RELEASE OF TRAPPED MARKER FROM LIPOSOMES BY THE ACTION OF PURIFIED COMPLEMENT COMPONENTS

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Abstract.—Liposomes containing trapped glucose marker were prepared from the chloroform-soluble fraction of sheep erythrocyte membranes. These liposomes release glucose when incubated with rabbit anti-sheep erythrocyte serum and a source of complement. Experiments with purified human complement components show that loss of marker is absolutely dependent on the presence of components 2 and 8. An absolute requirement for component 9 cannot be demonstrated, although it stimulates glucose release from the liposomes. These results establish a parallelism between the response of biological membranes and liposomal membranes to antibody and complement.

In recent years it has become clear that complement provides the protein components which impair membrane function in immune cytolysis. The complexity of biological membranes constitutes, at present, an obstacle to an understanding of the molecular basis of membrane damage caused by the terminal complement components. Possible circumvention of this difficulty with artificial membranes was indicated in previous studies using liposomes prepared from sheep erythrocyte membranes.[1, 2] These liposomes release trapped glucose marker when incubated with a combination of rabbit anti-sheep erythrocyte serum (as a source of antibodies) and fresh guinea pig serum (as a presumptive source of complement). The available evidence suggests that the alteration in the permeability of these structures mimics very closely the functional impairment of biological membranes following the action of antibody and complement.

Before liposomes can be properly used as a model system to investigate complement-membrane interaction, it is necessary to determine whether their impairment by antibody and fresh serum requires the participation of the same components which are essential for immune cytolysis in general, and immune hemolysis in particular. Classical immune cytolysis has been shown to require the sequential action of nine complement components (C1–C9).[3] The reaction is initiated by C1 which, after activation by interaction with antibody, catalyzes the assembly of the $\overline{C4,2}$ enzyme on the cell surface. This enzyme catalyzes binding of C3 and formation of the $\overline{C4,2,3}$ enzyme. Through the action of the $\overline{C4,2,3}$ enzyme, the next three components, C5, C6, and C7, are activated. Subsequent binding of C8 results in manifestation of membrane damage, the rate of which is increased by C9. The present investigation, in which purified human complement components were used, demonstrates a definite similarity between glucose release from liposomes and hemoglobin release from erythrocytes by antibody and complement.

Materials and Methods.—Chemicals: ATP and TPN^+ were obtained from the Sigma Chemical Co., St. Louis, Mo. Hexokinase (10 mg/ml) and glucose-6-P dehydrogenase (5 mg/ml) were purchased from Boehringer-Mannheim Corp., New York, N.Y., and dialyzed against distilled water before use.

Liposomes: The chloroform soluble fraction of sheep erythrocyte membranes (designated sheep fraction II*a*) was isolated by procedures which have been described previously.[1] Liposomes were prepared[1] by adding an aliquot of fraction II*a*, containing 2 μmoles of phospholipid, to 10-ml conical flasks and removing the solvent (chloroform) under reduced pressure. Marker solution (0.2 ml of 0.3 *M* glucose) was added to the flask and the dried lipid residue was dispersed by agitation with a Vortex mixer in the presence of a small quantity of glass beads (0.2 mm in diameter). The liposome preparation was subsequently dialyzed for 1.5 hr at room temperature against 150 ml of isotonic salt solution (0.075 *M* KCl–0.075 *M* NaCl) to remove most of the untrapped glucose.

Antisera: Rabbit anti-sheep erythrocyte serum (Baltimore Biological Laboratories, Baltimore, Md.) was diluted 10-fold with Veronal buffer (VB^{++}) prepared according to the technique of Mayer.[4] Rabbit anti-human C8 was prepared according to ref. 5. Both antisera were decomplemented by heating for 2 hr at 56°.

Complement components and reagents: The feasibility of using purified human components was suggested by experiments indicating that human serum can replace guinea pig serum as a source of complement necessary for release of glucose from liposomes.[6] The procedures for the preparation of the purified human complement proteins used in this study have been described in detail elsewhere: C2 (ref. 7), C4 (ref. 8), C8 (ref. 9), and C9 (ref. 10). C2 was oxidized with I_2 before use[11] because this procedure has been shown to stabilize and increase the efficiency of human C2 in immune hemolysis. Partially purified C1 was obtained according to Nelson *et al.*[12] and the C3–8 reagent was prepared as outlined previously.[10] The latter contained noncomplement components (primarily γ-globulin) accounting for approximately 50% of the total protein present.

The amounts of purified complement proteins added to the reaction mixtures (see below) were: $^{oxy}C2$ (5 μg), C4 (46 μg), C8 (17 μg), and C9 (13 μg). In terms of activity, 1 μg of $^{oxy}C2$, C4, and C8 correspond, respectively, to 1.3×10^{10}, 1×10^{10}, and 2×10^{11} hemolytically effective molecules.[13]

To neutralize C8 in the C3–8 reagent, the heated rabbit antiserum to human C8 was diluted 1:2 with Veronal buffer and then added to an equal volume of the C3–8 reagent. The precipitate formed after 15 hr at 4° was removed by centrifugation. C3–8 reagent treated in an identical manner with normal rabbit serum served as a control.

All preparations of complement components, the antiserum to C8, and the normal rabbit serum were dialyzed against Veronal buffer before use.

Assay: As in previous studies,[1, 2] loss of glucose from liposomes was determined by the change in absorbance at 340 mμ which resulted when the trapped marker was released and rapidly oxidized by TPN^+ in the presence of hexokinase, glucose-6-P dehydrogenase, and the necessary cofactors. Before each experiment, a stock assay reagent was prepared which contained: 100 mM Tris·HCl, pH 8.5, 129 mM NaCl, 3.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 2 mM ATP, 1 mM TPN, and approximately 80 μg of hexokinase and 40 μg of glucose-6-P dehydrogenase per ml. Dilution of the assay reagent with the other constituents of the reaction mixture (i.e., liposomes, rabbit antiserum, complement, Veronal buffer, etc.) reduced the concentration to the requisite levels.

In the present investigation, the liposomes were first treated with antiserum and subsequently incubated with the complement components in the order in which they react to produce immune cytolysis. At various times after addition of the components, the absorbance of the reaction mixture was measured to see whether any glucose had been released. Under the conditions of incubation (pH 8.5 at 30 or 37°), the enzymes in the assay reagent were slowly inactivated. Therefore, immediately prior to each absorbancy determination, the cuvettes received 2 μl of a mixture which contained approximately 5 μg of the dialyzed hexokinase and 2.5 μg of the dialyzed glucose-6-P dehydrogenase. This general procedure was varied to fit the requirements of a particular experiment, as

well as the availability of the purified complement components, and details are presented in the appropriate table legends.

Results.—Requirement for C2: The results recorded in Table 1 show that glucose release is dependent on the presence of C2. This experiment was performed in three stages. In the first stage, the liposomes were incubated with antiserum in the absence of complement. Under the conditions of the experiment, the absorbance at the end of this stage is a measure of the small amount of untrapped glucose contaminating the liposome preparation and light scatter by the liposomes. In the second stage of the experiment, the liposomes were incubated with a mixture of C1, C4, and ^{oxy}C2 (cuvette 1) or a mixture of C1 and C4 (cuvette 2). The last two columns of Table 1 indicate that glucose was not released, because these complement components did not produce an increase in absorbance over that observed at the end of stage I. In the third stage, C3–8 reagent was added. This reagent resulted in an appreciable release of the trapped glucose from liposomes which had been incubated with Cl, C4, and ^{oxy}C2 (cuvette 1). However, no marker was released from liposomes which had not been exposed to ^{oxy}C2 during the second stage (cuvette 2).

TABLE 1. *C2 requirement for glucose release.*

	Additions to:		Corrected Absorbance After Incubation of:	
Stage	Cuvette 1	Cuvette 2	Cuvette 1	Cuvette 2
I	Antiserum, liposomes	Antiserum, liposomes	0.319	0.321
II	C1, C4, C2	C1, C4, buffer	0.317	0.323
III	C3–8	C3–8	0.683	0.331
Net absorbance change for complete glucose release			1.038	1.038
Glucose released during stage III			35%	<1%

Stage I: Cuvettes contained initially 400 µl of Veronal buffer, 500 µl of assay reagent, 20.5 µl of diluted rabbit antiserum, and 10.2 µl of liposome preparation. These were incubated for 5 min at room temperature.

Stage II: The following components were then added in the order listed and the cuvettes were incubated at 30° for the times indicated in parentheses after each addition: 95 µl of C1 reagent (5 min); 40 µl of C4 (10 min); 50 µl of ^{oxy}C2 to cuvette 1 and 50 µl of Veronal buffer to cuvette 2 (10 min).

Stage III: 50 µl of C3–8 reagent were added to both cuvettes, which were then incubated for 20 min at 37°. The observed absorbancy values were corrected for any contribution by the antiserum and complement components, and are expressed on the basis of a final vol of 1 ml. The change in absorbance for complete glucose release was determined after lysis of the liposomes with 1% Triton.[1,2]

Requirement for C8: The requirement for C8 was investigated because it is the last of the components present in the C3–8 reagent to act in immune cytolysis. The availability of a specific antiserum to C8 prompted its use as an inhibitor in this experiment, which was also performed in a stepwise manner (Table 2). The first two stages involved incubation of the liposomes with antiserum and components C1, C4, and ^{oxy}C2. Under the conditions of the experiment, the absorbance at the end of stage II is a measure of untrapped glucose in the liposome preparation and light scatter by the liposomes, as well as any contribution by these complement components *per se*. Glucose was released from the liposomes during the third stage upon addition to cuvette 1 of the C3–8 reagent which had been treated with normal rabbit serum. In contrast, there was essentially no change in the absorbance of cuvette 2 to which had been added C3–8 reagent that

TABLE 2. *C8 requirement for glucose release.*

Stage	Additions to: Cuvette 1	Additions to: Cuvette 2	Corrected Absorbance After Incubation of: Cuvette 1	Corrected Absorbance After Incubation of: Cuvette 2
I	Antiserum, liposomes	Antiserum, liposomes	0.452	0.447
II	C1, C4, C2	C1, C4, C2	0.561	0.570
III	C3–8 plus NRS	C3–8 plus anti-C8	1.499	0.644
IV	C9	C9	1.638	0.695
V	Buffer	C8	1.698	1.479
Net absorbance change for complete glucose release			1.469	1.469
Glucose released during stage III			64%	5%
Total glucose released through stage V			77%	62%

Stage I: Cuvettes contained initially 170 μl of Veronal buffer, 500 μl of assay reagent, 20.5 μl of diluted rabbit antiserum, and 9.6 μl of liposome preparation. Incubation time: 5 min at room temperature.

Stage II: The following components were then added in the order listed and the cuvettes were incubated at 30° for the times indicated after each addition: 9.6 μl of C1 reagent (5 min); 40 μl of C4 (10 min); 50 μl of ^{oxy}C2 (10 min).

Stage III: 50 μl of C3–8 reagent treated with heated normal rabbit serum (NRS) was added to cuvette 1, and 50 μl of C3–8 reagent treated with anti-C8 was added to cuvette 2. Incubation time: 21 min at 37°.

Stage IV: 50.9 μl of C9 was added to both cuvettes, which were then incubated for 11 min at 37°.

Stage V: 50.9 μl of Veronal buffer was added to cuvette 1, and 50.9 μl of C8 was added to cuvette 2. Incubation time: 21 min at 37°. The observed absorbancy values were corrected for any contribution by the C3–8 reagents and C8, and are expressed on the basis of a final vol of 1 ml. The change in absorbance for complete glucose release was determined after lysis of the liposomes with 1% Triton.[1, 2]

had been treated with rabbit anti-C8. Furthermore, C9 did not induce glucose release when it was added to cuvette 2. The appreciable difference in absorbance between cuvettes 1 and 2 at the end of stage IV therefore indicates that C8 is required. Direct evidence for the involvement of C8 was obtained during the fifth stage of the experiment. Addition of this component to cuvette 2 restored the ability of the C3–8 reagent treated with anti-C8 to promote loss of marker from the liposomes.

Stimulation by C9: Marker was released without the addition of C9 in the preceding experiments. Figure 1 shows that addition of this component does, however, greatly increase the initial rate of glucose release.

Discussion.—These experiments constitute a preliminary study to determine which complement components are necessary for the release of trapped glucose from sheep fraction II*a* liposomes. Three of the nine components known to participate in immune cytolysis were tested. The results presented in Table 1 demonstrate an absolute requirement for C2 and for one or more of the components which can be supplied by a reagent containing C3, C5, C6, C7, and C8. The experiment described in Table 2 demonstrates an absolute requirement for C8. Furthermore, provided that the reagents used were not contaminated with traces of C9, the data indicate that there is no absolute requirement for C9 although this component can enhance the rate of glucose release from the liposomes (Fig. 1). These observations have their counterpart in recent studies which have shown that slow, but definite, hemoglobin release from erythrocytes occurs following completion of the reaction involving C8.[14, 3]

It has been well documented that C2 cannot participate in immune cytolysis when C1 and C4 are absent. C2 and C4 are the precursors of the indigenous

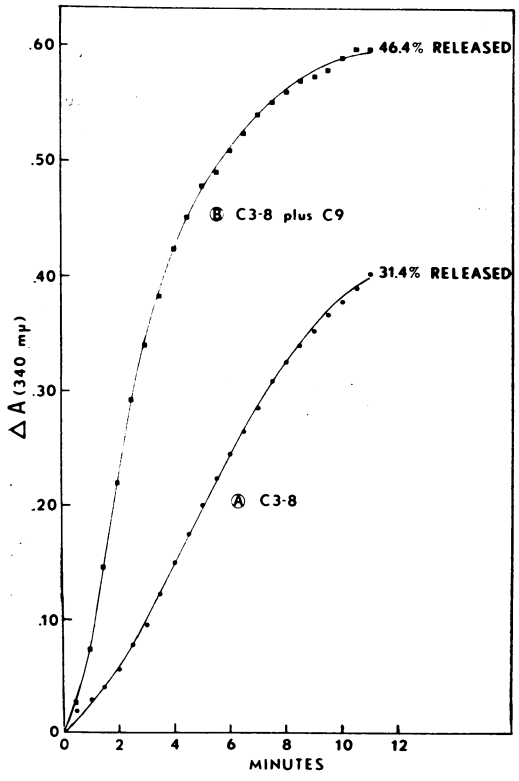

FIG. 1.—Stimulation of glucose release by C9. Cuvettes contained initially Veronal buffer (370 μl), assay reagent (500 μl), diluted rabbit antiserum (20.5 μl), and liposomes (9.6 μl). Each cuvette was incubated at 30° for 5, 10, and 10 min following the sequential addition of C1 reagent (10.3 μl), C4 (39.9 μl), and ^{oxy}C2 (50.9 μl). Enzyme mixture (10.3 μl) was then added and the "zero time" absorbance was determined. The abscissa indicates the change in absorbance after addition of a mixture of C3–8 reagent (25.1 μl) and Veronal buffer (50.9 μl) to cuvette *A*, and C3–8 reagent (25.1 μl) and C9 (50.9 μl) to cuvette *B*. Incubation temperature: 22°. All absorbance values were corrected for any contribution by the complement components, and are expressed on the basis of a final vol of 1 ml. Complete glucose release would have produced an absorbancy change of 1.286 as determined after lysis of the liposomes in the presence of 1% Triton.[1, 2]

complement enzyme $\overline{C4,2}$, formation of which is catalyzed by activated C1. The action of the subsequent components, including C7, depends on a functional $\overline{C4,2}$ enzyme. Similarly, interaction of C8 with cell membranes is contingent on prior reaction of C5,6,7, which in turn requires C4,2,3 for their activation. Thus, although an absolute requirement for glucose release from liposomes has so far only been demonstrated in the case of C2 and C8, present knowledge of the complement reaction sequence strongly implicates the other components (with the possible exception of C9) as essential participants.

Experiments, which are described in detail elsewhere,[15] suggest that Forssman antigen is partly responsible for the response of the sheep fraction II*a* liposomes to antibody and complement. These studies also demonstrate that liposomes can be prepared from a mixture of pure lipids (of known structure and composition) and Forssman hapten (*N*-acetyl-galactosamine (α1 → 3)-galactose (β1 → 3)-galactose (β1→ 4)-glucose-1-ceramide). These liposomes, like the II*a* liposomes used in the present investigation, release glucose when incubated with rabbit anti-sheep erythrocyte serum and fresh guinea pig serum. It now remains to be determined if, with these liposomes, the requirement for guinea pig serum can also be replaced by the purified complement components which are involved in immune cytolysis. Should this be the case, then these liposomes would constitute an extremely appropriate artificial membrane system for investigating the molecular basis of complement mechanism.

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