). CoF had no detectable effect on the early components. The apparent discrepency in the literature concerning the consumption of the six terminal components was thought to be due to variation in serum dilution and CoF concentration. The consumption of C5-C9 was dependent on the concentration of the reactants, whereas the consumption of C3 was not (41).

Other studies with guinea pig serum demonstrated that CoF and guinea pig serum would cause hemolysis of unsensitized erythrocytes (42,43). The complex formed would react with C-EDTA to cause depletion of the terminal components. The hemolytic activity of CoF

was dependent upon an intact complement system (42). Lysis of unsensitized erythrocytes by CoF and guinea pig serum was dependent on the formation of a complex between CoF and the proactivator. Hemolysis required the late-acting components. No lysis occurred in genetically C6-deficient rabbit serum or in zymosan-treated serum (39).

Plasmin and Trypsin. Incubation of C3 with small amounts of trypsin for short periods of time produces a cleavage product of C3 with identical properties to C3a (30). Plasmin can also cleave a fragment of 6,000 molecular weight from C3 which possesses chemotactic activity for polymorphonuclear leukocytes (44). This chemotactic factor is different from the previously described chemotactic factor derived from $\overline{C5,6,7}$ both in terms of requirements for generation and in physical properties (45). However, the biological properties of the various C3 cleavage products are identical (43).

Endotoxic Lipopolysaccharide. The terminal portion of the complement system is a potent source of biologically active by-products. These by-products perform functions which are very similar to those observed in studies on the biological activities mediated by endotoxins (46). The interaction of serum and endotoxic lipopolysaccharide (LPS) generates the complement-dependent biologically active products possessing neutrophil chemotactic activity and anaphylatoxin (47,48, 49). Following the administration of LPS to animals or man, several physiological changes take place, among which are contraction of smooth muscle and increased vascular permeability. The small amounts of LPS required to bring these alterations about has led to speculation that LPS does not act directly on the tissues, but indirectly through

a system of serum proteins, namely the complement system (46,47,50). Endotoxins when injected into animals consume complement or are anti-complementary (51,52,53,54). Endotoxic LPS has a potent ability to interact with complement during incubation in normal mammalian serum (55,56). Lesions indicative of terminal complement component activation appear on LPS after reactions with fresh serum (55), as well as on the bacterial cell from which the LPS was derived (49,57).

A correlation between the endotoxicity of LPS and its ability to consume complement exists (5). The complement-consuming ability of LPS is lost following several different modifications which result in loss of biological activity. Especially noticeable is the dramatic decrease in consumption of the terminal components during detoxification (56).

The most striking characteristic of LPS interaction with complement is that a preferential consumption of each of the six terminal components occurs (54). These findings support the hypothesis based on the previously described electron microscope data that the lesions observed on LPS are produced by activation of C9 in a manner similar to that required for the production of lesions on the erythrocyte membrane. LPS has little ability to consume substantial amounts of the early-acting components (C1,C4,C2) (51,55,56,58).

Because of the marked consumption of the terminal components with little or no consumption of the early-acting components by LPS, it was unclear whether the LPS activated the C3-C9 components via the usual mechanism of antibody-C1, C4, C2, or whether another pathway into the system existed. Studies with immunoglobulin-deficient sera showed

that bacterial LPS could induce complement consumption and that large amounts of antibody were not necessary for this interaction to occur (59,60). Extremely small quantities of antibody were hypothesized to suffice for the initiation of the complement sequence (60). LPS was hypothesized to interact with trace amounts of immunoglobulin in serum in a way as to lead to consumption of the terminal components, but to little, if any, consumption of the early-acting components (60).

The necessity for additional serum factors for the activation of the six terminal components by LPS was studied. Incubation of purified preparations of the six terminal components with LPS induced no complement consumption (54). Prior incubation of bacterial endotoxin in undiluted normal serum formed a serum-endotoxin intermediate, termed LPS-X, which contained at least six different serum proteins including a γ_2-globulin and C3 (61). LPS-X, but not LPS, destroyed purified C3, C5, C8 and C9. C3 was cleaved by LPS-X into two fragments, one of which was indistinguishable from C3a (61).

Even though LPS can efficiently activate the terminal components without detectable consumption of C1, C4 or C2 (54), the participation of the early-acting components, as well as antibody, cannot be excluded because LPS may promote an extremely efficient $\overline{C4,2}$ convertase formation (1). Experiments performed by Jensen using a specific C4 inactivator found in shark serum demonstrated that endotoxin failed to fix the terminal components when incubated with the C4 inactivator (62). These results would indicate that at least small quantities of the early-acting components are required to consume C3-C9. This data would support the hypothesis of an extremely efficient utilization of

the early-acting components by LPS.

The Properdin System. The properdin system emerged from studies on the mechanism of inactivation of the third component of complement (C3)[1] by zymosan, an insoluble carbohydrate derived from yeast cell walls. In 1954 Pillemer and associates isolated properdin and described its properties and role in the immune system (51). The properdin system consisted of a unique serum euglobulin, termed properdin, Mg^{++}, and serum factors resembling C1, C2 and C4 (51,63,64). Properdin reacted with zymosan at 17°C to form a complex (PZ) which in turn reacted with and inactivated C3 at 37°C (51). A method for the assay of properdin was devised based on the requirement of properdin for the inactivation of C3 by zymosan (65). Properdin could be eluted from the PZ complex (51,65,66) and this was its main means of isolation.

The inactivation of C3 by zymosan and properdin occurs in two stages (51). The first stage is the combination of properdin with zymosan at 17°C to form the PZ complex. Two other serum factors are required at this stage for formation of the PZ complex. The first of these two factors is Factor A, which is hydrazine-sensitive, but has been shown not to be identical with C4 (also hydrazine-sensitive) (67). The second required factor, Factor B, is heat-labile, but could not be identified with either C1 or C2, which are also sensitive to heat (68). Mg^{++} is also required for the formation of the PZ complex (51). Mg^{++} can be replaced by Co^{++} or Mn^{++}, but Mg^{++} is the most effective cation (69). The second stage is the inactivation of C3 by PZ (51). Mg^{++}

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[1] C3, as recognized in 1954, was the only complement component known to follow the C1,4,2 sequence. C3, therefore, refers to one or more of the components in the sequence beginning with C3 (C3,C5-C9).

is also required for this stage, but Factors A and B are not (51,67,68).

The properdin system was implicated in a variety of immunologic reactions. The properdin system was determined to be bacteriocidal against a variety of Gram-negative microorganisms (70,71). Removal of properdin from the serum also abolished bacteriocial activity. Addition of properdin to properdin-deficient serum restored activity against bacteria. Because of similarities in the requirements for properdin activity and the hemolysis of erythrocytes from patients suffering from paroxysmal nocturnal hemoglobinuria (PNH), properdin was determined to be required for the hemolysis of PNH cells (72). Properdin was also postulated to have a role in virus neutralization (73).

The concept of properdin as being a unique serum protein was challenged by several investigators (74,75,76). Nelson suggested that properdin was an antibody or group of antibodies to zymosan, present in small amounts in serum, and fixed complement and destroyed C3 in a manner similar to that of an antigen-antibody complex (74,77). However, several important differences existed between a typical antigen-antibody reaction and the reaction of properdin and zymosan. The formation of PZ was very dependent on environmental conditions, complexing only in a very small termpeature and pH range, requiring Mg^{++}, Factors A and B, and being very dependent on ionic strength (51, 78,79). Also PZ had very little effect on the early-acting components, whereas antigen-antibody complexes inactivate C1, C2 and C4, with little effect on C3 (79). From these differences properdin was designated as a unique serum protein and not antibody to zymosan.

One of the reasons for the controversy over the existence of properdin was the heterogeniety of the properdin preparation (51,65, 66,80). More than a decade after the first description of properdin, a report appeared which discussed the properties of a highly purified properdin (79). This purified properdin consisted of a single protein band in electrophoresis, contained no antigenic determinants to IgM, IgG or IgA, or to any of the complement components, agglutinins to zymosan or antibody to the test bacterial strain. It was thus re-established that properdin was not an antibody. It could be distinguished from immunoglobulin in both its physiochemical and antigenic properties and by its behavior in an immunologic system. It was reconfirmed that properdin was not a member of the complement system, performing none of the functions of complement in the assay system. The purified properdin had a sedimentation coefficient of 5.2S and a molecular weight of 223,000.

Recently the LPS-X factor has been related to properdin (81). Both LPS and properdin interact with the complement system through an intermediate (LPS-X or PZ). At this time, however, the relationship between X and properdin cannot be defined. Also it has been established thatthe C3 proactivator (C3PA) is Factor B of the properdin system (82). Identity of the other factors of the C3PA alternate pathway with factors of the properdin system have yet to be determined.

Part III

Liposomal Model Membranes

One of the most successful systems for the study of lipid bilayers and their biological importance is the liposomal model membrane (83). To form these model membranes dry lipids are swollen in an aqueous solution to form liquid crystalline structures, which are called liposomes (83). Low molecular weight substances can be trapped in the aqueous interiors of the liposomes (83). With the aid of electron microscopy, the polyene antibiotic filipin was shown to produce pits in erythrocyte membranes and lecithin-cholesterol liposomes similar to the lesions observed in erythrocytes after immune hemolysis with antibody and complement (84). Based on these observations the liposomes were investigated as a model membrane system with which to study the mechanism of complement action (85). Liposomes prepared from the phospholipid extract of sheep erythrocyte membranes released their trapped marker (glucose) when incubated with both rabbit antiserum prepared against sheep erythrocytes (as the source of antibody) and guinea pig serum (as the source of complement) (85). A spectrophotometric assay based on the detection of the released glucose with hexokinase, ATP, glucose-6-phosphate dehydrogenase and NAD was developed (85). Other studies indicated that liposomes prepared from the phospholipid extract could bind (neutralize) antibodies in rabbit hemolysin and fix guinea pig complement (86).

The antigen that confers immune sensitivity to the liposomes appears to be the Forssman antigen (87). This antigen is found in

sheep erythrocyte membranes and is also present in the phospholipid extract of these membranes. The proposed structure for the Forssman antigen is:

N-Ac-Gal(α1-3)Gal(β1-3)Gal(β1-4)-Glc-1-ceramide (87).

Since the Forssman antigen is present both in the intact sheep erythrocyte and in the phospholipid used to prepare the liposomes, antiserum prepared against the Forssman antigen may be used as the source of antibody for the complement- and antibody-mediated lysis of liposomes. This antiserum is called amboceptor or hemolysin.

Modification of the preparation of the liposomes and of the spectrophotometric assay produced more stable liposomes and provided for a less complicated enzymatic assay for the detection of the trapped marker, galactose (88). With these stable liposomes, it was shown that the degree of lysis of the liposomes was dependent on the amount of complement added to the system (88). Electron micrographs revealed the liposomes to consist of vesicles bounded by several bilayers (88).

Studies on the mechanism of liposome lysis by complement and antibody indicated that lipids alone may serve as the substrate for complement (87). It did not appear that cell membranes contained any unique and specific receptor sites. Additional studies revealed that no degradation of the phospholipid occurred during liposome lysis (89). The complement-dependent damage to liposomes did not appear to occur by the enzymatic rupture of covalent bonds in the phospholipids. The use of purified human complement components for the lysis of liposomes indicated that loss of the marker was dependent on the presence of C2

and C8 (90). C9 did not appear to be an absolute requirement for liposome lysis although its presence enhanced marker release.

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ARTICLE 1

Quantitative Measurement of Guinea Pig Complement with Liposomes

By

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QUANTITATIVE MEASUREMENT OF GUINEA PIG COMPLEMENT WITH LIPOSOMES[1, 2]

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Liposomes were investigated as an alternative reagent to sheep erythrocytes for quantitative titration of whole complement activity. The liposomes were prepared with various concentrations of phospholipid and were sonicated for more effective dispersion. It was found that reproducible complement titrations could be performed with sonicated liposomes prepared with 1 or 2 μmol of phospholipid per milliliter of marker solution (0.3 M galactose). Electron micrographs of liposomes treated with complement and hemolysin showed no discrete lesions, but rather aggregates of liposome fragments.

Lipid dispersions, termed liposomes, can be used as a model membrane system to study lytic mechanisms. Low molecular weight substances can be trapped in the aqueous regions of the liposomes (1). Using electron microscopy Kinsky *et al.* (2) showed that the antibiotic filipin produced pits in erythrocyte membranes and lecithin-cholesterol liposomes similar to the holes observed in erythrocytes after immune hemolysis with complement and antiserum. Based on these observations, Haxby *et al.* (3) investigated the feasibility of using liposomes as an artificial membrane system to study the mechanism of complement action. Liposomal membranes were prepared from sheep erythrocytes, and a spectrophotometric assay was devised for the detection of the trapped marker (glucose) released by the lytic action of complement and antiserum. Further studies by Alving *et al.* (4) indicated that these liposomes could bind (neutralize) antibodies in rabbit hemolysin as well as fix whole guinea pig complement.

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We were interested as to whether liposomes could be routinely used to quantitatively measure complement activity. Our experiments indicated that stable liposomes could be prepared by sonicating the lipid derived from sheep erythrocytes in the marker solution. Furthermore, these liposomes could be specifically lysed by complement and hemolysin, and the extent of lysis was dependent on the complement concentration. Examination of complement and antibody-treated liposomes indicated that no discrete holes were produced, but rather the liposomes seemed to fragment during lysis.

MATERIALS AND METHODS

Complement. Guinea pig blood was obtained by cardiac puncture from retired breeders donated by the Michigan State Department of Health, and the blood was allowed to clot at 0°C overnight. The serum was clarified by centrifugation at 23,500 × G to remove any lipid present. Any natural hemolytic antibody was removed by absorption with washed erythrocytes (5). The serum was then dialyzed overnight against cold 0.1 M Tris-HCl buffer, pH 7.4, containing 0.001 M Mg^{++} and 0.00015 M Ca^{++} to remove endogenous galactose. After dialysis the guinea pig serum (GPS) was titrated for complement (C) activity with 5×10^8/ml sensitized erythrocytes (5). The titer was 360 CH_{50} units/ml.

Antisera. Commercial anti-sheep hemolysin (whole hemolysin) prepared against washed whole sheep erythrocytes was obtained from

Baltimore Biological Laboratory (Baltimore, Md.). Anti-Forssman hemolysin was prepared against boiled sheep erythrocyte stromata as described by Mayer (5). Two hundred milliliters of sera from two rabbits were pooled and the globulins were precipitated by adding 100% saturated $(NH_4)_2SO_4$ to achieve a final concentration of 40% saturation. IgM and IgG immunoglobulins were separated by gel filtration on a Bio-Gel A 1.5 column (4 x 100 cm). Fractions containing the IgM were pooled, concentrated by dialysis against dry sucrose, dialyzed and frozen at −40°C until use. The IgM antiserum was heated at 56°C for 30 min before use.

Anti-Forssman antibody concentration. Quantitative precipitin assays were carried out with 50 μl of whole hemolysin or IgM with 0.0025 to 7.0 μg N Forssman antigen in a volume of 0.25 ml. The tubes were incubated at 37°C for 60 min and 7 days at 0°C with daily mixing. The micro-Kjeldahl technique described by Shiffman *et al.* (6) was used to determine nitrogen concentration in the washed precipitates. The antibody nitrogen cencentrations were 34.4 μg N/ml and 101.8 μg N/ml for whole hemolysin and IgM respectively.

Preparations, extraction and fractionation of membranes. Sheep blood was collected in Alsever's solution and aged at least 3 weeks before use. The erythrocyte membranes were isolated by the method of Dodge *et al.* (7). The membranes were washed or dialyzed as outlined by Haxby *et al.* (3) and Kinsky *et al.* (8). The extraction procedure was that of Bligh and Dyer (9) with experimental details provided by Haxby *et al.* (3) and Kinsky *et al.* (8). Organic phosphate was determined by analysis for total phosphorus (P) by acid hydrolysis (10). Determination of total phosphorus is a reliable measurement of organic phosphate, because most of the latter is present in the chloroform soluble phospholipids (3).

Liposome preparation. Liposomes were prepared as described by Haxby *et al.* (3). Portions of the chloroform soluble fraction containing 2 μmol phosphorus were taken to dryness by vacuum evaporation or by a stream of nitrogen gas. All preparations were further dried by evacuation in a desiccator for 1 hr.

The phospholipid was rehydrated in 0.3 M galactose in the ratios of 10 μmol P/ml galactose, 2 μmol P/ml galactose and 1 μmol P/ml galactose. The dried phospholipid residue was dispersed by a glass bead during agitation with a Vortex mixer.

Sonication of liposomes. Sonication was carried out with modifications of the procedure reported by Huang (11). A Bronwill U-20 Biosonik (Will Corporation, Rochester, N. Y.) was used for all sonication. The sonicating chamber contained 70 ml of the buffer solution used in the spectrophotometric assay with the addition of 0.3 M galactose. The dispersed phospholipid was transferred to a dialysis bag and both the bag and chamber buffer were flushed with nitrogen gas. Sonication of the liposomes was carried out for 2.5 hr with circulating ice water (0°C) as a coolant for the sonication chamber. Dialysis of the liposomes was carried out at 4°C againt 1000 ml of isotonic salt solution (0.075 M KCl-0.075 M NaCl) for 5 hr to remove untrapped marker from the liposome preparation.

Spectrophotometric assay. The assay using liposomes was based upon the oxidation of the released galactose with the nicotinamide adenine dinucleotide (NAD)-dependent β-galactose dehydrogenase. The reduction of the NAD was followed spectrophotometrically at 340 nm.

β-Galactose dehydrogenase suspended in 2.2M $(NH_4)_2SO_4$ (EC 1.1.1.48) and NAD were obtained from Boehringer-Mannheim (New York, N. Y.). The enzyme was diluted in water to 1 M $(NH_4)_2SO_4$ (2.27 mg/ml) before use. NAD was prepared to a concentration of 10 mg/ml (15 mM). The buffer solution contained 0.1 M Tris-HCl, pH 7.5, 0.058 M NaCl, 0.001 M $MgCl_2$ and 0.00015 M $CaCl_2$ ($\mu = 0.15$).

A typical reaction mixture contained 1.2 ml Tris-HCl buffer, 0.05 ml NAD and 10 μl β-galactose dehydrogenase. Preliminary experiments had shown that these concentrations of enzyme and cofactor were sufficient to oxidize all the galactose released. Appropriate quantities of liposomes (20 μl), GPS and antibody (AS) (20 μl) were added. The GPS and AS were not added until the residual galactose (i.e., that not removed by dialysis) had been oxidized. All assays were performed at 25°C using a Shimadzu multi-convertible double 40 Model S spectrophotometer and a Sargent Model SR recorder.

A modification of the spectrophotometric assay was used for some experiments. Liposomes, GPS and AS were incubated at 37°C for 60 min after which time a sample was removed and assayed for the galactose released. A typical reaction mixture consisted of: 30 μl liposomes, 0.1 ml GPS, 0.2 ml IgM anti-Forssman antibody and 0.04 ml Tris-HCl buffer in a total volume of 0.37 ml. After incubation 0.25 ml was transferred into

a cuvette and 0.05 ml NAD, 0.9 ml Tris-HCl buffer and 10 μl of enzyme were added. The reaction was allowed to progress until there was less than a 5% change in absorbance over a period of 1 min.

Total galactose. Total galactose was determined by lysis of the liposomes in Triton X-100 (Rohm and Haas Co., Philadelpha, Pa.). To 20 μl of liposomes was added 0.1 ml of 10% Triton X-100 and the mixture was incubated at room temperature for 15 min. Tris-HCl buffer (1.1 ml), NAD (0.05 ml) and β-galactose dehydrogenase (10 μl) were added and the reaction followed spectrophotometrically.

Trapped galactose. The amount of trapped galactose was defined as the total galactose (as determined by lysis with Triton X-100) minus the free galactose remaining in the liposome preparation after dialysis against isotonic salt solution. The concentration of galactose was calculated using a molar extinction for NADH of 6.22×10^6 and assuming that for each mol of NADH formed, 1 mol of galactose was oxidized.

Degree of trapped marker released. The degree, Y, of trapped galactose released by the addition of C and AS was determined from the total amount of marker released divided by the amount of trapped marker. A correction was made for the free galactose present in the liposome preparation.

Electron microscopy. Untreated liposomes, liposomes lysed with Triton X-100, and the spectrophotometric reaction mixtures of liposomes lysed with C and AS were examined in a Philips EM 300 electron microscope operated at 80 kv. A drop of the liposome preparation was placed on a collodion-carbon-coated grid and the excess was drained off after 20 sec. In the case of the liposomes lysed with C and AS, the drop remained on the grid for 10 min to insure the deposition of a sufficient number of liposomes to the grid. A drop of phosophotungstic acid, pH 7.0, was added and after 20 sec the excess was removed.

RESULTS

Galactose release. Liposomes containing galactose could be made from a chloroform extract of sheep erythrocyte membranes, and the trapped marker could be released by the action of C and AS (Table I). No lysis of the liposomes occurred with GPS that had been heated at 56°C for 30 min.

Liposome preparations which were not sonicated were found to be unstable, containing no trapped galactose 24 hr after preparation. The instability caused a greater amount of liposome leakage (galactose released independent of C and AS). This spontaneous leakage of galactose interfered with the detection of the complement-mediated lysis of liposomes, and the liposomes could not be used for titration of C for more than 1 day. Sonication of the liposomes, however, proved to be a very effective means of greatly increasing the stability of the liposomes. Sonicated liposomes were stable for at least a month with little leakage of galactose during that time.

When non-sonicated liposomes prepared with a concentration of 1 μmol P/ml galactose were used, galactose release was dependent on the C concentration and the response was sigmoidal with the addition of AS (Fig. 1). The lag in release of marker occurred consistently and was similar to the lag observed in the hemolytic assay (5). The galactose release from sonicated liposomes prepared in the ratios of 2 μmol P/ml galactose and 1 μmol P/ml galactose was also dependent on the C concentration and the response was sigmoidal with the addition of AS (Fig. 2). The lag period observed with these sonicated liposomes was shorter than that seen with the non-sonicated preparations (2 min compared to 6 min for 40 CH_{50} units for sonicated and non-sonicated liposomes respectively).

The data shown in Figures 1 and 2 were used to determine the CL_{50} (the number of C units required to release 50% of the trapped marker) and 1/n (the slope of the plot of log X, μl of GPS, *vs* log Y/(1 − Y)) (5) (Table I).

The CL_{50} values of the GPS titrated with liposomes and either whole hemolysin or IgM anti-Forssman antibody were compared. Since purified IgM released little or no galactose at 25°C over a period of 30 min with 10 CH_{50} to 50 CH_{50} units/20 μl of liposomes, the liposomes were incubated in the presence of C and AS for 60 min at 37°C. Larger liposome concentrations were used to increase the sensitivity. Despite the differences in conditions and concentrations, if the reaction mixtures were normalized to contain the same concentration of liposomes, the same amount of GPS would cause 50% lysis (Table I).

Electron microscopy. Electron micrographs of the liposomes prepared with the various phospholipid concentrations revealed strikingly different arrangements. Liposomes prepared with 10 μmol

TABLE I

Complement titrations with hemolysin and IgM anti-Forssman antibody[a]

Antibody	Lipsome Preparation	CH_{50} Added	% Galactose Released	CL_{50}	1/n
Whole hemolysin: 10 μl = 6.88 μg antibody N	1 μmol P/ml of 0.3 M galactose; mechanical mixing; 28.78 nmol galactose trapped/10 μl (10 μl used for assay)	50	64.6	11.5	0.647
		40	69.7		
		30	54.5		
		20	43.3		
		10	0.1		
		Heated GPS	1.7		
20 μl = 13.76 μg antibody N	1 μmol P/ml of 0.3 M galactose; 22.03 nmol galactose trapped/20 μl; sonicated (20 μl used for assay)	50	72.3	21.3	0.794
		40	70.1		
		30			
		20	40.2		
		10	25.6		
		Heated GPS	13.9		
		Liposomes only	9.5		
20 μl = 13.76 μg antibody N	2 μmol P/ml of 0.3 M galactose; sonicated; 56.43 nmol galactose trapped/20 μl (20 μl used for assay)	100	55.3	11.9	0.931
		50	56.1		
		40	51.3		
		30	47.7		
		20	29.1		
		10	18.5		
		Heated GPS	5.7		
		Liposomes only	5.7		
IGM: 0.2 ml = 40.22 μg antibody N	1 μmol P/ml of 0.3 M galactose; mechanical mixing; 17.12 nmol galactose trapped/10 μl (10 μl used for assay)	16.7	43.1	3.2	0.914
		13.3	49.3		
		10.0	43.3		
		6.6	25.6		
		3.3	14.5		
0.2 ml = 40.22 μg antibody N	2 μmol P/ml of 0.3 M galactose; sonicated; 39.55 nmol galactose trapped/10 μl (10 μl used used for assay)	16.7	41.6	2.1	0.704
		13.3	34.0		
		10.0	20.2		
		6.6	15.9		
		3.3	27.7		

[a] Reactions carried out with whole hemolysin were performed at 25°C for 30 min after the addition of AS; those involving IgM were performed at 37°C for 60 min. The percentage of trapped galactose released by C and AS has been corrected for the nonspecific leakage of galactose from the liposomes. The calculations were made from plots of log X (μl of GPS) *vs* log Y/(1-Y).

P/ml marker solution without sonication were composed of monolayers with no definite closed structure, i.e., they consisted of sheets of what appeared to be stacked membranes. Most of the stacks were composed of about 16 layers. The liposomes seem to be in the forms Thompson and Henn (12) described for hand shaken lipid dispersions. Liposomes made with this same ratio of P to marker solution with sonication were still composed of many layered sheets frequently in convoluted formations. These sheets were mostly about 9 layers thick, although they ranged from 8 to 20 layers in thickness. Smaller, more closed structures consisting of five layers were seen with sonicated liposomes prepared with 2 μmol P/ml galactose (Fig. 3). Liposomes lysed with C and AS revealed opaque spherical aggregates composed of smaller particles of approximately 188 Å in diameter (Fig. 4). In contrast the Triton-lysed liposomes appeared as debris which had agglutinated into spheroid masses (Fig. 5).

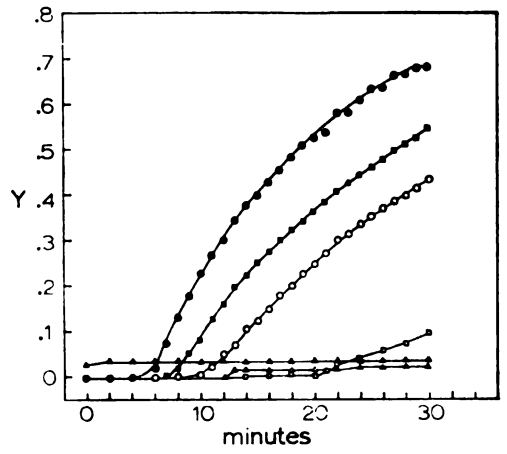

Figure 1. Dependence of galactose release on complement concentration. ●, 40 CH_{50}; ■, 30 CH_{50}; ○, 20 CH_{50}; □, 10 CH_{50}; ▲, heated GPS; and △, liposomes only. Spectrophotometric assay was performed at 25°C for 30 min following the addition of whole hemolysin.

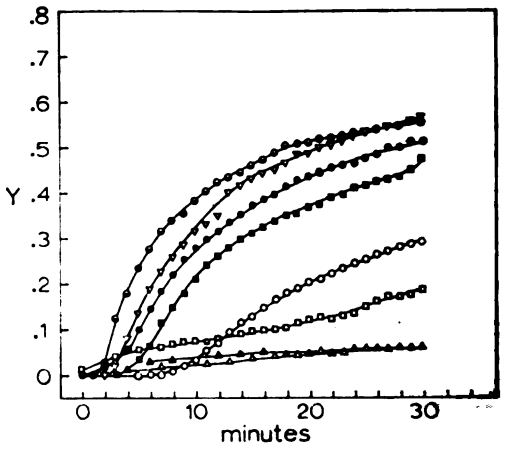

Figure 2. Galactose release with sonicated liposomes. ⊖, 100 CH_{50}; ▽, 50 CH_{50}; ●, 40 CH_{50}; ■, 30 CH_{50}; ○, 20 CH_{50}; □, 10 CH_{50}; ▲, heated GPS; and △, liposomes only. The spectrophotometric assay was performed at 25°C for 30 min following the addition of whole hemolysin.

DISCUSSION

The purpose of this investigation was to test complement-mediated lysis of liposomes as an alternative system to the classical hemolytic assay for the quantitative measurement of whole guinea pig complement. We have shown that the complement-mediated release of trapped galactose from liposomes is similar to the lysis of sheep erythrocytes by complement in the presence of antibody, and have confirmed the results of Haxby *et al.* (3).

From a technical standpoint we found sonicated liposomes to be a good substitute for erythrocytes in the assay of complement. The liposomes obtained by sonication of the lipid dispersion are stable for at least a month, and perhaps longer. Since galactose can be detected with one enzyme and one cofactor, the substitution of galactose for glucose has greatly simplified the previously published procedures for detecting the marker (3). Small volumes of serum (30 to 140 μl) and antibody (20 μl) are used, thereby making it possible to conserve valuable reagents. A single assay uses only 20 μl of the liposome preparation. This means that as a minimum it is possible to get 4000 assays from 125 ml of whole sheep blood. Because the extent of lysis is measured using enzymatic techniques, it might also be possible to automate the whole system for routine measurement of complement activity.

We found that the ratio of phospholipid to marker solution during suspension of the lipid dispersion and the method of suspension of the lipid in the marker solution were both extremely critical. By increasing the amount of galactose so there was 1 ml per 1 to 2 μmol of phosphorus (representing phospholipid), and sonicating the lipid and sugar mixture in an inert atmosphere, we were able to reproducibly prepare liposomes which did not spontaneously release galactose, but were sensitive to lysis by complement in the presence of hemolysin. Decreasing the amount of galactose with or without sonication of the lipid solutions resulted in liposomes which were insensitive to lysis by complement, or which spontaneously released galactose so quickly that they were not suitable for assay purposes.

A possible explanation for these results is suggested by the electron microscopic studies of the liposomes. We noted that the electron micrographs of liposomes prepared with 10 μmol of P/ml of marker solution with mechanical agitation on a Vortex mixer contained no discrete vesicles but appeared as randomly layered sheets consisting of predominantly 16 layers. The absence of discrete vesicles would explain the continued leakage of "trapped" galactose. Sonication of an identical preparation gave discrete vesicles, predominantly nine layers thick. These preparations, however, were also not lysed by complement and hemolysin. We suspect therefore that

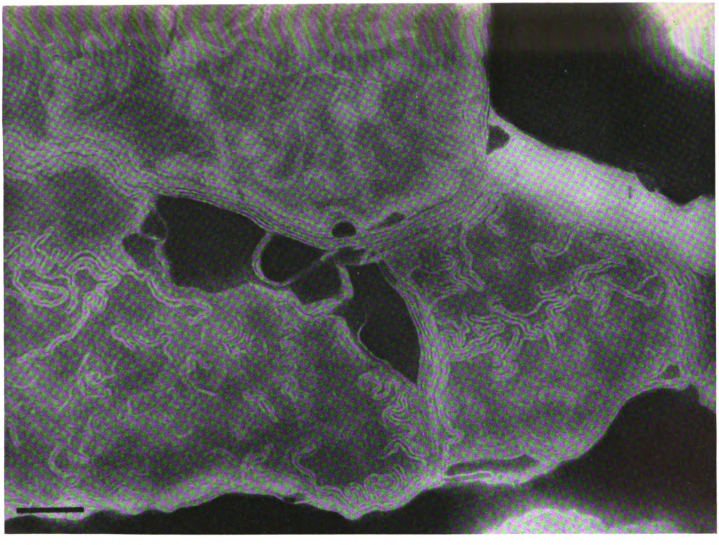

Figure 3. Untreated liposomes prepared with a concentration of 2 μmol P/ml marker solution and sonicated for 2.5 hr. Negatively stained with 2% phosphotungstic acid. Marker = 1000 Å (× 218,000).

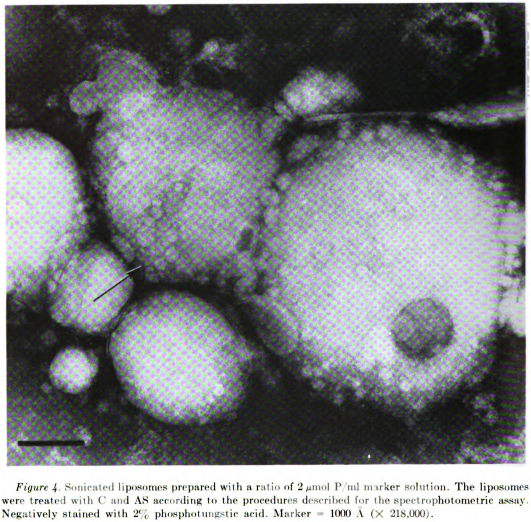

Figure 4. Sonicated liposomes prepared with a ratio of 2 μmol P/ml marker solution. The liposomes were treated with C and AS according to the procedures described for the spectrophotometric assay. Negatively stained with 2% phosphotungstic acid. Marker = 1000 Å (× 218,000).

our technical difficulties with mechanically mixed preparations were due to the galactose not being completely trapped. In the sonicated preparations which could not be lysed by complement but contained trapped galactose, all of the complement was utilized before the liposome was lysed. This hypothesis is further supported in that the preparations of liposomes which were lysed with complement and antibody were discrete vesicles with five layers.

Liposomes treated with hemolysin were a sensitive reagent for quantitatively measuring whole complement activity. An individual assay was done at 25°C and was complete in 30 min. We noted that in no instance was it possible to cause 100% release of the marker with complement. The most trapped galactose which could be released was between 50% and 70% (see Fig. 2). If we call the degree of lysis at which the system is saturated with complement 100% lysis, normalize the other points to this value and recalculate the 50% endpoint, we find there are approximately 190 to 200 CL_{50} units/ml of serum, a figure close to the figure of 360 CH_{50} units/ml determined for this lot of serum. Both of these facts indicate that liposomes can be substituted for erythrocytes in complement-mediated immune lysis without loss of sensitivity, and the values obtained for the CL_{50} and 1/n probably are a property of this particular reagent. The suitability of the system

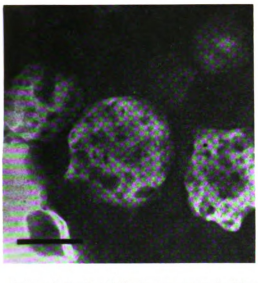

Figure 5. Sonicated liposomes prepared with a ratio of 2 μmol P/ml marker solution and lysed with 1.0% Triton X-100. Negatively stained with 2% phosphotungstic acid. Marker = 1000 Å (× 218,000).

for quantitatively measuring activity of the individual components will need to be tested in further experiments.

Complement action on whole erythrocytes results in 85 to 110 Å holes (13) and, we were curious as to the nature of the lesions in liposomes caused by complement and antibody. To examine this question we undertook an electron microscopic study of complement-antibody and detergent lysed liposomes. The picture seen with complement- and antibody-treated liposome preparations was very different than that seen with the Triton-lysed liposomes. There were no regular sized lesions or "holes" in the antibody-complement-treated liposomes. Although the multilayered structures seen in the untreated liposomes were gone, there still were large globular structures present which upon close examination seemed to be an association of fragments of the phospholipid layers which were released from the liposome (see *arrow*, Fig. 4). At this time we hypothesize that the large structures are liposome "ghosts" and that complement action destroyed the original liposome layers by releasing by as yet an unknown chemical process variable size pieces of the original phospholipid layer. Once released, these small pieces tend to mass in solution much in a manner similar to that in which the liposomes were originally formed. As the lipid masses are only small segments of what was once a long sheet of phospholipid, they do not form layered structures, but they remain associated with the liposome "ghost" by virtue of nonspecific hydrophobic bonding. The size of the piece of liposome released by complement can apparently vary, as the size of the small globules varied widely in size (92 to 322 Å). In any case, we feel that the small globules may represent the product of complement action on antibody-treated liposomes. We are currently designing experiments to test this hypothesis.

Acknowledgment. We thank Stuart Pankratz for his assistance in the electron microscopy.

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ARTICLE 2

Evidence for Alternate Mechanisms for Immune Lysis of Liposomal Model Membranes

By

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(Manuscript to be submitted to

Journal of Experimental Medicine)

INTRODUCTION

Liposomes prepared from the phospholipid extract of sheep erythrocyte membranes have been used as a model for investigation of the mechanism of complement[1] action in immune cytolysis (1,2,3,4). Liposomes can be lysed by the sequential addition of appropriate antibody and all of the highly purified human complement proteins, C1-C9, and this lysis is dependent on the addition of C2 and C8, but not on C9 (4). There are no data, however, that indicate which components of complement or what other factors cause the lysis of liposomes by fresh serum. This kind of information is necessary before it can be concluded that liposomes are lysed by C1-C9 in serum by the same mechanism which causes immune lysis of antibody-treated sheep red blood cells. Our studies on fresh serum-mediated lysis of liposomes, as well as the binding of functionally purified complement components to the liposomal model membranes, indicated that at least two mechanisms were operating simultaneously to cause lysis of the liposomes. The first of these mechanisms appeared to be the classical complement reaction sequence requiring all nine complement components, antibody, and the divalent cations, Ca^{++} and Mg^{++}. The second mechanism appeared to require the late-acting complement components (C3-C9) and Mg^{++}, and liposome lysis was postulated to be mediated by the alternate pathway involving the C3 proactivator system (5,6). In this paper

[1]The complement terminology is that recommended by the World Health Organization. (Bull. World Health Organ. 1968. 39: 939.)

evidence for the role of this alternate pathway in the lysis of liposomes is presented. Also investigated were the requirements of antibody and a diisopropylfluorophosphate-sensitive factor present in the amboceptor preparation for lysis of the liposomes.

MATERIALS AND METHODS

Antisera. Amboceptor prepared against washed sheep erythrocytes and stored in 50% glycerol was obtained from Behring Diagnostics (Woodbury, NY). The amboceptor was dialyzed overnight against 0.1M Tris-HCl, 0.056M NaCl buffer, pH 7.5, ionic strength 0.15. Treatment with diisopropylfluorophosphate (DFP) was done by mixing equal volumes of amboceptor and 10^{-3}M DFP, followed by incubation at 37°C for 60 minutes and dialysis overnight against the same Tris-HCl-NaCl buffer.

Monospecific goat antisera to human C3, C4 and C3 proactivator (C3PA) were generously provided by Dr. Neil Cooper (Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, CA). These antisera were heated at 56°C for 30 minutes prior to use.

Antiserum to C1, designated anti-Fraction E, was obtained from a rabbit immunized with human C1 prepared as described below. Although this antiserum was not monospecific for C1, it contained antibody to C1 as demonstrated by its ability to inhibit the formation of EAC1,4 from EAC4.

Human Serum. Blood was collected from a healthy male donor and allowed to clot at room temperature for 2 hours and then at 4°C for 8 hours. The serum was harvested by centrifugation at 1500 X g and immediately frozen in aliquots at -40°C. The serum was titrated for complement activity with $5x10^{8}$/ml sensitized erythrocytes (7). The

titer was 384 CH_{50} units/ml. This serum (HS) was used as the source of complement (C), C-EDTA and C-MgEDTA.

Pooled human serum used for the preparation of C1 was obtained from the Michigan State Public Health Laboratories (Lansing, MI).

Guinea Pig Serum. Guinea pig blood was obtained by cardiac puncture from retired breeders donated by the Michigan State Department of Health (Lansing, MI), and the blood was allowed to clot at 4°C overnight. The serum was harvested and clarified by centrifugation at 23,500 X g to remove any lipid present.

Liposome Preparation. The preparation, extraction and fractionation of the sheep erythrocyte membranes were carried out as previously reported (8). Liposomes containing 2 μmoles phosphorus/ml of 0.3M galactose were prepared from the chloroform-soluble membrane fraction and sonicated for 3 hours. The liposomes were dialyzed 5 hours against 2 liters of isotonic salt solution (0.075M NaCl-0.075M KCl) to remove the untrapped marker from the preparation. The methods for determination of total galactose, amount of trapped galactose, and the degree of trapped marker released have been described (8).

C1 Preparation. Human C1 was obtained by precipitation of pooled human serum with eight volumes of 0.02 ionic strength acetate buffer, pH 5.5 to give a final serum pH of 6.4 and ionic strength 0.03 (9). This precipitate was designated Fraction E. Guinea pig C1 and C2 were prepared by the method reported by Nelson (10).

Galactose Release Assay. The details of the spectrophotometric assay for the detection of released galactose from the liposomes has been previously described (8). Twenty μl of liposomes were used in

all assays. However, in the galactose release assays reported here, the assay buffer contained no metals. In the assays involving C-EDTA, human serum was diluted 1:2 in veronal-buffered NaCl containing 0.01M EDTA (VBS-EDTA)(11). Human serum was diluted 1:2 in veronal-buffered NaCl containing 1.5×10^{-3}M MgEDTA (VBS-MgEDTA)(9) for use in the assays requiring C-MgEDTA.

In the experiments involving the antisera to complement components, varying amounts of antisera were preincubated with 0.2ml of either HS or C-MgEDTA at 37°C for 20 minutes. Samples of 0.15ml of these mixtures were used in the galactose assay.

Before the addition of the complement reagent to the liposome, buffer, NAD, and galactose dehydrogenase mixture, any untrapped galactose present in the liposome preparation was oxidized by the enzyme system. The complement reagent was then added and the reaction continued for 3 minutes. Amboceptor (20µl) was then added and the subsequent release of galactose was followed spectrophotometrically at 340nm for 15 minutes. In all cases, the amount of amboceptor used was that concentration which promoted maximal lysis as determined by prior amboceptor titration with a saturating amount of serum. All assays were done with a Shimadzu multi-convertible double 40 Model S spectrophotometer and a Sargent Model SRG recorder.

Binding of C1 to Liposomes. Liposomes (50µl) were added to two centrifuge tubes designated LAC and LC. Amboceptor (50µl) was added to LAC and both tubes incubated 30 minutes at 37°C followed by 30 minutes at 4°C. Human C1 (0.1ml) was then added to both tubes and the reaction mixtures incubated 20 minutes at 30°C. The mixtures were

made to volume (0.5ml) with 0.095 ionic strength Tris-acetate-NaCl buffer (12), centrifuged at 4100 X g for 15 minutes and the precipitates washed twice with buffer. The final precipitates were resuspended in veronal-buffered NaCl (11) (0.1ml) and assayed for C1 by the C1 transfer test (13). The same procedure was used for the binding of guinea pig C1 except that 200µl of liposomes, 200µl of amboceptor, and 0.2ml of C1 were used in the reaction in a total volume of 1.0ml.

Liposome Lysis by Functionally Pure Complement Components. Liposomes (20µl) were added to three 5ml centrifuge tubes. Amboceptor (20µl) was added to two of the tubes. Isotonic buffer (0.075M NaCl-0.075M KCl) was added to the third tube. All tubes were incubated at 37°C for 20 minutes. To tubes designated LAC and LC 0.2ml of human C1 was added. The third tube, designated LA, received 0.2ml of buffer. All tubes were incubated 15 minutes at 30°C and 10 minutes at 0°C. The samples were centrifuged at 4°C at 4100 X g. In separate studies it had been noted that the liposomes could be centrifuged and washed with an isotonic salt solution with little non-specific release of the trapped galactose marker[2]. The supernatants were removed and saved for later analysis. The LAC, LC and LA pellets were washed twice with buffer. To LAC and LC was then added 0.1ml of C-EDTA, prepared by diluting HS 1:20 in VBS-EDTA and to LA was added an equal volume of VBS-EDTA. The tubes were incubated at 0°C for 15 minutes. After centrifugation the pellets were washed once with VBS-EDTA and once

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[2]Knudson, K.C. and D.H. Bing. A simplified method for the centrifugation of liposomes. Manuscript in preparation.

with isotonic buffer. Human C1 (0.2ml) was added to LAC and LC and the tubes incubated at 30°C for 15 minutes. Again LA was brought to volume with buffer. C2 (50µl) was added to the samples and the mixtures incubated 15 minutes at 30°C. C-EDTA (0.15ml), prepared by diluting HS 1:2 in VBS-EDTA, was added to LAC and LC. An equal volume of buffer was added to LA. The tubes were incubated at 37°C for 40 minutes and then centrifuged. To the LAC and LC pellets was added 0.15ml of C-MgEDTA, prepared by diluting HS 1:2 in VBS-MgEDTA. LA received 0.15ml of buffer. After 15 minutes at 37°C the supernatants were removed by centrifugation and 0.1ml of 10% Triton (Rohm and Haas, Philadelphia, PA) was mixed with the pellets to release any remaining trapped galactose. All of the supernatants obtained during the procedure were assayed for released galactose.

Preparation of Properdin Reagents. The isolation of properdin (P) from pooled HS was done following the procedure of Todd, Pillemer and Lepow (14). Zymosan (Z) was obtained from Nutritional Biochemical Corp. (Cleveland, OH), heated for 60 minutes in a boiling water bath as directed (15), and suspended in 0.15M NaCl to a concentration of 50mg/ml. The reagent deficient in properdin (RP) and serum deficient in the terminal components (R3) were prepared according to Todd et al. (14). The titer of the properdin extract was determined by the zymosan assay for properdin (15). The titer of the properdin extract was 16 units/ml.

RESULTS

Lysis of Liposomes by Human Serum. Liposomes were lysed by fresh human complement and the lysis was dependent on the addition of amboceptor (Table 1). Omission of the amboceptor or substitution by normal rabbit serum which had been treated identically to the amboceptor preparation resulted in essentially no lysis of the liposomes by human serum. Heating of the serum at 56°C for 30 minutes or treatment with $5x10^{-4}$M DFP resulted in a substantial reduction in the degree of liposome lysis. Prior treatment of the amboceptor with DFP caused about a 50% reduction in the lysis of liposomes, but heating of the amboceptor for 30 or 120 minutes at 56°C had no effect on the degree of liposome lysis. No lysis occurred with the combination of heated HS and DFP-treated amboceptor. In all assays the amount of amboceptor used was the concentration which produced maximal lysis with a saturating concentration of serum. Since heating the amboceptor resulted in no reduction in lysis of the liposomes, but DFP treatment did (Table 1), it was reasoned that some other factor in the amboceptor, sensitive to DFP, but stable to heat, might be at least partially responsible for the liposome lysis and that antibody might not be an absolute requirement for complement-mediated lysis of liposomes. This hypothesis was tested in the next experiments.

C1 Binding to Liposomes. To determine whether antibody was necessary for the binding of C1 to liposomes, liposomes were mixed

TABLE 1

Lysis of Liposomes by Human Serum

Amboceptor	Human Serum	Lysis[a]	Prior Treatment
(µl)	(µl)	(%)	
20	50	49.3	Amboceptor was dialyzed overnight against 0.1M Tris-HCl-0.056M NaCl, pH 7.5
20	100	44.8	
20	150	40.1	
20	200	49.0	
0	150	0	
20 NRS[b]	150	2.5	NRS was diluted in glycerol and treated identically to the amboceptor
20	100	43.6	Amboceptor heated 56°C, 30 min
20	100	47.0	Amboceptor heated 56°C, 120 min
20	150	22.5	Amboceptor reacted with $5x10^{-4}$M DFP
20	150	8.8	Human Serum heated 56°C, 30 min
20	150	15.5	Human Serum reacted with $5x10^{-4}$M DFP
20	150	5.9	Human Serum and Amboceptor heated 56°C, 30 min
20	150	0	Human Serum heated 56°C, 30 min; Amboceptor reacted with $5x10^{-4}$M DFP

[a] 15 minutes after the addition of amboceptor
[b] NRS = normal rabbit serum

with C1 in the presence and absence of antibody. Liposome-antibody-C1 (LAC1) and liposome-C1 (LC1) complexes were prepared with amboceptor and functionally pure human or guinea pig C1, and the amount of C1 bound to the liposomes was determined by the C1 transfer test (13). As shown in Table 2, C1 was bound to liposomes both in the presence and absence of amboceptor (LAC1 and LC1) when either human or guinea pig C1 was used. Addition of amboceptor doubled the amount of human C1 bound to the liposomes, as compared to the amount of C1 bound in the absence of any antibody. Approximately the same amount of guinea pig C1 was bound in the presence and absence of amboceptor.

TABLE 2

C1 Binding to Liposomes

Complex	effective molecules/ml bound to liposomes	
	human C1	guinea pig C1
LAC1	21.0×10^{11}	9.1×10^{11}
LC1	12.8×10^{11}	7.0×10^{11}

Lysis of Liposomes by Complement Components. Since C1 was able to bind to liposomes in the absence of antibody, liposomes were tested for their ability to be lysed by the sequential addition of functionally pure complement components in the presence and absence of antibody. Liposomes, either untreated or treated with amboceptor, were pretreated with C1 and C-EDTA and washed as described in Materials

and Methods. A control for non-specific liposome lysis consisted of a liposome-antibody (LA) complex to which no complement components or serum reagents were added. Neither the LAC nor the LC liposome mixture reacted in this fashion could be lysed by the step-wise addition of C1, C2 and C-EDTA (Table 3). But the addition of C-MgEDTA could cause lysis of the pretreated liposomes in the presence or absence of amboceptor. The addition of amboceptor in this procedure did enhance the lysis of liposomes by C-MgEDTA; there was 33% lysis in the presence of antibody as compared to 23% lysis in the absence of antibody. It was noted that this decrease was similar to the decrease seen in the lysis of liposomes by untreated serum and DFP-treated amboceptor (Table 1), again suggesting the participation of the DFP-sensitive factor in the amboceptor-mediated lysis of liposomes.

Lysis of Liposomes by C-EDTA and C-MgEDTA. Next, the ability of C-EDTA and C-MgEDTA to directly lyse liposomes in the presence and ansence of amboceptor was examined. The concentration of amboceptor used was the amount which caused maximal lysis of the liposomes. A marked reduction in the degree of liposome lysis occurred when human serum was diluted in VBS-EDTA (Table 4). With an equivalent amount of serum, a 85% reduction in the degree of liposome lysis occurred when C-EDTA was substituted for untreated human serum.

In contrast to the poor ability of C-EDTA to cause galactose release from liposomes, human serum diluted in VBS-MgEDTA was a good complement reagent for lysis of the liposomes in the presence of amboceptor (Table 5). Identical results were obtained if the HS was diluted in veronal-buffered NaCl containing 1.5×10^{-2}M MgEDTA.

TABLE 3

Binding of Complement Components to Liposomes[a]
Galactose Release Assay

Supernatants	LAC lysis	LC lysis	LA lysis
	(%)	(%)	(%)
C1	3.5	5.1	16.3
Wash 1	4.6	6.4	4.5
Wash 2	12.3	5.5	6.9
C-EDTA	18.0	16.1	3.6[b]
EDTA Wash	7.4	3.7	4.5
NaCl-KCl Wash	3.5	3.7	4.5
Subtotal	49.3	40.6	40.1
C1, C2, C-EDTA	6.0	6.0	7.3[b]
Subtotal	55.3	46.6	47.6
C-MgEDTA	32.8	23.0	4.5[b]
Subtotal	88.0	69.6	52.0
Triton	12.0	30.4	48.0
Total	100.0	100.0	100.0

[a]See Materials and Methods for experimental procedure.

[b]Human serum omitted from the buffer.

TABLE 4

Lysis of Liposomes by C-EDTA

Amboceptor	C-EDTA[a]	Lysis[b]
(µl)	(µl)	(%)
20	50	19.8
20	100	14.0
20	150	10.7
20	200	8.6
20	300	5.9
0	150	0

[a]Human serum diluted 1:2 in VBS-EDTA.

[b]15 minutes after the addition of amboceptor.

TABLE 5

Lysis of Liposomes by C-MgEDTA

Amboceptor	C-MgEDTA[a]	Lysis[b]	Prior Treatment
(µl)	(µl)	(%)	
20	50	26.1	Amboceptor was dialyzed over-
20	100	49.4	night against 0.1M Tris-
20	150	58.0	HCl-0.056M NaCl, pH 7.5
20	200	57.7	
20	300	49.3	
0	150	2.5	
20	150	15.8	Amboceptor reacted with $5x10^{-4}$M DFP
20	150	2.5	Human serum reacted with $5x10^{-4}$M DFP
20	150	32.3	Amboceptor heated 56°C, 30 min
20	150	29.6	Amboceptor heated 56°C, 120 min

[a]Human serum diluted 1:2 in VBS-MgEDTA.

[b]15 minutes after the addition of amboceptor.

Comparison of equivalent amounts of complement reagent revealed that similar degrees of lysis occurred with HS and C-MgEDTA. It should be noted that in the case of C-MgEDTA, the only source of Mg^{++} was the VBS-MgEDTA buffer. Also seen in the experiments with untreated human complement (Table 1), treatment of the C-MgEDTA reagent or amboceptor with DFP resulted in a reduction in the lysis of the liposomes. Heated amboceptor was less potent in mediating lysis by C-MgEDTA than by HS. The effect of DFP on the C-EDTA-mediated lysis was not tested.

The results obtained with C-EDTA and C-MgEDTA indicated that the early-acting complement components might not be required for lysis of liposomes. The role of several components in lysis was tested using antisera prepared against complement components and against the C3 proactivator.

Inhibition of Liposome Lysis by Antisera to Complement Components. Preincubation of either HS or C-MgEDTA with antisera to complement proteins and the C3 proactivator prior to lysis of the liposomes by the addition of amboceptor produced the results seen in Tables 6,7,8, and 9. Again the amount of amboceptor added was the amount which caused maximal lysis of the liposomes with a saturating concentration of serum. The concentration of complement reagent used was the optimal volume of HS and C-MgEDTA previously determined to cause maximal lysis of the liposomes (Tables 2,5).

Anti-C4 caused more inhibition of liposome lysis when preincubated with HS than when preincubated with comparable amounts of C-MgEDTA. Comparable degrees of lysis inhibition were noted when HS and C-MgEDTA were preincubated with the anti-C3, anti-C3 proactivator, and

TABLE 6

Inhibition of Liposome Lysis with Anti-C4
Preincubated with Human Serum and C-MgEDTA

Anti-C4	HS		C-MgEDTA[a]	
	Lysis[b]	Inhibition[c]	Lysis[b]	Inhibition[c]
(µl)	(%)	(%)	(%)	(%)
0	36.2	----	37.1	----
20	31.3	13.5	29.5	20.5
40	20.4	43.6	30.4	18.1
60	20.7	42.8	----	----

[a]Human serum diluted 1:2 in VBS-MgEDTA

[b]15 minutes after the addition of amboceptor

[c] $100\left(1 - \frac{\text{antiserum treated}}{\text{control}}\right)$

TABLE 7

Inhibition of Liposome Lysis with Anti-Fraction E
Preincubated with Human Serum and C-MgEDTA

Anti-E[b]	HS		C-MgEDTA[a]	
	Lysis[c]	Inhibition[d]	Lysis[c]	Inhibition[d]
(µl)	(%)	(%)	(%)	(%)
0	36.2	----	37.1	----
40	14.3	60.5	11.8	68.2
60	10.6	70.7	9.1	75.5

[a]Human serum diluted 1:2 in VBS-MgEDTA

[b]E is Fraction E, the precipitate of human serum obtained by dilution of HS with 8 volumes of 0.02 ionic strength acetate buffer, pH 5.5.

[c]15 minutes after the addition of amboceptor

[d] $100\left(1 - \frac{\text{antiserum treated}}{\text{control}}\right)$

TABLE 8

Inhibition of Liposome Lysis with Anti-C3 Preincubated with Human Serum and C-MgEDTA

Anti-C3	HS		C-MgEDTA[a]	
	Lysis[b]	Inhibition[c]	Lysis[b]	Inhibition[c]
(ul)	(%)	(%)	(%)	(%)
0	36.2	----	37.1	----
20	29.5	18.5	29.5	20.5
40	22.2	38.7	18.8	49.5
60	20.7	42.8	17.3	53.4

[a]Human serum diluted 1:2 in VBS-MgEDTA

[b]15 minutes after the addition of amboceptor

[c] $100\left(1 - \frac{\text{antiserum treated}}{\text{control}}\right)$

TABLE 9

Inhibition of Liposome Lysis with Anti-C3 Proactivator Preincubated with Human Serum and C-MgEDTA

Anti-C3PA	HS		C-MgEDTA[a]	
	Lysis[b]	Inhibition[c]	Lysis[b]	Inhibition[c]
(ul)	(%)	(%)	(%)	(%)
0	36.2	----	37.1	----
20	10.3	71.6	21.0	43.4
40	8.1	77.6	12.6	66.3
60	9.1	74.9	11.8	68.2

[a]Human serum diluted 1:2 in VBS-MgEDTA

[b]15 minutes after the addition of amboceptor

[c] $100\left(1 - \frac{\text{antiserum treated}}{\text{control}}\right)$

anti-Fraction E antisera. In addition to antibody against C1, anti-Fraction E also contained antibodies to other proteins present in the serum precipitate used to prepare the antiserum. Since the C3 pro-activator has recently been shown to be Factor B of the properdin system, the properdin system was investigated as another possible mechanism for liposome lysis.

Effect of the Properdin System on Liposome Lysis. First, the supernatants from the zymosan assay for properdin (15) were tested for their ability to lyse liposomes. Little lysis of the liposomes occurred with the supernatant from the reaction of properdin (P), zymosan (Z), RP and Mg^{++} for 60 minutes at 37°C (Table 10, Expt. A). However, lysis of the liposomes did occur with the control supernatants of RP + Mg^{++} and Z + RP + Mg^{++}. In all cases no lysis of the liposomes occurred until the amboceptor was added to the cuvette.

Second, the preincubation of liposomes, properdin, RP and Mg^{++} at 37°C for 60 minutes in the absence of amboceptor demonstrated that liposomes can substitute for zymosan to cause the inactivation of factors in RP responsible for lysis of liposomes (Table 10, Expt. B). That is, similar degrees of lysis occurred with the preincubation mixtures containing either liposomes or zymosan (Table 10, Expt. A vs. Expt. B).

Third, the direct action of the properdin reagents on liposome lysis in the presence of amboceptor was tested. When amboceptor was present, RP caused release of trapped galactose (Table 10, Expt. C). However, no increase in the amount of lysis occurred when properdin was added back into the RP reagent. Also, little lysis occurred with

only properdin and amboceptor. These experiments suggested that a liposome-properdin complex was formed, analogous to the zymosan-properdin complex, which inactivated the components in RP responsible for liposome lysis. That is, RP alone or RP + P in the presence of amboceptor lysed liposomes, but incubation of liposomes with RP + P for 60 minutes prior to addition of amboceptor resulted in a 65% reduction in lysis (Table 10, Expt. B and C).

TABLE 10

Effect of the Properdin System on Lysis of Liposomes

Experiment	Treatment of Liposomes	% Lysis[a]
A	P + Z + RP + Mg^{++}	6.5
	RP + Mg^{++}	22.9
	Z + RP + Mg^{++}	27.3
B	P + Lip + RP + Mg^{++}	10.6
	P + Lip + RP + Mg^{++}	8.7[b]
C	RP + Mg^{++}	29.8
	RP + P + Mg^{++}	28.7
	P + Mg^{++}	10.9
	C-MgEDTA	37.4
	C- MgEDTA + P	38.3

[a] 15 minutes after the addition of amboceptor

[b] amboceptor treated with 5×10^{-4}M DFP

Experiment A: Supernatants from the zymosan assay for properdin. 0.01ml P, 0.25ml RP, 0.01ml Z, 0.0003M Mg^{++}; incubated 60 minutes at 37°C; 0.15ml sample assayed.

Experiment B: Preincubation of properdin reagents with liposomes in the absence of amboceptor. 0.025ml P, 0.15ml RP, 0.0005M Mg^{++}, 0.02ml lipsomes; incubated 60 minutes at 37°C.

Experiment C: Direct action of properdin reagents on liposomes in the presence of amboceptor. 0.025ml P, 0.15ml RP, 0.0005M Mg^{++}, 0.15ml C-MgEDTA added sequentially.

DISCUSSION

Based on the data presented it is hypothesized that at least two mechanisms are operating simultaneously in the lysis of liposomal model membranes. The first mechanism is the classical complement effector system using the components C1-C9 to bring about lysis of the liposomes. Both Ca^{++} and Mg^{++}, as well as antibody, are required. The second mechanism appears to use the terminal complement components, C3-C9, and does not require the early-acting components (C1, C4, C2). Since Mg^{++} is required, but not Ca^{++}, the role of C1 in this mechanism is probably minimal. With the removal of Ca^{++} by MgEDTA and the resulting dissociation of the C1 macromolecule, the liposomes are lysed equally well with either human serum or serum diluted in MgEDTA. However, when both divalent cations are removed by EDTA, no significant lysis of the liposomes occurs. Additional data to support the hypothesis that the early components are not required were obtained with the anti-C4 antiserum. Preincubation of this antiserum with C-MgEDTA caused only a 20% decrease in the amount of liposome lysis. This indicated that at least C4 could be by-passed.

Preincubation of the anti-C3 proactivator with HS and C-MgEDTA demonstrated that the C3 proactivator can participate in the lysis of the liposomes. The alternate pathway of the C3 proactivator system was thus implicated in the lysis of liposomes (5). Since the C3 proactivator has been identified as Factor B of the properdin system

(16), other factors in the properdin system were examined for their ability to cause lysis of the liposomes. RP caused lysis of the liposomes, but no enhancement of lysis occurred when properdin was added to RP. However, the amount of lysis with RP was about 25% less than that of human serum.

In addition to these two mechanisms outlined above, it appears that there may be other mechanisms operating in the immune lysis of liposomes by human serum. First, it was noted that there is a DFP-sensitive, heat-stable factor present in the amboceptor that apparently participates in lysis of liposomes by HS and C-MgEDTA.

Second, antibody enhances the lysis of liposomes by complement, but it is not required for the binding of C1 to liposomes or for the lysis of liposomes treated sequentially with C1, C2, and C-EDTA prior to the addition of C-MgEDTA. It could be that some unknown factor present in the amboceptor can act on the liposomes in a way that permits lysis by complement components. In the experiment in which liposomes were pretreated with complement components prior to addition of C-MgEDTA (Table 3), this factor might have been replaced by C1 or other non-specific substances in human serum. The enhancement effect of amboceptor on liposome lysis is demonstrated more clearly in the assay in which the liposomes are not isolated by centrifugation prior to analysis for release of the trapped marker. This supports the hypothesis that this factor present in the amboceptor preparation must act before other complement components can participate in lysis.

Third, the monospecific antisera against C3 does not completely abolish the ability of either HS or C-MgEDTA to lyse the liposomes.

Other workers have noted that C5, C6, C7, C8, and C9 can lyse erythrocytes in the absence of antibody if some C3 convertase ($\overline{C4,2}$) is present in the fluid phase or on the surface of other cells (5,17). Although this mechanism of lysis is very inefficient when compared to the classical complement-mediated cytolysis, it is possible that the residual lysis of liposomes observed here is due to this attack mechanism.

Finally, it is somewhat disconcerting that the role of the properdin system in the lysis of liposomes could not be definitively established. It was shown, however, that liposomes could substitute for zymosan in the inactivation of factors in RP responsible for liposome lysis. It may be that other factors are involved and that until liposomes are examined with highly purified preparations of properdin, Factor A, C3 proactivator (Factor B), C3 and the other terminal components, it will only be possible to demonstrate participation of the alternate pathway in a lytic system by undiluted and untreated fresh human serum.

These results extend the observations of other investigators concerning the use of liposomes as an alternative system in the study of immune lysis of membranes by complement (1,2,3,4,8). It does appear that the system is somewhat more complicated and technically difficult to use than the erythrocyte system because two or more mechanisms are operating simultaneously to cause release of the trapped marker. The interactions between these mechanisms will require the use of highly purified complement proteins and antibody, before there is a thorough understanding of the mechanism of immune complement-mediated lysis of liposomal model membranes.

SUMMARY

This paper presents evidence for the role of the alternate mechanisms in the lysis of liposomal model membranes by the complement system. The C3 proactivator system is implicated since Mg^{++} is required and anti-C3 and anti-C3PA antisera markedly reduce the ability of human serum and C-MgEDTA to mediate marker release from the liposomes. Although the C3PA has been identified as Factor B of the properdin system, experiments designed to test the role of the properdin system in liposome lysis were inconclusive. However, liposomes were able to substitute for zymosan in the inactivation of the terminal complement components. Complete inhibition by the anti-complement component antisera in conjunction with the complement reagents never occurred, and it is possible that additional factors and/or lytic mechanisms are involved in the lysis of liposomes.

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ARTICLE 3

A Simplified Method for the Centifugation of Liposomes

By

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(Manuscript to be submitted to Immunochemistry)

Recently Alving and Kinsky (1971) reported that during centrifugation of liposomes a substantial portion of the trapped marker was lost due to destruction of the liposomal integrity. To avoid this problem they resorted to ultracentrifugation in a sucrose or lactose solution to keep the liposomes intact. Harvesting the liposomes after centrifugation required careful removal of the subnatant solution underneath the layer of liposomes which had floated to the top of the sucrose or lactose solution.

This communication describes a method for collection of intact liposomes by centrifugation in a desk top centrifuge and ordinary 5ml centrifuge tubes. In this instance centrifugation was carried out in a Sorvall Model M desk top centrifuge placed in a cold room and run at a speed of 5500 rpm (4100 X g) for 15 minutes.

It has been observed that liposomes alone do not pellet very well, unless some source of protein is added (Alving and Kinsky, 1971; Knudson, unpublished observations). In the experiments described below it was noted that the function of the protein was non-specific, as normal serum could substitute for antiserum (hemolysin) against antigenic structures on the liposome surface in the centrifugation procedure.

Liposomes containing galactose were prepared by the method of Knudson, Bing and Kater (1971). The liposomes were treated with hemolysin (Behring Diagnostics, Woodbury, NY) and human serum, and

the resulting liposome-antibody-complement complex (LAC) was removed by centrifugation. This complex was washed several times and assays for released galactose (Knudson et al., 1971) were done on all of the supernatants. Several buffers were tested for their suitability as the wash solution.

The first buffer, veronal-buffered saline, pH 7.4, ionic strength 0.15 (VBS)(Mayer, 1961) proved to be unsatisfactory as a wash solution. Two VBS washes caused the release of 38% of the trapped galactose marker solution.

A 310 milliosmolar phosphate buffer, pH 7.4 (Dodge, Mitchell and Hanahan, 1963) was tried next. This buffer was also not satisfactory. The LAC complex did not pellet as completely or as quickly as it had with the VBS buffer. Because of the loss of liposomes to the supernate, assays for the amount of released galactose could not be done.

The third buffer tested was the isotonic salt solution (0.075M NaCl-0.075M KCl) used for the dialysis of the liposomes (Haxby, Kinsky and Kinsky, 1968). This buffer proved to be the best of the three buffers tested. Two washes of the LAC complex released only 9.7% of the trapped galactose. Complexes of liposomes and antibody (LA) and liposomes and complement (LC) could also be centrifuged and washed with this procedure. The properties of these complexes are described in another report (Knudson and Bing, manuscript in preparation).

An explanation for the non-specific loss of marker during centrifugation of liposome complexes reported by Alving and Kinsky (1971) may be related to the method of liposome preparation. It was

previously reported (Knudson et al., 1971) that sonication of the liposomes for 2 1/2 to 3 hours yielded preparations which were extremely stable in terms of non-specific marker release, and still contained trapped marker at least two months after preparation. Electron micrographs revealed that unsonicated liposome preparations consisted almost exclusively of many layered sheets rather than discrete vesicles. Sonication of these lamellar sheets gave the more characteristic vesicle structures. If the liposome preparation used by Alving and Kinsky in their work consisted of the sheet structures, it is possible that these structures could not withstand the mechanical manipulation that is an integral part of the centrifugation and wash procedure.

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