Biochimica et Biophysica Acta, 736 (1983) 92–98
Elsevier

BBA 71889

MEASUREMENT OF ANTIBODY-DEPENDENT BINDING, PROTEOLYSIS, AND TURNOVER OF C1s ON LIPOSOMAL ANTIGENS LOCALIZES THE FLUIDITY-DEPENDENT STEP IN C1 ACTIVATION

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(Received May 16th, 1983)

Key words: Liposome; Complement activation; Antibody dependence; Membrane fluidity

The antibody-dependent binding and activation of the first component of human complement (C1) by liposomes containing nitroxide spin-label lipid haptens have been simultaneously measured. The liposomes were either fluid (dimyristoylphosphatidylcholine) or solid (dipalmitoylphosphatidylcholine) at the temperature of the experiments (32°C). In 10 minutes fluid liposomes activate 40% of the C1 whereas solid liposomes only activate 10% of the C1. The fraction of C1 bound at the end of the activation incubation is approx. 2% for fluid liposomes and approx. 4% for solid liposomes. This binding is consistent with the relative amounts of antibody which bind to these two types of liposomes. These results demonstrate turnover of C1 or $C1r_2s_2$ on the liposome surface. It is concluded that the differential activation of C1 is due to a difference in the rate of activation of C1 after it is bound to the liposome surface. Lower limits for the activation rate constant for C1 bound to fluid and solid liposomes are estimated to be $8 \cdot 10^{-2}$ s^{-1} and $1 \cdot 10^{-2}$ s^{-1}, respectively.

Introduction

Previous studies have shown that the antibody-dependent activation of complement by lipid hapten containing liposomes is sensitive to the physical state of the liposomal membrane [1–4]. At 32°C the fluid membranes of dimyristoylphosphatidylcholine (DMPC) were found to activate complement more efficiently than the solid membranes composed of dipalmitoylphosphatidylcholine (DPPC). In a number of studies aimed at understanding the mechanism of this differential activation we have found (1) that approximately the same amount of C1q binds to both fluid and solid liposomes [5], (2) that the first component of complement (C1) is the step of the complement cascade that is sensitive to the physical state of the liposome [6], and (3) that the difference on C1 activation is kinetic [7]. Fluid membranes activate C1 with a pseudo-first-order rate constant approximately four times that for solid membranes [7]. The fact that similar quantities of C1q bind to the fluid and solid liposomes yet activation of C1 in these two systems is kinetically distinct lead to the conclusion that the actual rate of activation of the C1 molecule once bound to the liposome is the rate-limiting step in this interaction. The evidence that C1q binding to antibodies is different from C1 binding [8] prompted the present study. In this

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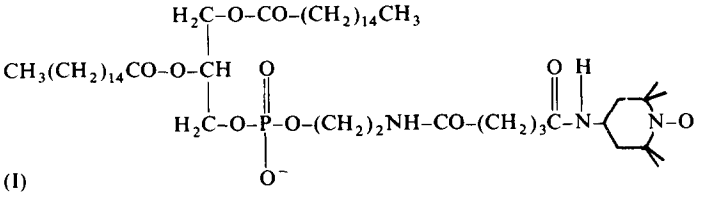
Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC. dipalmitoylphosphatidylcholine; NBD-PE, *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine; veronal-buffered saline, 4.95 mM barbital, 0.145 M NaCl, pH 7.4; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate. Complement components are identified according the WHO Recommendations (1968). A bar over the number of a complement component represents an activated state not normally expressed by the native protein molecule.

study we have measured the binding and activation of C1 using the same reagent and in addition we have measured directly the binding of antibodies to both fluid and solid liposomes.

Materials and Methods

Antibody and hapten preparation. Anti-nitroxide antibodies were prepared as an IgG fraction from rabbit antisera as previously described [9]. This antibody preparation was determined to be approx. 2 μM in specific anti-nitroxide antibodies from measurements on the binding of soluble nitroxide haptens. This 2 μM value referred to throughout the paper as the 1 : 1 antibody dilution is probably a lower limit for the actual antibody concentration as our measurements are not sensitive to low affinity antibodies. This preparation was centrifuged at 100000 × *g* for 20 min immediately prior to use in order to remove IgG aggregates. The nitroxide spin-label phospholipid hapten (I) was synthesized by the procedure of Brûlet and McConnell [2].



(I)

Liposome preparation. Required amounts of phospholipid, DMPC or DPPC, were dissolved in methanol and mixed with 1 mol% nitroxide spin-label hapten (I) and a trace quantity, approx. 10^{-4} mol% ^{3}H-DPPC. For some experiments 0.1 mol% of the fluorescent phospholipid *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE) was added. The lipid solutions were evaporated to dryness in 10-ml round-bottom flasks. A sufficient quantity of veronal-buffered saline containing 2% ficoll 70, 0.05% NaN_3, and 1 mM $CaCl_2$ was added to the flask to give a final lipid concentration of 15 mM. The flasks were incubated for 15 min at 15°C above the chain melting phase transition of the particular lipid and liposomes were formed by vigorous vortexing. By transferring the flasks from a water bath to ice, the liposomes were cycled four times through their chain melting phase transition temperature and then placed in a refrigerator for two days. These procedures allow the liposomes to anneal and insure high recovery in the following centrifugation steps. After annealing, the liposomes were centrifuged for 5 min at 4000 × *g* in a Fisher Model 59 centrifuge at 32°C. The liposomes were resuspended in veronal-buffered saline, washed three times by centrifugation at 32°C and finally resuspended to a concentration of 10 mM based on the radioactivity of the ^{3}H-DPPC.

C1 reagent. ^{125}I-C1 was prepared by adding partially purified ^{125}I-labeled C1s to whole serum followed by ultracentrifugation on sucrose density gradients as described previously [10]. The ^{125}I-C1 reagent was made 2 mM in EDTA by the addition of an appropriate volume of 0.1 M EDTA solution. The sample was then dialyzed overnight against veronal-buffered saline containing 1 mM EDTA, to remove the sucrose and frozen in liquid nitrogen. Immediately prior to use, this ^{125}I-C1 reagent was adjusted to 2 mM $CaCl_2$ by addition of 0.1 volumes of a 20 mM $CaCl_2$ solution. C1 activation was determined by monitoring cleavage of ^{125}I-C1s as previously described [10].

C1 binding. The binding of C1 to liposomes was determined in the following manner. Specified amounts of liposomes, antibody, and C1 reagent were incubated for the specified amount of time in 150-μl conical-bottom polyethylene centrifuge tubes at 32°C. The tubes were then placed in carrier tubes in a Fisher Model 59 centrifuge and centrifuged at 6000 × *g* for 5 min at 32°C. Immediately after centrifugation, the supernatant fluid was removed from the pellet with a 100 μl Hamilton syringe. The bottom of the centrifuge

tube containing the liposome pellet was cut off and placed in a test tube. The supernatant fluid and the rest of the centrifuge tube were placed in a second test tube where 75 μl of a solution of 2% sodium dodecyl sulfate, 8 M urea, and 2% 2-mercaptoethanol was added to wash down the sides of the centrifuge tube and stop further C1 activation. Radioactivity in the pellet and supernatant fluid was quantitated in a Beckman 4000 gamma counter. The samples were then boiled and loaded on SDS-polyacrylamide gels for determination of C1 activation [10]. This assay allows for the measurement of both C1 binding and activation on the same sample. The recovery of liposomes in the pellet after centrifugation was determined by measuring the fluorescence of 0.1 mol% NBD-PE initially incorporated into the liposomes. After stopping the C1 reaction by addition of the urea solution to the pellet and supernatant fluid the fluorescence of NBD-PE was quantitated for both solutions on a Spex Industries spectrofluorometer. In all cases 99% of the fluorescence was recovered in the liposome pellet.

Protein assay for antibody binding. Quantitation of the amount of IgG bound to the liposomes was performed essentially by the method of Butcher and Lowry [11] using acid hydrolysis of the protein followed by reaction of the amino acids with orthophthalaldehyde. Briefly, the liposomes and antibody are mixed, incubated at 32°C and centrifuged to a pellet as previously described for the C1 binding experiments. After removal of the supernatant fluid the centrifuge tube is placed in a Speed Vac concentrator (Savant Industries) to completely dry the pellet. 6 μl of 6 N HCl is added to the dried pellet, a drop of mineral oil is used to cover the acid solution and hydrolysis is performed in an autoclave for 6 h. The remaining procedure was essentially that of Butcher and Lowry [11]. Samples with no antibody, and with antibody but no spin-label hapten in the liposomes were used to control for fluorescence contributed by hapten in the liposomes, and nonspecific antibody binding, respectively.

Results

In previous studies we have demonstrated the activation of C1 by liposomes containing 0.1 mol% spin-label hapten in the presence of anti-spin-label antibodies [6,7]. Although we had previously demonstrated that the antibody-dependent binding of C1q to these fluid and solid liposomes was approximately the same [5], we decided to attempt to measure directly the binding of the C1 reagent used to measure C1 activation since it has been shown that the binding to antibodies of C1 is quantitatively different from that of C1q [8]. Initial attempts to measure antibody-dependent binding of C1 to liposomes containing 0.1 mol% spin-label hapten resulted in no detectable binding.

Activation of C1 at higher hapten density

In order to optimize the assays for binding we decided to increase the hapten concentration to 1 mol% and increase antibody and liposome concentrations until binding could be observed. It was first necessary to demonstrate that at 1 mol% hapten and high liposome and antibody concentrations the differential activation of C1 by fluid and solid liposomes still obtains. Table I shows the activation of C1 by fluid (DMPC) and solid (DPPC) liposomes containing 1 mol% spin-label hapten as a function of both antibody and liposome concentration. At all concentrations of liposomes and antibodies except one, DMPC liposomes activate C1 faster than DPPC liposomes. The concentrations representing a 1 : 1 dilution of liposomes and a 1 : 10 dilution of antibody were chosen for the binding study because they resulted in a large differential activation between fluid and solid liposomes and represented a relatively high concentration of reagents as compared to those used in previous studies [6,7].

Binding and activation of C1

In order to study C1 binding to liposomes an experiment was designed such that both the amount of C1 bound to liposomes and the amount of C1 activated could be measured on the same sample (see Methods for details). Table II shows the binding and activation of C1 by DMPC and DPPC liposomes under a variety of conditions. Two striking results emerge from this experiment. First, the number of C1 molecules activated far exceeds the number of C1 molecules bound both in the case of DMPC and DPPC liposomes. And second, although the DMPC liposomes activate

TABLE I

C1 ACTIVATION AS A FUNCTION OF LIPOSOME AND ANTIBODY CONCENTRATION

Each assay tube contained 12.5 μl of liposomes, 12.5 μl of antibody, and 50 μl of the ^{125}I-C1 reagent. The 1:1 concentration of liposomes is 10 mM total lipid, and the 1:1 concentration of antibody is approx. 2 μM specific anti-nitroxide antibody as judged from measurements of binding to soluble nitroxide haptens. The concentration of C1 in the C1 reagent is estimated from dilutions during preparation to be approximately one-fourth the normal serum concentrations or approx. 40 nM. The numbers in the table represent the percent of C1 activated minus the percent of C1 activated at that antibody dilution in the absence of liposomes. The activation by antibody alone ranged from 32% at a 1:1 dilution to 22% at a 1:1000 dilution. The incubation was for 10 min at 32°C. (C1, component of complement; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine)

Liposome dilution	Antibody dilution			
	1:1	1:10	1:100	1:1000
DMPC				
1:1	44	29	0	0
1:10	36	40	7	2
1:100	10	26	21	0
1:1000	0	0	2	0
DPPC				
1:1	34	6	0	0
1:10	23	21	0	0
1:100	10	11	7	0
1:1000	1	0	1	0

significantly more C1 than DPPC liposomes in the 10-min incubation, the binding of C1 to DMPC liposomes is lower than it is to DPPC liposomes. Several controls were performed in this experiment. The use of liposomes without haptens demonstrates that both activation and binding of C1 require that the antibodies be specifically bound to the liposomes. Chelation of calcium by EDTA inhibits activation as expected by dissociating the C1 complex, and in addition it demonstrates that the binding of ^{125}I-C1s to the liposomes depends on the integrity of the C1 complex.

Independence of binding on the degree of activation of C1

It was hypothesized that the C1 molecule may dissociate upon activation and thus release ^{125}I-C1s from the surface of the liposome. If this occurred then the greater activation of C1 by DMPC liposomes might explain the lower binding of radioactivity to these liposomes. To test this hypothesis, binding measurements were carried out in the presence of the protease inhibitor *p*-nitrophenyl-*p'*-guanidinobenzoate (NPGB). Because it was necessary to add the NPGB as a concentrated solution in dimethyl formamide, the solvent alone was tested as well. As shown in Table II, DMF alone inhibited C1 activation to a certain extent but the same relationship between activation and binding was still observed with DMPC and DPPC liposomes. Addition of NPGB resulted in background C1 activation values for both DMPC and DPPC but binding of C1 was still greater for DPPC liposomes than for DMPC liposomes. Thus activation-dependent dissociation of the C1 complex cannot explain the differential binding of C1 to DMPC and DPPC liposomes.

TABLE II

ACTIVATION AND BINDING OF ^{125}I-C1 BY ANTIBODIES SPECIFICALLY BOUND TO FLUID (DMPC) AND SOLID (DPPC) LIPOSOMES

Both activation and binding measurements were made on the same sample (see Methods for details). All samples contained 12.5 μl of 10 mM liposomes, 12.5 μl of a 1:10 dilution of antibody, and 50 μl of C1 reagent. All numbers refer to the percent of total ^{125}I-C1 in the assay tube. Each value is the average of three separate determinations with the standard error of the mean included. Assays were performed at 32°C for a total of 10 min (incubation plus centrifugation time). (C1, component of complement; DMPC, dimyristoylphosphatidylcholine; DPPC dipalmitoylphosphatidylcholine; DMF, dimethyl formamide; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate)

	Percent activated	Percent bound
DMPC	52.3 ± 4.4	2.6 ± 0.55
DPPC	20.2 ± 0.66	4.7 ± 1.3
DMPC – hapten	11.5 ± 0.81	0.72 ± 0.01
DPPC – hapten	10.5 ± 1.0	0.86 ± 0.08
DMPC + EDTA	7.2 ± 0.61	0.52 ± 0.14
DPPC + EDTA	7.0 ± 0.25	0.76 ± 0.06
DMPC + DMF	26.0 ± 5.8	1.2 ± 0.15
DPPC + DMF	15.9 ± 0.57	4.3 ± 0.22
DMPC + NPGB	11.6 ± 0.53	1.1 ± 0.07
DPPC + NPGB	10.5 ± 0.81	3.6 ± 0.38

C1s does not turn over in the C1 complex on the time scale of these experiments

The fact that more C1 is activated by the liposomes than is bound at any point in time suggests

TABLE III

C1 VERSUS C1s ACTIVATION

All samples contained 12.5 μl of 10 mM liposomes, and 12.5 μl of a 1 : 10 antibody dilution. Rows 1–5 indicate: (1) addition of 50 μl of C1 followed by a 60-min incubation, (2) addition of 50 μl of C1 followed by a 60-min incubation followed by addition of 15 μl of veronal-buffered saline and an additional 60-min incubation, (3) addition of 15 μl of ^{125}I-C1s, which contained an amount of radioactivity equal to that in 50 μl of C1, plus 45 μl of veronal-buffered saline followed by incubation for 60 min, (4) addition of C1 followed by incubation for 60 min followed by addition of 15 μl of ^{125}I-C1s, and (5) addition of C1 followed by incubation for 60 min, addition of ^{125}I-C1s followed by an additional 60-min incubation. All incubations were carried out at 32°C. The maximum amount of C1 that could be activated in this preparation was approx. 60%. Rows 4 and 5 contain calculated values for C1 activation assuming no C1s turn over. These values are the average of rows 1 and 3, and 2 and 3, respectively (C1, complement of component; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine)

Antibody and liposomes with the following incubation conditions	DMPC		DPPC	
	Percent activated	Values calculated [a]	Percent activated	Values calculated [a]
C1, 60 min	59	0	39	0
C1, 60 min, buffer, 60 min	57	0	51	0
C1s, 60 min	6	0	8	0
C1, 60 min, C1s	32	33	24	24
C1, 60 min, C1s, 60 min	33	32	29	30

[a] Values calculated assuming no C1s turn over.

that the C1 is turning over on the surface of the liposome. Since our C1 reagent is labeled in the C1s subunit several possibilities exist for the mechanism of turnover. The entire C1 molecule may bind, activate, and release from the liposome surface, exposing a site for another C1 molecule to bind. The $C1r_2s_2$ complex may bind, activate, and dissociate from a relatively permanently bound C1q subunit. Or lastly, C1s may cycle through a bound $\overline{C1qr_2}$ complex. Although the latter of these possibilities is unlikely, it was readily testable. Table III gives the results of incubating labeled C1 in the presence of liposomes followed by the addition of an amount of ^{125}I-C1s equal to the amount of ^{125}I-C1s contained in the C1 reagent initially added. These data demonstrate that the addition of more ^{125}I-C1s to the C1 liposome mixture does not result in acitvation of that additional C1s. Therefore C1s is not turning over on a bound $\overline{C1qr_2}$ complex or being activated in solution by $\overline{C1r}$.

The binding of C1 to liposomes is an equilibrium value

In order to determine the step in the activation process that is sensitive to the physical state of the liposome membrane it is necessary to determine whether the C1 binding to the liposomes is a saturation or equilibrium phenomenon. The number of antibody molecules bound to the surface of the liposomes was determined as described in Materials and Methods. For the assay 12.5 μl of 10 mM liposomes was mixed with 12.5 μl of a 1 : 10 dilution of the stock anti-nitroxide IgG solution (approx. 0.38 μg) and 50 μl of veronal-buffered saline containing 1 mM $CaCl_2$ was added. Binding of antibody to liposomes was complete as fast as we could measure it (approx. 5 min). DMPC liposomes bound 0.12 μg IgG and DPPC liposomes bound 0.22 μg IgG. These levels of bound antibody should provide enough sites to bind significantly more ^{125}I-C1 than that which is observed in the experiments reported in Table II (see Discussion for details). If saturation of liposome-bound antibody by C1 were occurring, then dilution of the C1 reagent in the assay should result in a shorter half life for the unactivated C1. Table IV shows this not to be the case. Both 2- and 4-fold dilutions of the C1 reagent resulted in activation rates similar to that of the stock C1 reagent for both DMPC and DPPC liposomes. This is strong evidence that the bound C1 is in equilibrium with that free in solution and that it is not saturating antibody sites on the liposome surface.

TABLE IV

ACTIVATION AS A FUNCTION OF C1 DILUTION

All samples contained 12.5 μl of 10 mM liposomes, 12.5 μl of a 1 : 10 antibody dilution, and 50 μl of the various dilutions of C1 indicated. Incubations were carried out for 15 min at 32°C. (C1, component of complement; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine)

C1 dilution	Percent C1 activation	
	DMPC	DPPC
No dilution	57	27
1 : 2	54	34
1 : 4	51	32

Discussion

Since the finding of Brûlet and McConnell [1,2] that the efficiency of antibody-dependent complement depletion by haptenated liposomes is dependent on the physical state of the liposome membrane, we have been studying the mechanism(s) underlying this phenomenon. We have shown that (1) fluid and solid phase liposomes bind essentially the same amount of ^{125}I-C1q in an antibody-dependent manner [5], (2) the difference in complement depletion by fluid and solid liposomes is at the level of activation of the first component of complement, C1 [6], and (3) the difference in C1 activation by these two types of liposome is kinetic, the pseudo-first-order rate constant for C1 activation by fluid liposomes being approximately four times greater than that for solid liposomes [7]. From these studies two major hypotheses emerged. One hypothesis states that C1 binds to the antibody on the liposome surface, activates, and remains bound due to the probable multivalency of binding. The difference between activation rates on fluid and solid liposomes would be due to differences in the activation step. A second hypothesis states that C1 binds, activates, and dissociates, and that the differential activation rate on fluid and solid liposomes may be due to differences in the rate of the activation step and/or in the rate of turnover of C1 on the liposome surface. The present study clearly shows that there is turnover of C1 on the liposomal surface. The level at which turnover occurs, however, is not certain. Table III demonstrates that the turnover is not at the level of C1s (e.g., C1s associating with, activating, and dissociating from a complex on the liposome containing C1q and C1r). The two remaining possibilities are (1) that the entire C1 molecule ($C1qr_2s_2$) is turning over on the liposomal surface or (2) that the $C1r_2s_2$ complex is turning over on C1q molecules which remain bound to the liposome. The present study cannot resolve these two possibilities. In addition, both types of turnover may be occurring simultaneously.

Recently, Kilchherr et al. [12] demonstrated turnover of the $C1r_2s_2$ complex on C1q bound to chemically cross-linked IgG dimers and studied the kinetics of C1 activation by these dimers. They determined a rate constant of $2 \cdot 10^{-3}$ s^{-1} for the rate-limiting step in the activation of C1 in their system. This is similar to the rate constant of $2.9 \cdot 10^{-3}$ s^{-1} for the spontaneous activation of pure C1 in the absence of C1INA observed by Ziccardi [13]. For the antibody-dependent activation of C1 bound to fluid and solid liposomes, we can set a lower limit for this rate constant of approx. $8 \cdot 10^{-2}$ s^{-1} and $1 \cdot 10^{-2}$ s^{-1}, respectively. These rate constants are based on the fact that the equilibrium binding of C1 is 2% for fluid liposomes and 4% for solid liposomes and the activation of C1 in 10 min is 40% and 10%, respectively. The determination of these rate constants is dependent on an equilibrium binding of C1 to the liposomes as stated above. The data in Table IV strongly argue that we truly have equilibrium binding of C1 to the liposomes. In support of this are the following calculations. Accounting for a 4-fold dilution of the physiologic serum concentration of C1 [13] in our preparation we have approx. 2 pmol of C1 per assay tube. Thus we have 40 fmol and 80 fmol of C1 bound to fluid and solid liposomes, respectively. There is sufficient liposomal surface area to accommodate more than 2000 fmol of C1. This is based on a 500 Å diameter for C1, 60 Å^2 surface area per lipid molecule, and the fact that only 5% of the total liposomal lipid is exposed to the extracellular aqueous compartment [14]. In addition, from our protein determinations, on liposome-bound antibody, there are 760 fmol and 1500 fmol of antibody bound to the fluid and solid liposomes, respectively. These

calculations demonstrate that there is available space and antibody molecules to bind significantly more C1 than was actually determined to be bound in our assays. This lends additional support to the data which indicate that, under these conditions, the C1 binding is indeed an equilibrium measurement. The present study therefore shows that it is the actual rate of activation of C1 once bound to the liposome surface that is altered by changes in liposome fluidity. Thus the activation rate of C1 is determined by some quality factor for the IgG antigen complex to which it is bound. In this case the quality factor almost certainly involves motional freedom of the antibodies on the liposome surface. Our results are totally consistent with those of Füst et al. [15] who show that there is a complete lack of correlation between binding and activation of C1 for a variety of immune activators. Our study extends this observation to show that the lack of correlation between binding and activation of C1 can be demonstrated using the same antibody molecules attached to physically dissimilar but chemically identical haptens.

In summary we have shown that the difference in antibody-dependent activation of complement by fluid and solid haptenated liposomes is due to a difference in the rate of the conversion of C1 to $\overline{C1}$ once bound to the liposome surface. This difference in the internal conversion rate of C1, due only to a difference in the physical state of the liposomal antigen, makes this an ideal system in which to study the mechanism of activation of C1.

Acknowledgments

This work was supported by grant AI-16810 from the National Institutes of Health. We thank Dr. D. Lyles for helpful discussion in preparing this manuscript.

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