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Immunology

Membrane-controlled depletion of complement activity by spin-label-specific IgM

(hapten spacing/cholesterol/membrane fluidity/electron paramagnetic resonance)

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ABSTRACT **Complement depletion mediated by high molecular weight (IgM) rabbit antibodies specifically bound to spin-label lipid haptens dispersed in model membranes is controlled by various physical attributes of those membranes other than the total number of exposed determinants that they provide. Carrier lipids used at 32° were (*i*) a "fluid" phosphatidylcholine (PC), (*ii*) a "solid" PC, and (*iii*) a cholesterol/PC mixture. The concentration of hapten in the plane of the membranes (two-dimensional concentration) was varied while the overall hapten molarity (three-dimensional concentration) was kept constant.**

Both specific binding and the efficiency of depletion by IgM are markedly enhanced by systematically decreasing the average distance between haptens ($\infty \rightarrow 26$ Å). Heterogeneous distribution was found to be more favorable than a random homogeneous distribution of the same number of haptens in the same total quantity of lipids. IgM efficiency is also markedly increased by the inclusion of cholesterol in PC membranes, an effect thought to result from enhanced projection of the determinant from the surface of the membrane and hence increased accessibility to the antibody-binding site. Furthermore, the efficiency of IgM was increased by using haptens dispersed in fluid rather than in solid PC membranes.

The results are consistent with the hypothesis that IgM molecules must be bound to a critical multiple of antigenic determinants at a membrane surface in order to induce complement-mediated attack and that subtle variation of the physical state of membrane antigens can be the crucial factor in determining the outcome of this type of efferent immune response.

The relative cytotoxic efficiencies of different effector components of the immune system against the same target membrane can differ by several orders of magnitude. For example, erythrocyte cytolysis by antibody-dependent monocytes is typically about 10^3 times more efficient per IgG antibody molecule than is cytolysis of the same cells with the same antibody in the presence of complement (C) (1, 2). Likewise, specific antierythrocyte antibodies of the IgM class are about 10^3 times more efficient in C-mediated lysis than are specific IgG antibodies (3, 4). On the other hand, efficiencies of the same effector components against different target membranes can differ widely (5–10). In attempting to elucidate mechanistic differences in molecular terms, we have turned to the use of model membranes (liposomes or vesicles) to serve as targets for these various components of the immune system (7–12). Because the physical and chemical properties of such target membranes can be varied, we can hope to discern which special characteristics of an immune response are directly related to properties of components of the immune system and which characteristics are related to properties of the target membrane.

In recent work, we have shown that the capacity of the target membrane to induce IgG-mediated complement depletion depends on the lateral mobility of spin-label haptens in the plane of the membrane (7, 8). Our results thus far are consistent with the classical studies of Borsos and Rapp (13), Humphrey and Dourmashkin (3), Cohen (14), and Hyslop *et al.* (15), in that pairs (or higher multiples) of hapten-bound (or antigen-bound) IgG appear necessary for C1 activation; however, we have updated these early results to take into account the significant fact that many antigenic determinants of some cells (but not the erythrocytes on which the early work was based) have a high lateral mobility, and this in turn modifies the immune effector function.

The purpose of the present paper is to describe studies of C fixation with a spin-label hapten and specific IgM antibodies. As will be noted later, a number of the results described here are similar to or corroborate those reported earlier by other investigators using other haptens/antigens. However, the use of spin-label haptens for the studies in question has certain important advantages for defining the physical state of the haptens and the target membrane (7, 16), which is absolutely essential in our approach to these problems.

MATERIALS AND METHODS

Preparation of Liposomes. Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and cholesterol (chol) were as used before (9). The spin-label lipid hapten (SLH II) was a product of the reaction between dipalmitoylphosphatidylethanolamine and *N*-(1-oxyl-2,2,6,6-tetramethylpiperidinyl)-4-iodoacetamide (TEMPO-iodoacetamide) prepared as described (8). When a series of liposome preparations having the same quantity of hapten per unit volume of suspension (i.e., overall molarity) but differing in the hapten density in the plane of the membranes was required, it was prepared by first drying down, as before (9), equal total weights of lipids of the required composition, suspending these in equal volumes of Veronal-buffered saline (VBS) (17), and finally diluting them to the appropriate extent with VBS. Liposomes were prepared from SLH II together with (*i*) DMPC, (*ii*) DPPC, and (*iii*) 50 mol% chol plus $50 - x$ mol% DPPC (in which x = mol% SLH II).

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Abbreviations: C, complement; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; chol, cholesterol; SLH II, spin-label lipid hapten; TEMPO, *N*-(1-oxyl-2,2,6,6-tetramethylpiperidinyl; VBS, Veronal-buffered saline; EPR, electron paramagnetic resonance; ASCF activity, antigen-specific complement-fixing activity; PC, phosphatidylcholine.

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We made the assumption that the fraction of SLH II available at the surface of the liposomes varied little or not at all for the range of SLH II concentrations used (i.e., 0–10 mol% SLH II with respect to total lipid).

C-Fixation Assay and Determination of C-Fixing Efficiency of IgM. C was pooled fresh guinea pig serum absorbed with sheep cells as described by Kabat and Mayer (17) and stored in liquid N_2. The C-fixation assay was that of Wasserman and Levine (18), with minor modifications, and has been described before (10). The fixation step was invariably for 2 hr at 32°, a temperature intermediate between the principal gel-to-liquid crystalline phase transition temperatures of DPPC (41°) and DMPC (23°). Liposome preparations neither depleted C activity in the absence of antibodies specific for the hapten nor destabilized indicator cells.

By varying the amount of IgM (see next section) supplied to fixed amounts of liposome preparations, we have graphically determined the weight, in micrograms, of IgM required to give 50% of the maximum inhibition of indicator-cell lysis ($\Delta A_{413\,nm}$). The reciprocal of this value is proportional to C activity fixed per molecule of IgM and has, therefore, been termed the "C-fixing efficiency" of the IgM.

Antibody Preparation and Characterization. Polymerized flagellin was prepared from *Salmonella adelaide* (a gift of L. Valdivig, P. Howell, and B. Stocker) by the method of Ada *et al.* (19). To 14 ml of an aqueous solution of the protein (0.3 mg/ml, pH 8.5) was added 1 ml of ethanolic 0.1 M TEMPO-iodoacetamide. The mixture was stirred at room temperature for 19 hr at pH 8.5. After subsequent precipitation of the protein with cold saturated ammonium sulfate solution, the mixture was dissolved in 3 ml of distilled water and dialyzed extensively, first against distilled water and finally against 0.145 M NaCl/0.01 M $NaPO_4$, pH 7.3. Computer integration of the electron paramagnetic resonance (EPR) spectrum generated by the product and determination of the protein concentration by the method of Lowry *et al.* (20) indicated that the label was bound at a ratio of approximately 1 per 100,000 daltons; similar low levels of hapten substitution have been found to be effective for induction of hapten-specific IgM by dinitrophenylated polymerized flagellin (21).

New Zealand White rabbits were each injected subcutaneously with 200 μg of spin-labeled flagellin in Freund's complete adjuvant and bled 5, 7, 9, and 11 days later. Sera were heated at 56° for 30 min and precipitated three times with 33% saturated ammonium sulfate. The final precipitates, derived from about 70 ml of serum, were redissolved in the minimum volume of VBS, pooled, and separated (in batches) by passage over Sepharose 6B with VBS as the solvent.

This procedure has been used with many different sera raised against antigens such as galactocerebroside, human erythrocyte stroma, and spin-labeled hemocyanin (7–12). Invariably, three major protein fractions (I, II, and III) are obtained. Fraction I, which elutes at the void volume, is frequently slightly opaque and, whereas addition of antigen may demonstrate some antigen-specific C-fixing (ASCF) activity, it has always been found to deplete C activity appreciably by itself. Although no systematic study has been made to determine the nature of fraction I, we assume that it includes immune complexes and otherwise aggregated immunoglobulins and routinely discard it. The bulk of the ASCF activity has always been found in one or both of the other fractions.

Fraction II elutes at a volume that corresponds to 600,000 daltons as determined by reference to the dextran-calibrated curve supplied by Pharmacia (22) or 1,500,000 daltons as determined by reference to their protein-calibrated curve. Fraction II contains much of the ASCF activity of sera raised against galactocerebroside, human erythrocyte stroma, or spin-labeled polymerized flagellin but contains negligible activity of late sera to spin-labeled hemocyanin. Immunodiffusion of fraction II versus goat anti-rabbit-μ-chain and goat anti-rabbit-IgG gives results characteristic of IgM (tests kindly performed by G. M. Iverson with fraction II of a serum raised against stroma).

Fraction III elutes at a volume closely corresponding to 150,000 daltons and contains essentially all of the ASCF activity in sera from rabbits immunized with spin-labeled hemocyanin; it has been used to prepare, characterize, and study spin-label-specific IgG (7–12).

Central portions of fraction II from the various immunoglobulin batches were pooled and passed over a small column of Sepharose 4B (10-ml bed volume) to which a low concentration of spin label, 0.05 μmol/ml, had been bound. (The column material was the gift of J. T. Lewis.) The column was washed with 0.2 M NaCl/0.01 M $NaPO_4$, pH 7.3, until 50 ml of eluate contained no detectable protein. About 60% of the protein, including all detectable ASCF activity, remained on the column. About 70% of this activity was removed by 30 ml of 3 M KSCN/0.1 M $NaPO_4$, pH 6.0. The first 5 ml of eluate, being devoid of protein, was discarded and the next 25 ml was immediately placed in a dialysis bag and concentrated, in 2 hr, to about 8 ml by use of Aquacide II (Calbiochem). Parallel studies have shown that the pH of buffered KSCN solution remains constant during this procedure but that the concentration of SCN^- inside the dialysis bag, as determined by titration with Ag^+, drops to between 2.5 and 2.7 M. It is highly unlikely that much TEMPO-specific IgG was included with the protein because of the cautious manner in which fraction II was selected and the low ASCF activity of IgG in the original sera (i.e., about 10% of that in the IgM fraction on an equal weight basis). At pH 6.0 and at the concentration of SCN^- used, aggregation of IgG is not observed (23), and even aggregation that can be observed at 4.5 M SCN^- is reversible on removal of that ion. Concentration of the protein was immediately followed by its passage over a Sephadex G-25 column to remove KSCN. It was then precipitated with ammonium sulfate, redissolved in the minimum volume of VBS, and refractionated on a Sepharose 6B column equilibrated and eluted with VBS. The elution profile is shown in Fig. 1. Approximately 13 mg of protein was retained from the central portion of the main peak and used as the standard IgM preparation for the experiments which will be described (except where stated otherwise). This represented 35% of the original fraction II material, and the ASCF activity per milligram of protein had been almost doubled.

After affinity column purification, the average intrinsic affinity of the IgM preparation for monovalent haptens [as judged by EPR analysis (11, 12)] was $<3 \times 10^4$ liters/mole, indicating that many molecules having extremely low intrinsic affinities for TEMPO were bound to the column. Furthermore, adsorption of the preparation with liposomes containing 10 mol% SLH II, 40 mol% DPPC, and 50 mol% chol removed only about 7% of the protein but virtually all of the ASCF activity, as tested by using other liposomes (of the same composition) as the antigen (see Fig. 2); some of the protein binding is likely to be nonspecific, and so the value given represents the maximum quantity of antibody effective for C fixation with liposomes of the highest hapten density used.

Immunodiffusion in 0.6% agarose versus goat anti-rabbit-serum gave a single precipitin line close to the IgM well, easily distinguishable from the single precipitin line situated midway

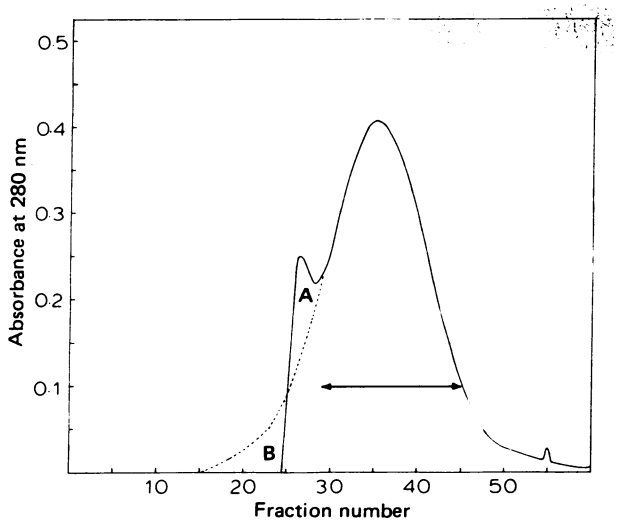

FIG. 1. Profile of the final elution of the principal IgM preparation from a Sepharose 6B column is shown by the solid line; the dotted line is the mirror image of the descending limb of the main peak. Almost all the protein eluted as a single peak whose leading edge was intersected by the void volume, fraction number 25; note that the areas A and B are approximately equal. About 0.2% of the protein eluted at fraction 55, the volume characteristic of IgG. Fractions were 2.7 ml each. The double-headed arrow encompasses those fractions that were retained and used for the experiments described.

between the IgG and antiserum wells in the same dish. IgG and IgM were both supplied at 0.34 mg/ml.

In parallel studies with IgG and IgM specific for TEMPO or human erythrocyte stroma, equal depletion of ASCF activity by 0.03 M ethanethiol (24) was observed with the two IgMs but none with either IgG.

C fixation by hapten-bearing liposomes together with a constant weight of IgG antibodies can readily by inhibited by the presence of too many or too few liposomes (7, 10). This can be understood theoretically on the basis of the requirement for two or more IgG molecules in close proximity on the surface of a membrane to trigger C1 activation (13). C fixation by IgM, on the other hand, should not be so readily inhibited by increased particulate antigen (with a concomitant increase in surface area) because single antibody molecules, suitably bound, are effective (25). For the system under investigation, $\Delta A_{413\ nm}$ was found to vary only between 30% and 50% of the maximum value when a certain fixed quantity of IgM was mixed with five consecutive serial dilutions of liposomes.

A low level of non-ASCF activity was observed with the IgM preparation alone. On the basis of protein weight, it was at a level about 3% of that reported by Brown and Koshland (26) using an almost identical assay system.

Adsorption of IgM with Liposomes. Liposomes prepared in the usual way were washed three times with VBS, with light centrifugation between washes to remove small particles. IgM was incubated for 2 hr at 32° with liposomes at a ratio of 20 μmol of total lipid per milligram of protein. After removal of the liposomes by centrifugation, the supernatants were assayed for protein by the Lowry *et al.* (20) method and for C-fixing efficiency in the usual way. All liposomes used in this type of experiment contained 50 mol% chol together with DPPC and SLH II.

Liposomes containing high concentrations of SLH II (e.g., 10 mol%) were visibly agglutinated during the adsorption procedure.

In one experiment, adsorption of C-fixing activity was tested at intervals over a period of 3 hr and found to be at the characteristic level by 30 min. or less.

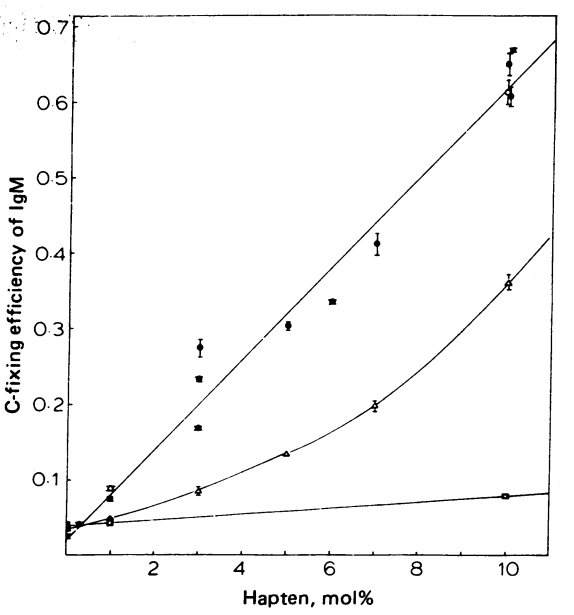

FIG. 2. The C-fixing efficiency of IgM as a function of spin-label hapten (SLH II) concentration in the plane of liposomal membranes; *the (three-dimensional) overall molarity of hapten was constant* (0.5 μM). Serial dilutions of IgM were used to determine the weight, in micrograms, required to give the 50% endpoint of C depletion; C-fixing efficiency of IgM is numerically equal to the reciprocal of this value. The data shown were obtained by using the principal IgM preparation without prior absorption (●), or after absorption with liposomes containing 0 mol% (○), 1 mol% (△), or 10 mol% (□) SLH II. Removal of 4, 10, and 7% of the protein accompanied adsorption with liposomes containing 0, 1, and 10 mol% SLH II, respectively, a result probably reflecting the erratic nature of nonspecific adsorption with liposomes. See *text* for further details.

RESULTS

Effect of Hapten Density in the Plane of the Membrane. As illustrated by Fig. 2, the efficiency of C fixation by the unadsorbed IgM preparation increased approximately linearly with hapten density in chol–DPPC membranes, even though the number of antigenic determinants was kept constant; an increase in efficiency with hapten density was also observed when DMPC or DPPC alone was used as the carrier lipid.

The EPR spectra of liposome preparations indicate that SLH II does not form a separate phase at any concentration up to 10 mol% when included in phosphatidylcholine (PC) or in PC together with 50 mol% chol (see Fig. 3 and ref. 16), and so we conclude that hapten distribution is random within the plane of the PC or PC–chol bilayers for all preparations studied.

Effect of Carrier Lipid Fluidity on C Fixation by IgM. The C-fixing efficiency of IgM, as determined at 32° with 3, 6, and 10 mol% SLH II, was greater when "fluid" DMPC rather than "solid" DPPC was used as the carrier lipid—e.g., with 10 mol% SLH II, the efficiency is approximately doubled by using DMPC rather than DPPC.

Effect of Cholesterol Concentration. The efficiency of IgM in the presence of liposomes containing 50 mol% chol was far higher than that with liposomes containing SLH II and either DMPC or DPPC alone. This has been observed for all SLH II concentrations studied and may be illustrated by citing the example that for 10 mol% hapten dispersed in (*i*) DMPC, (*ii*) DPPC, and (*iii*) 50 mol% chol plus DPPC, efficiencies were approximately (*i*) 0.06, (*ii*) 0.04, and (*iii*) 0.65.

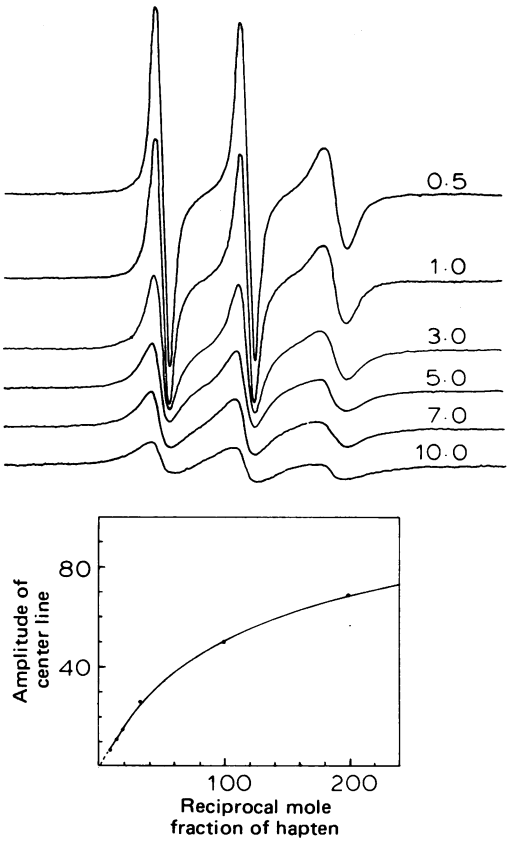

FIG. 3. Spectral consequence of decreasing the spacing of SLH II in DPPC membranes with 50 mol% chol; EPR spectra of liposomes at 32° containing *equal amounts* of hapten at the various molar percentages (with respect to total lipid) are indicated. Also shown is a plot of the amplitude of the center line (in arbitrary units) versus the reciprocal of the mole fraction of hapten present in the bilayers. This latter parameter is proportional to the average area of membrane available to each hapten. The data indicate a random, monomolecular dispersion of this hapten in chol–DPPC bilayers of the chosen composition. Spectra were obtained with a Varian E12 spectrometer interfaced with a Digital PDP8/e computer. Samples were prepared as specified but such that all had the same *overall* concentration of nitroxide (i.e., total lipid per ml of liposomes suspension was varied). Even so, because of the heterogeneous nature of lipid suspensions, spectra were integrated and normalized to the same spin concentration and then plotted at these new normalized values in order to measure comparable spectral parameters.

If chol content was systematically varied in the range of 0–50 mol%, with SLH II at 3 mol% and DPPC as the other lipid, then for all chol concentrations the efficiency of IgM was increased over that observed in the absence of chol. Although, in general, there is a positive correlation between chol content and ability to induce C fixation, an anomalous increase in efficiency at concentrations around 30–40 mol% chol has been observed but the effect is erratic.

Effect of Heterogeneous versus Homogeneous Distribution of Haptens. If a fixed quantity of SLH II (e.g., 0.3 nmol) was presented in a fixed quantity of carrier lipids (e.g., 4 nmol of chol and DPPC) but in one case a single liposome type having 7 mol% SLH II was used and in another case an appropriate mixture of liposomes containing 5 and 10 mol% SLH II, then the observed C-fixing efficiency of the IgM was typical of that with liposomes containing 7 mol% hapten in the first case and of that with liposomes containing 10 mol% hapten in the second.

Effect of Adsorption of the IgM Preparation with Liposomes. Although some binding of IgM to liposomes lacking hapten could be detected by use of the Lowry *et al.* protein assay, the C-fixing efficiency of unbound IgM was not affected when calculated with respect to protein weight as described. However, adsorption with liposomes containing SLH II markedly decreased the efficiency of the principal IgM preparation. The effectiveness of liposomes used as adsorbents increased with hapten concentration. Furthermore, the percent decrease in efficiency was always greater when tested against lipsomes having a low hapten density than it was when tested against those having a higher hapten density (see Fig. 2).

Even when IgM was adsorbed with liposomes containing only 0.1 mol% SLH II, a level at which ASCF activity was scarcely detectable when such liposomes were used as antigens, a significant decrease in efficiency was observed—e.g., approximately 25% decrease when tested versus liposomes having 7 mol% SLH II. For this latter experiment, an IgM preparation from a single 9-day bleeding was used and, although the protein was fractionated twice on a Sepharose 6B column, it was not separated on the affinity column. Its behavior was the same as that of the principal preparation except that the measured efficiencies were all about 30% less because of the presence of more nonspecific IgM.

DISCUSSION

The results illustrated by Fig. 2 provide strong evidence that when the IgM molecules used in this study are specifically bound to multiple haptens at a membrane surface they are particularly effective in inducing C depletion; the data do not, however, prove that specific binding to more than one hapten is a necessary condition to induce C depletion. [For the case of haptenated proteins, it has been variously reported that a single IgM bound to a single hapten is (26) or is not (27) sufficient to induce C depletion.] Note that IgM-mediated depletion is still increasing strongly at 10 mol% hapten in the target membrane. The perimeter of a membrane-bound IgM is approximately 300 Å (28), enclosing an area of about 6700 Å^2. For the case of 10% SLH II, about 10 haptens would be included in this area. Subtle geometric factors may be involved in antibody binding that leads to C fixation. Increased lipid hapten content has been shown, by Six *et al.* (29), to increase the C-mediated marker molecule release from a fixed quantity of liposomes by a fixed quantity of IgM. The adsorption studies illustrated by Fig. 2 and described in the *Results* section suggest to us that, particularly at the lower hapten densities (0.1–1.0 mol%), specific IgM binding takes place that is relatively inefficient for C depletion—i.e., certain IgM that are capable of fixing C at high hapten densities are less likely to do so when specifically bound at lower hapten densities.

In the present study and in previous work (8, 10), we have found that inclusion of chol greatly increases the ability of spin-label lipid haptens dispersed in PC membranes to induce both IgG- and IgM-mediated C depletion. The inclusion of chol in such membranes results in changes in the EPR spectra of SLH II, indicating increased motion, which we interpret as due to enhanced projection of the nitroxide moiety from the surface of the membrane and hence increased accessibility to the antibody binding site (8, 10).

Physical chemical (J. Sheats and H. M. McConnell, unpublished data) and immunochemical (8, 10) studies are consistent with a very low rate of lateral molecular diffusion in the plane of PC membranes containing 50 mol% chol, the carrier/lipid mixture used for most of the experiments reported here. Erythrocytes, whose membranes have commonly been used as

targets for immune attack, are similar in that rates of lateral molecular diffusion are very low (30). When SLH II, initially randomly distributed in fluid DMPC membranes, was compared with SLH II, randomly distributed in solid DPPC, the former was found to be more effective than the latter for induction of IgM-mediated C depletion.

Our work to date with IgG and IgM directed against model membranes containing lipid haptens whose physical state is directly amenable to definition indicates that antibody-C-mediated attack can be critically controlled by subtle variation of physical factors pertaining to the membrane. We deduce that attack against a biological cell could, similarly, be controlled by such variation. The relative efficiencies of different effector immune systems may be strongly influenced by the target membranes; an example of this may be the case of IgM/IgG directed against natural erythrocyte membrane antigens compared to the case of IgG/IgM directed against passively sensitized erythrocytes (31, 32). The relative hemolytic inefficiency of IgM in the latter circumstance has been ascribed to "non-C-fixing IgM" but this conclusion might well be reexamined with the view that it is the antigenic structure that is critical in determining the outcome of such an attack. An equivalent conclusion for the case of "soluble" compared to "particulate" antigens was reached by Ishizaka *et al.* (33) and is further corroborated by the work of Cunniff and Stollar (27). The total number of appropriate antigenic determinants present at a cell surface need not be the crucial factor in determining susceptibility to the cytotoxic action of antibodies and C. Our results suggest that C activation is strongly favored only when a critical number of accessible determinants, x, is contained in a critical area of membrane, y, in which $2 \leq x <$ some upper limit appropriate for the particular system, and y is equal to the membrane area occupied by one bound IgM or a small group (≥ 2) of IgG (13, 27). Physical variations of the membrane that would favor C activation in such a model and that have been shown to do so in this or previous work with model membranes include (*i*) appropriate change (usually increase) in the rate of lateral diffusion of antigenic determinants, (*ii*) decrease in spacing of determinants (within certain limits), (*iii*) changes in their distribution (homogeneous ↔ heterogeneous) where this results in a more favorable spacing, and (*iv*) increased accessibility of determinants to antibody-binding sites. These observations are relevant to both IgG and IgM. We have noted a difference between these two antibodies, as used in model systems: the efficiency with which IgG activates C is more susceptible to diminution caused by using excessive amounts of membrane than is that observed by using IgM, a result that doubtless reflects the requirement for dimeric or larger multimeric associations of IgG for C activation.

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