Lateral hapten mobility and immunochemistry of model membranes
(complement fixation/spin-label hapten/membrane fluidity)

PHILIPPE BRULLET* AND HARDEN M. McCONNELL†
Stauffer Laboratories for Physical Chemistry, Stanford University, Stanford, California 94305

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ABSTRACT  A study has been made of the interaction of specific anti-nitroxide (anti-spin label) IgG antibodies, Fab fragments, complement, and liposomes containing dimyristoylphosphatidylcholine, dipalmitoylphosphatidylycholine, and dipalmitoylphosphatidylethanolamine plus 0–50 mole % cholesterol, together with various concentrations of a head group spin-labeled phospholipid (0.5–0.01 mole % in the plane of the membrane). The spin-labeled lipid is the amide formed from the reaction of an iodoacetamide spin label, N(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide, with dipalmitoylphosphatidylethanolamine. We have reached two conclusions: (i) complement fixation depends on the lateral mobility of the lipid hapten at low hapten concentrations in the plane of the membrane (≤0.1 mole %) and does not depend strongly on this mobility at high hapten concentrations; (ii) cholesterol may have two distinct effects on complement fixation, one arising from an enhancement of hapten exposure to antibody binding sites and a second due to a modulation of membrane fluidity.

There is much evidence to suggest that the lateral motions and distributions of membrane components may play significant roles in the biological functions of cell membranes (1–6). Perhaps the most interesting question is the extent to which such motions and distributions are involved in cell surface recognition. With these broad problems in mind, we have undertaken a study of the immunochemistry of model membranes (e.g., liposomes) in which the structure, motion, distribution, and concentration of membrane hapten can be established by physical and chemical methods (7, 8). Our studies take advantage of the discovery by Kinsky and colleagues that hapten-sensitized liposomes can be damaged by specific antibodies and complement (9, 10). The present work provides strong evidence that the fixation of complement by liposomes containing lipid hapten spin labels and specific antibodies to spin labels depends on the lateral mobility of the hapten when the hapten concentration in the plane of the membrane is low. The present work also clarifies and extends our previous report (11) of the enhancement of complement fixation by the inclusion of cholesterol in liposomal membranes. The inclusion of cholesterol enhances antibody binding, probably by increasing hapten exposure at the membrane surface, in addition to modulating membrane fluidity.

MATERIALS AND METHODS

Spin-Label Lipid Hapten. The spin-label lipid hapten I was the reaction product of an iodoacetamide spin label, N(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide, and L-α-dipalmitoylphosphatidylethanolamine.

Six hundred milligrams of the iodoacetamide spin label (a gift of Dr. B. J. Gaffney) were dissolved in 10 ml of CHCl₃:MeOH (1:1, vol:vol). One milliliter of this solution was added to 100 mg of dipalmitoylphosphatidylethanolamine dissolved in CHCl₃:MeOH (1:1). Forty microliters of distilled triethylamine were added to the mixture and the reaction was allowed to proceed for 72 hr at room temperature. The mixture was then dried under reduced pressure and showed four spots on silica gel thin-layer developed in CHCl₃:MeOH:H₂O 65:25:4. Purification was achieved on a silica gel plate chromatograph 1 mm thick (Analtech). When CHCl₃:MeOH:H₂O 65:25:4 was used as solvent, two bands; in addition to the front, were visible under ultraviolet light and were isolated; each was extracted twice with 200 ml of CHCl₃:MeOH (1:1), dried under reduced pressure, redissolved in EtOH, dried as before, and stored in EOH. Paramagnetic resonance spectra of the fastest running band showed the characteristic fine lines of a nitroxide biradical. The phosphate content gave 16 μmol by the method of McClure (12). The phosphate content of the nitroxide I (the slower band) gave 32.5 μmol. The paramagnetic resonance assay gave 31 μmol, in excellent agreement.
Anti-Nitroxide Specific IgG and Fab. New Zealand rabbits were immunized as described before (7, 8). The sera obtained were assayed qualitatively by 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo) binding (see below).

In a typical experiment, 50 ml of heat-inactivated serum, pooled from three successive bleedings, were passed through an affinity column. The affinity column matrix was prepared, in collaboration with Dr. P. Rey, from the reaction product of 4-amino-tetramethylpiperidine-1-oxyl and activated-CH Sepharose 4B. The bed, 5 ml, was washed with phosphate-buffered saline (PBS) until the effluent absorbance at 280 nm was less than 0.02. The specific antibodies were eluted with 20 ml of a 20 mM \( N,N \)-dimethyl-\( N-(2',2',6',6'-\)tetramethyl-4'-piperidinyl-1'-oxyl)-2-hydroxyethylammonium (Tempo-choline) chloride solution. The fraction eluted by Tempo-choline was concentrated by vacuum dialysis prior to being passed through a Sepharose 6B column (5 cm \( \times \) 30 cm) and eluted with PBS. This column separates IgM from IgG and removes unbound Tempo-choline chloride. The IgG fraction was concentrated by vacuum dialysis and dialyzed against 6 liters of PBS for 10 days at room temperature. Specific IgG (14.57 mg) were recovered by this procedure. Fifty microliters of a working solution of 1.94 mg of IgG per ml reduces the Tempo signal intensity of the binding assay by 80% (see below).

Further elution with 20 ml of 0.1 M acetic acid, neutralized with 1 M NaOH, yields an additional 2.3 mg of proteins with binding activity, after filtration and centrifugation to remove denatured proteins. This fraction was not mixed with the one eluted with the small hapten and not used in the experiments reported here. In other experiments, immunoglobulins were separated by fractionation with \( (NH_4)_2SO_4 \) and the IgG fraction separated on a Sepharose 6B column was passed through the affinity column. Similar yields were obtained by the two methods.

Specific Fab fragments were prepared by the method of Porter (13). Specific IgG (5.82 mg) was digested with 1% of mercuripapain in 2.6 ml of 0.1 M sodium phosphate buffer at pH 7.5, containing 4 mM EDTA, for 20 hr at 37\(^\circ\). The products of the digestion, after dialysis against PBS, were separated on a Sephadex G-150 column (2 cm \( \times \) 32 cm). The Fab fraction was concentrated by vacuum dialysis and further dialyzed against PBS. No attempt was made to separate the Fab from the Fc fragments. A total of 4.01 mg of protein was recovered. Fifty microliters of a working solution of 1.6 mg/ml reduced the Tempo signal intensity in the binding assay by 79% (see below).

Preparation of Liposomes. The desired amounts of lipids and hapten spin label were mixed in organic solvent and the solvent was removed by evaporation under vacuum. Buffer was added [PBS, except for the studies with complement, where we used Veronal-buffered saline (14)]. The hydration was carried out in a water-equilibrated chamber at 60\(^\circ\) and the flask was gently shaken. The liposomes were centrifuged at low speed (3000 X \( g \)), the supernatant was removed, and the pellet was resuspended in buffer. Liposomes were examined under a light microscope and showed a broad distribution of size and shape from 1 \( \mu m \) to several tens of \( \mu m \).

Antibody Binding Assay. Antibodies, lipid membranes, and other reagents were mixed on ice in a small test tube, shaken, and incubated at the required temperature at a final volume of 100 \( \mu l \). The stock liposome suspension had a concentration of 40 \( \mu mol \) of lipids per ml of PBS.

The binding of antibody to a membrane was assayed by evaluation of the antibodies unbound to the membrane. Prior to the experiment the stock solution of specific IgG (1.94 mg/ml) was assayed with a small hapten. For this purpose \( x \) \( \mu l \) of IgG (\( x = 0-90 \)) was added to 10 \( \mu l \) of a 50 \( \mu M \) solution of Tempo or Tempo-choline chloride in PBS. The volume was made up to 100 \( \mu l \) and the intensity of the low-field peak was recorded. The binding was always complete within the dead time of the experiment. The plot of spectral intensity versus the amount of antibody provided a standard for titration of unbound antibodies.

The assay consists of incubating \( x \) \( \mu l \) of antibodies with \( y \) \( \mu l \) of lipids, increasing the volume to 90 \( \mu l \), and incubating, usually at 32\(^\circ\), for 30 min. The membranes were centrifuged at 3000 \( X \) \( g \) for 10 min and the supernatant was carefully removed. Ten microliters of 50 \( \mu M \) Tempo solution was added to each supernatant and unbound antibody was assayed as above.

The controls involve membrane without hapten and membrane with hapten and nonspecific IgG. When 20 \( \mu l \) of stock suspension of lipid were used, 15–20% of IgG were found absorbed on the surface in a nonspecific manner. Direct inspection of the supernatant after centrifugation, with or without antibodies, showed the presence of small vesicles. The intensity was usually small enough as to be negligible. Due to the volume of the pellet and the various manipulations, the signal of free Tempo, after centrifugation, and without antibody, was higher than the signal of the same quantity of Tempo in exactly 100 \( \mu l \).

Complement Fixation Assay. The procedure for the complement fixation studies has been described previously (8). Some experiments were made with fresh guinea pig serum. In others the complement was purchased from Difco or from Miles. Again, controls involved mixture of lipids without hapten, or with hapten-lipids but nonspecific IgG.

Paramagnetic Resonance Spectra. The paramagnetic resonance spectra of I in all the liposomes used in the present work showed no evidence of spin exchange and dipolar broadening due to clustering. This is a critical point, since the resonance spectra of other amphiphilic labels show temperature-, concentration-, and lipid-composition-dependent clustering (P. Rey and H. M. McConnell, to be published).

RESULTS

Specific IgG anti-nitroxide antibodies prepared by affinity chromatography as described in Materials and Methods have rather low affinities for monovalent nitroxide hapten in aqueous solution, \( K \approx 10^5 \) liters/mole. These low affinities are due to the fact that the higher affinity antibodies are retained on the affinity column.

Antibody Binding. Fig. 1 summarizes the results of our study of the binding of specific IgG antibodies and Fab fragments to 0.5 mole % I in dipalmitoylphosphatidylcholine (DPPC)-cholesterol membranes containing 0-50 mole % cholesterol. These data show a strong dependence of IgG and Fab binding on cholesterol concentration, the binding increasing significantly for cholesterol concentrations above 20–33 mole %. The relative binding of IgG and Fab to the liposomal membranes are compared in Fig. 1, as follows. When the concentration of \( binding sites \) in an IgG solution is equal to the concentration of \( binding sites \) in a Fab solution, and each of these solutions is separately allowed to interact with a given hapten-sensitized liposomal preparation, the loss of Tempo-binding capacity in the solution after removal of liposomes by centrifugation is the same for Fab and IgG. Since the number of exposed hapten in these liposomal systems is not known, these binding data alone do not permit us to say whether or not all externally ex-
rigid (DPPC) membrane. This can be seen by comparing the curves (i.e., in the range at which $\Delta A_{113}$ is linear with respect to complement supplied (15)) at a fixed degree of complement depletion, i.e., that corresponding to $\Delta A_{113} = 0.25$. In the case of the fluid DMPC liposomes the same degree of complement depletion is seen when [c] is decreased by a factor of five (from $2.5 \times 10^{-3}$ to $0.5 \times 10^{-3}$) and the IgG concentration is increased by a factor of about 4. On the other hand, in the rigid membrane, complement depletion corresponding to $\Delta A_{113} = 0.25$ is the same when [c] is decreased by a factor of 2.5 (from $2.5 \times 10^{-3}$ to $1 \times 10^{-3}$) and IgG is increased by a factor of approximately 6.7. Thus, IgG is much more effective for complement fixation in a fluid membrane as the hapten concentration is decreased.

**DISCUSSION**

A major objective of the present work has been to show that the lateral mobility of membrane haptens is important for complement fixation by hapten-sensitized liposomes and specific IgG when the hapten concentration in the plane of the membrane is low. This objective stemmed from a desire to demonstrate a membrane-surface recognition event that depended on the lateral mobility of membrane components. We were encouraged in these experiments by many observations on intact cells that suggested the possibility of a lateral-motion-, or distribution-function relationship (1–5, 16).

It will be seen from the data in Fig. 2 that complement fixation falls off rapidly in a rigid membrane (DPPC at 32°, below its chain-melting transition temperature, 42°, where the rate of lateral diffusion is presumably very low).

We may make a rough, order-of-magnitude estimate of the critical concentration $[c]^*$ for a rigid membrane, as follows. We assume that for complement binding (C1 activation), two IgG molecules must be bound to the membrane "next to" one another (16) and that the binding sites of both IgG molecules must be occupied by hapten. If two membrane haptens separated by a distance between $R$ and $R + \Delta R$ are effective in divalent binding of IgG, then the fraction of membrane haptens having this spacing is roughly $2\pi R\Delta R [c]/A$ where $A \approx 60 \text{ Å}^2$ is the area of a lipid molecule. The critical concentration $[c]^*$ then corresponds to the concentration [c] where $2\pi R\Delta R [c]/A$ becomes less than one, i.e., $[c]^* \sim A/(2\pi R\Delta R)$. For a typical separation of IgG binding sites $R \sim 50 \text{ Å}$, and a rough estimate of $\Delta R \sim 30 \text{ Å}$, we obtain $[c]^* \sim 10^{-3}$. Note that $\Delta R$ must be restricted by the segmental flexibility of the IgG molecule, as well as by the orientation of the active sites within the molecule. Appropriate values of $\Delta R$ may depend on the chain length separating the hapten from the plane of the membrane. The apposition of adjacent IgG molecules in a rigid membrane is also expected to become highly probable at this same value of $[c]^*$ when the IgG molecules are in excess.

The origin of the strong dependence of specific IgG and Fab binding on cholesterol concentration is not known. The cholesterol dependence of the binding parallels changes in other physical properties of binary mixtures of phosphatidylcholines and cholesterol (ref. 17, and further references therein). The enhanced binding at higher cholesterol concentrations might be merely due to enhanced membrane surface area. However, there are a number of reasons to believe that this cholesterol dependence of IgG and Fab binding to the membrane is due to a local, molecular effect that enhances the exposure of hapten to antibody at the molecular level. (i) Studies similar to those in Fig. 1 using other spin-label haptenes in which the chain length separating the nitroxide group and the phos-
Fig. 2. Complement fixation by liposomes as a function of hapten concentration in the plane of the membrane, lipid composition ("fluidity"), and antibody concentration. The 1:1 specific IgG concentration is 0.9 μM, the total lipid concentration is 17 μM, and the numbers by each curve give the hapten-lipid ratio, [c], × 10^2. Maximum lysis with the amount of complement used yields an absorbance at 413 nm of 0.570; total lysis yields an absorbance of 0.61–0.62. Experiments were at 32°C.

A: In Experiment A the host lipid is DMPC (a "fluid" membrane), B: in Experiment B the host lipid is DPPC (a "rigid" membrane), and C: in Experiment C the lipid is a 50:50 mole ratio of DPPC to cholesterol.

Phosphodiester linkage is varied show a strong dependence on chain length (P. Brület and H. M. McConnell, to be published) analogous to that observed earlier by Six et al. (10). (ii) Spin-label paramagnetic resonance data, as well as 13C nuclear resonance data, show that increasing cholesterol concentration in phosphatidylycholine bilayers has a marked effect of enhancing molecular motion in the polar head group region (P. Brület and H. M. McConnell, to be published). This evidence, taken together, indicates to us that cholesterol enhances the accessibility of "short" lipid haptens to antibodies. [This may well contribute to the reported marked enhancement of complement fixation by anti-cardiolipin antibodies by cholesterol in phosphatidylycholine membranes containing about 3 mole % of the lipid hapten cardiolipin (11).] In view of the above discussion, one must clearly consider the possibility that cholesterol has at least two effects on complement fixation in liposomal membranes,
one related to enhancement of hapten exposure, and one due to the effect of cholesterol on membrane fluidity, particularly in membranes where the hapten concentration is low. It is not possible to compare directly this IgG and Fab binding data, and the complement fixation data, for the specific IgG antibodies used in the present work. This follows from the facts that the IgG and Fab binding to the liposomal membranes are essentially equal to one another, whereas Fab has no inhibitory effect on complement fixation by IgG under the conditions of our experiments. Also, the reader will note that IgG and hapten concentrations effective for complement fixation in Fig. 2 are significantly lower than those given for the binding data. A sub-population of high-affinity antibodies may be responsible for much of the complement fixation reported in Fig. 2.

The mobility-dependent complement fixation is most pronounced for hapten concentrations of the order of or less than 0.1 mole%. This corresponds to a two-dimensional hapten concentration of the order of 50,000 to 100,000 Å² per hapten. A number of monovalent haptens are present in cell membranes at concentrations of this order, for example, the Rh antigens in erythrocytes (18). It can be anticipated that factors that affect the lateral mobility of such antigens will be reflected in a change in the susceptibility of these cells to complement-mediated lysis.

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