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Antibody Interaction with a Membrane-Bound Fluorescent Ligand on Synthetic Lipid Vesicles†

Robert Luedtke‡ and Fred Karush*

ABSTRACT: The interaction of membrane-bound ligand with bivalent and monovalent fragments of monoclonal antibody was studied by fluorescence and precipitation analysis using synthetic lipid vesicles. The ligand N^{ϵ}-[5-(dimethylamino)-naphthyl-1-sulfonyl]lysine was linked to the hydrophobic anchor dipalmitoylphosphatidylethanolamine and ranged between 0.01 and 1 mol % of the membrane components. The effects of cholesterol on the specific interaction were observed over the range of 0-50 mol %. A precipitation assay was developed to evaluate various factors related to the cross-linking of small unilamellar vesicles by bivalent antibody. The cholesterol content was critical for this process as demonstrated by the increased efficiency of precipitation over the range of 0-40 mol % of this component. Fluorescence analysis yielded the parallel finding of increased accessibility of the ligand to the antibody with greater cholesterol content. Increased surface density of the ligand also was found to enhance the intervesicle interaction. Finally, a comparison of the kinetics by fluorescence analysis of the binding of monovalent and bivalent fragments indicated that the bivalent interaction involved primarily the cross-linking of vesicles in accord with published findings of the interaction of monoclonal antibody with cell membrane antigens.

Synthetic lipid vesicles (liposomes) provide model membranes for the analysis of antibody interactions at surface membranes. The defined composition of such membranes and their characterization with respect to the motion and distribution of the components of the membrane (Brûlet & McConnell, 1977) render feasible the effort to relate the quantitative aspects of antibody interaction to individual properties of the membrane. An early and useful demonstration of the utility of liposomal membranes for immunological study emerged from the finding that hapten-sensitized liposomes were damaged by interaction with specific antibody and complement (Kinsky, 1972; Six et al., 1973). This observation has been effectively exploited by McConnell and colleagues with liposomal membranes bearing a spin-label hapten reactive with specific antibody [e.g., see Parce et al. (1978)]. The present study was designed to compare bivalent and monovalent antibody fragments in bulk phase with respect to their interaction with a fluorescent ligand attached to a

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component of the liposomal membrane. The ligand N^{ϵ}-[5-(dimethylamino)naphthyl-1-sulfonyl]lysyl (Dns-Lys)[1] was linked to dipalmitoylphosphatidylethanolamine. Most of the experiments were done with monoclonal anti-Dns murine IgG1 antibody and fragments derived therefrom.

Two types of experimental techniques were employed to characterize the specific interactions. The first was based on the cross-linking of liposomes by bivalent antibody to form aggregates that could be readily sedimented. This method constitutes the liposomal equivalent of the traditional precipitin reaction used with multivalent antigens. The second technique was based on fluorescence methodology and served to provide detailed quantitative information about the formation of complexes between the antibody site and the specific ligand.

Materials and Methods

Antibody Preparation. The anti-Dns hybridoma cell line (44-26.10) used for this study was generously provided to us by Dr. L. Herzenberg and V. T. Oi from the Department of Genetics, Stanford University School of Medicine. The hybridoma cell line was derived from fusion with the NS-1 myeloma cell line. The hybrid anti-Dns hybridoma had lost the ability to secrete the NS-1 K chain. Ascites tumors were grown in BALB/c mice, and the pooled ascites fluid was precipitated twice with 50% saturated ammonium sulfate. After dialysis of the redissolved product vs. 0.01 M PO_4, 0.15 M NaCl, and 0.01% NaN_3, pH 7.2 (PBS), the sample was passed through a Sephadex G-200 column in PBS to select for the 7S component. This preparation was further chromatographed on a DEAE-cellulose column (Whatman) in 0.02 M Tris-HCl buffer at pH 8.0. A NaCl gradient was applied, in the same buffer system, with a range of 0.0–0.25 M NaCl. The major portion of the sample eluted from 0.05 to 0.07 M NaCl. This fraction was dialyzed vs. PBS and kept at 4 °C.

Ouchterlony immunodiffusion analysis with goat antisera specific for murine immunoglobulin isotypes (Bionetics) indicated that the major component was IgG1 (κ), although IgG2a and IgG2b were also present. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis in nonreducing gels indicated a single component which migrated identically with normal rabbit IgG (Pentex). In reducing gels, two components were observed which corresponded to rabbit IgG heavy and light chains.

Upon titration of this preparation of anti-Dns antibody with Dns-lysine, fluorescence enhancement of the Dns-lysine emission was observed as reported by Parker et al. (1967) for rabbit anti-Dns IgG. It was estimated that approximately 80% of the protein was specific for the Dns ligand by comparing the protein concentration obtained from OD_{283}, using $E^{1\%,1cm} = 14.0$, with the extrapolated value for antibody obtained by titrating Dns-lysine into protein solutions in PBS in which the Dns ligand was bound stoichiometrically.

$F(ab')_2$ fragments of the anti-Dns antibody were generated by pepsin digestion (Nisonoff et al., 1960). Briefly, 0.6 mL of 1.7 M sodium acetate buffer, pH 4.0, was added to 25 mg of IgG in 4.4 mL of 0.02 M Tris-HCl, pH 8.0. Porcine pepsin (0.5 mg) (3× crystallized, Calbiochem) was then added (2% pepsin by weight), and the digestion was carried out for 24 h at 35 °C. The sample was chromatographed on a Sephadex G-200 column in PBS to select for the $F(ab')_2$ component.

Fab′ anti-Dns was prepared by reduction of the $F(ab')_2$ product. A final concentration of 0.02 M DTT was used to reduce the $F(ab')_2$ preparation at a concentration of 2.5 mg/mL in 0.14 M Tris-HCl and 0.15 M NaCl solution, pH 8.0, for 1.0 h at room temperature. The sample was immediately alkylated with 0.05 M iodoacetamide for 1.0 h at room temperature. Both operations were performed under a nitrogen atmosphere. The Fab′ fragment was then selected by chromatography on Sephadex G-100 in PBS.

Rabbit anti-Dns IgG purification has been previously described by Luedtke et al. (1980). The anti-Dns was eluted from a Dns-BSA–Sepharose 4B column with 4.0 M urea and 1.0 M propionic acid. The IgG fraction was selected by chromatography on a Sephadex G-200 column in PBS.

Preparation of Lipid Vesicles. Lipid vesicles were prepared by using synthetic dimyristoylphosphatidylcholne (DMPC, Sigma), cholesterol (Δ^5-cholesten-3-ol, Sigma), [7(n)-^{3}H]-cholesterol in toluene at 5 Ci/mmol (Amersham), and *N*-(N^{ϵ}-Dns-lysyl)dipalmitoylphosphatidylethanolamine (DLPE). DLPE was synthesized by Dr. Dipali Sinha as previously described (Sinha & Karush, 1979). For the preparation of vesicles by sonication, a total of 0.01 mmol of lipid was dissolved in 1.0–2.0 mL of chloroform and the solvent evaporated under N_2. The lipid film was further dried in a desiccator with P_2O_5 at room temperature for at least 18 h. A total of 4.0 mL of PBS was added to the dried lipid, and the sample was vortexed for 5 min at the maximum setting. The sample was then sonicated, using a Model LS75 Branson ultrasonic sonifier equipped with a Model S75 probe at maximum settings, at room temperature for 3 min. The sample was cooled in an ice bath and resonicated for 3 min. The vesicles were then centrifuged at 12000*g* for 15 min to remove particulate material.

Lipid vesicles were also prepared by the solvent evaporation technique by using an apparatus described by Schieren et al. (1978). A total of 0.01 mmol of lipid was dissolved in 1–2 mL of chloroform. The solvent was evaporated by a stream of N_2 and the lipid film dissolved in a total of 20 mL of CS_2. This lipid solution was continuously added at a rate of 0.15 mL/min to 7.0 mL of PBS held in a condenser in which 65 °C water was circulated. This preparation was then centrifuged at 5000*g* for 15 min and the precipitate discarded.

For the preparation of both types of vesicles, the total concentration of lipid (DMPC and cholesterol) was determined by analytical weighing. Generally, a total of 0.01 mmol of lipid was used for each preparation. In addition, 10 μL of tritiated cholesterol (corresponding to 2×10^{-6} mmol of cholesterol) was added to standardize the preparation. The assumption was made that a decrease in radioactivity after centrifugation represented a decrease in the total amount of lipid, rather than a preferential loss of cholesterol. The concentration of DLPE was determined by using $\epsilon_{330} = 4.18 \times 10^3$ in a chloroform solvent on the basis of the aqueous extinction coefficient of 4.57×10^3 for *N*-Dns-lysine (Parker et al., 1967).

Precipitation of Vesicles with Anti-Dns Antibody. The general procedure followed for the precipitation of lipid vesicles with bivalent antibody was as follows. The antibody solution (100 μL) and 50 μL of the vesicle solution were mixed in a 1.5-mL polyethylene microfuge tube by vortexing. The samples were then incubated at room temperature for 1 h and at 4 °C for 1 h. After centrifugation in a Beckman microfuge B for 5 min, 50 μL of the solution was removed and added

[1] Abbreviations: Dns, 5-(dimethylamino)naphthalene-1-sulfonyl; Dns-Lys, N^{ϵ}-Dns-L-lysine; PBS, 0.01 M phosphate buffer, 0.15 M NaCl, and 0.01% NaN_3, pH 7.2; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; BSA, bovine serum albumin; DMPC, dimyristoylphosphatidylcholine; DLPE, *N*-(N^{ϵ}-Dns-lysyl)dipalmitoylphosphatidylethanolamine; Dnp, 2,4-dinitrophenyl; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

to 10 mL of scintillation fluid. The scintillation fluid used was either Scintisol (Isolab, Inc., Akron, OH) or a mixture of 10% Scintisol and 90% Formula 949 (New England Nuclear, Boston, MA). The kinetic analysis of precipitation of vesicles with bivalent antibody was performed by using 0.5 mL of the vesicle solution and 1.0 mL of the antibody solution. The two solutions were mixed and incubated at the appropriate temperature. Aliquots (100 μL) were removed in duplicate at the desired time points and centrifuged in the Beckman microfuge B. Fifty microliters of the supernate was then removed and counted for radioactivity in order to detemine the amount of vesicles which remained in solution.

Fluorescence Spectroscopic Analysis. Steady-state fluorescence analysis was performed by using a Perkin-Elmer Model 512 double-beam fluorescence spectrophotometer in the ratio mode with diffuse plates in the reference chamber. This arrangement permits the corrected excitation spectra and the uncorrected emission spectrum to be recorded. The specific interaction between the anti-Dns antibody and free Dns-lysine or DLPE associated with lipid vesicles was monitored by using an excitation wavelength of 280 nm and an emission wavelength of 480 nm. The excitation and emission slits were 10 and 20 nm, respectively. A glass filter was also placed between the sample and the emission window in order to minimize the detection of scattered excitation radiation. Aliquots of antibody solution were always titrated into a 0.5-mL solution of vesicles in PBS. Stirring was accomplished by repeated inversion of the cuvette, and the temperature was maintained at 25 ± 1.0 °C by a water bath which circulated thermostated water through the metal block which held the cuvette.

The observed relative fluorescence, F, is defined as

$$F = F_0(1 - x) + F_\infty(x) \quad (1)$$

where F_0 is the relative fluorescence of the unbound species, F_∞ is the relative fluorescence of the bound species, and x is the fraction of ligand bound. For the interaction between anti-Dns antibody and the ligand Dns-lysine, the value for F_0 was small compared to the relative value of F_∞ when excitation was at 280 nm; therefore, the value for F_0 was assumed to be zero. Consequently, the equation simplified to

$$x = F/F_\infty \quad (2)$$

In the case of the DLPE associated with lipid vesicles, the value of F_0 was significant compared to F_∞; consequently, the fraction of ligand bound was calculated by using

$$x = (F/F_0 - 1)/(F_\infty/F_0 - 1) \quad (3)$$

The values for F_∞, when titrations utilized Dns-lysine, or F_∞/F_0, when titrations utilized DLPE associated with lipid vesicles, were obtained from the maximum observed fluorescence at the end of the titration. When the interaction between anti-Dns Fab′ or F(ab′)$_2$ and vesicle-associated ligands was monitored, the value for F_∞/F_0 was obtained by linear regression analysis of double-reciprocal plots of F/F_0 vs. the total protein concentration, P_0, i.e., F_0/F vs. $1/P_0$. The appropriateness of the values selected for F_∞ was judged by the criterion that for monovalent interaction the value of the index of heterogeneity for the binding of a homogeneous antibody, analyzed by using the Sips distribution function, should be unity (Karush, 1962). The values obtained for the observed fluorescence were corrected for both protein and vesicle scattering. This was accomplished by simultaneously titrating protein into a vesicle solution which was prepared without the DLPE probe.

The total concentration of active anti-Dns combining sites was obtained by titrating Fab′, F(ab′)$_2$, and IgG preparations into 0.5 mL of 3.3 × 10^{-7} M Dns-lysine. Since the binding was stoichiometric, the concentration of active sites was obtained by extrapolation of the linear portion of the binding curve to F_∞. Vesicles used for the binding titrations were prepared by sonication as previously described. DLPE was present in the vesicle preparations at 0.1% on a molar basis. The concentration of vesicle-associated ligand available for binding to antibody was assumed to be 70%. This assumption was based upon previous studies which indicated that lipid vesicles prepared by sonication are small unilamellar vesicles in which 70% of the lipid was located in the outer leaflet (Pagano & Weinstein, 1978).

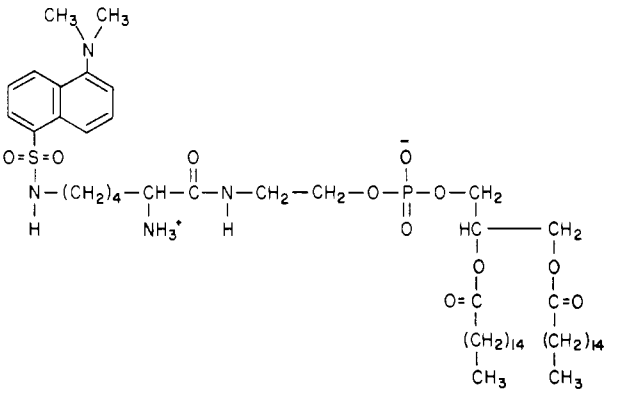

FIGURE 1: Structure of the dansyl-containing phospholipid *N*-[N^ϵ-[5-(dimethylamino)naphthyl-1-sulfonyl]lysyl]dipalmitoylphosphatidylethanolamine (DLPE).

Results and Discussion

Previous studies on the interaction of IgG antibody specific for 2,4-dinitrophenyl (Dnp) or paramagnetic nitroxide (spin-label) ligands which were attached to a phosopholipid in the liposomal membrane indicated that a spacer between the phospholipid head group and the antigenic determinant facilitated the binding of specific antibody (Six et al., 1973, 1974; Brûlet & McConnell, 1976, 1977; Humphries & McConnell, 1977). We, therefore, synthesized a probe which incorporated lysine as a spacer in order to separate the phospholipid head group and the haptenic group by 10–12 Å. The structure of the probe, *N*-(N^ϵ-Dns-lysyl)dipalmitoylphosphatidylethanolamine (DLPE), is shown in Figure 1.

The studies by Parker et al. (1967) of the interaction between Dns-lysine and rabbit anti-Dns IgG demonstrated a dramatic increase in quantum yield and a 60–70-nm blue shift in the Dns emission spectrum. In addition, these authors presented evidence for resonance energy transfer between protein side-chain chromophores and the protein-bound Dns ligand. Accordingly, we investigated the spectral perturbations of DLPE (0.1 mol %) associated with DMPC/cholesterol (7:3) lipid vesicles, prepared by sonication, in the presence of either mouse anti-Dnp or anti-Dns monoclonal IgG (Figure 2). Dns-lysine in aqueous solvents exhibits a broad uncorrected emission maximum of 540–550 nm when excited at 340 nm. The liposomal-associated DLPE exhibited an uncorrected emission maximum of 490 nm with a greater quantum yield than the Dns-lysine in solution (Figure 2A). It appears, therefore, that the vesicle-associated Dns fluorophore is in an environment with a lower dielectric constant than that of the aqueous compartment, most likely at the surface of the lipid vesicle. In the presence of anti-Dns, an increase in quantum yield and a 20-nm blue shift in the Dns uncorrected emission spectra were observed (Figure 2A). A comparison of the corrected excitation spectra of the liposomal-associated DLPE in the presence of either anti-Dnp or anti-Dns antibody in-

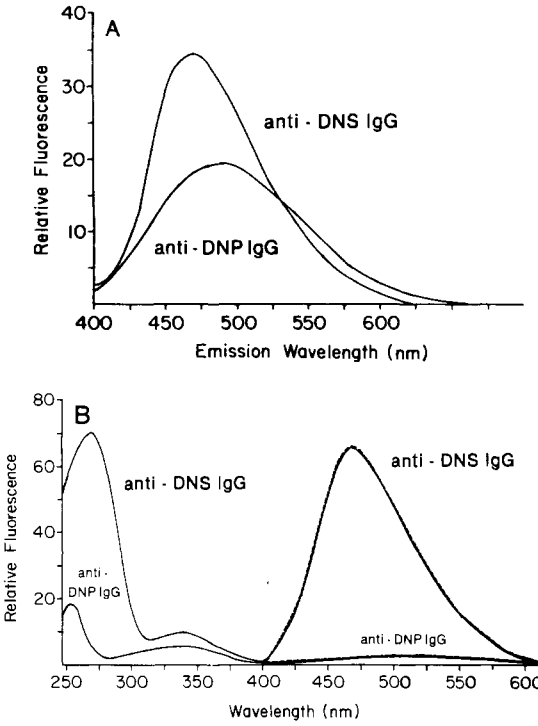

FIGURE 2: (A) Emission spectra of 0.1% DLPE associated with lipid vesicles composed of dimyristoylphosphatidylcholine and cholesterol (DMPC:cholesterol = 7:3) in the presence of either monoclonal murine anti-Dnp or anti-Dns IgG1. Excitation was at 340 nm. Lipid vesicles were prepared by sonication. The concentration of available DLPE was 8×10^{-8} M, assuming 70% in the outer leaflet. The concentration of IgG was 6×10^{-7} M. (B) Corrected excitation spectra and uncorrected emission spectra of DLPE associated with lipid vesicles composed of dimyristoylphosphatidylcholine and cholesterol (DMPC:cholesterol = 7:3) in the presence of either anti-Dnp or anti-Dns IgG. For the excitation spectra, the emission wavelength was 480 nm. For the emission spectra, the excitation was at 280 nm. The lipid vesicles were prepared by sonication with 8.0×10^{-8} M DLPE available for binding, assuming 70% in the outer leaflet. The concentration of IgG was 6×10^{-7} M.

dicated that the binding of the Dns moiety by specific antibody could be monitored by utilizing the resonance energy transfer property of the system. Figure 2B shows both the corrected excitation spectrum and the emission spectrum of free and bound liposomal-associated Dns with excitation at 280 nm. Because of the dramatic difference in the emission properties of the Dns probe when free or bound to antibody, all subsequent fluorescence spectroscopic experiments designed to monitor the primary interaction between liposomal-associated Dns and anti-Dns antibody utilized the resonance energy transfer binding assay with excitation and emission wavelengths set at 280 and 480 nm, respectively.

Effect of Vesicle Composition upon Antibody Binding. In the course of our studies, we observed that DMPC/cholesterol (2:1) lipid vesicles, prepared by sonication, which contained 1 mol % DLPE formed precipitin bands in the presence of bivalent antibody by Ouchterlony analysis (not shown). This analysis established two important properties of our system: (1) The lipid vesicles which contain the DLPE behave as multivalent antigens. Furthermore, the fluorescent hapten maintained its association with the lipid vesicle even when bound by antibody. (2) The ability to precipitate vesicles containing antigenic determinants indicates that there was extensive cross-linking of the vesicles by antibody. Therefore, a substantial fraction of the bivalent antibody molecules must be involved in the intervesicle interactions in contrast to binding to two antigenic determinants located on the same vesicle.

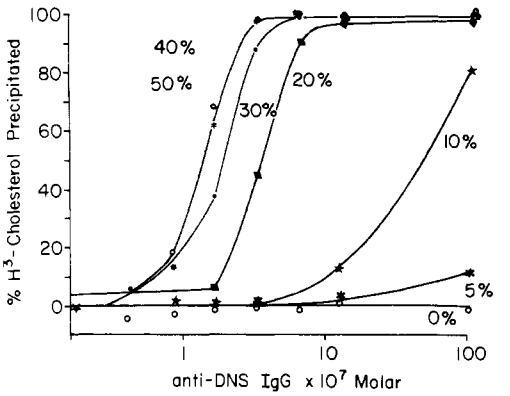

FIGURE 3: Dependence on the concentration of cholesterol of the precipitation of DMPC/cholesterol vesicles prepared by sonication as a function of murine anti-Dns IgG is shown. The concentration of available Dns groups is 1.25×10^{-5} M, assuming 70% of the probe is located in the outer leaflet of the vesicles. The amount of cholesterol is expressed as mole percent. Each point is the average of duplicates.

A precipitin assay was developed in order to establish an index of the ability of bivalent antibody to cross-link lipid vesicles. It was observed that while it was not possible to pellet the lipid vesicles alone by centrifugation, in the presence of antibody specific for the vesicle-associated ligand, aggregates developed which could be pelleted in a microfuge. A tritiated cholesterol component was incorporated into each vesicle preparation in an amount which did not exceed 0.02% of the total lipid used in the preparation of the lipid vesicles. After a vesicle preparation was incubated with an antibody solution, as described under Materials and Methods, the precipitate was centrifuged, and an aliquot of the supernatant was analyzed for radioactivity to determine the amount of lipid which remained in solution. This assay has the unusual property of utilizing a radiolabel which is noncovalently associated with the antigen. The obvious limitation of the method is that there may be antigen–antibody complexes in which vesicles are cross-linked by bivalent antibody but are not removed by centrifugation. Nevertheless, we were able to use the precipitin assay to delineate how the vesicle composition influences the binding of bivalent antibody with liposomal-associated ligands.

The ability of bivalent monoclonal murine anti-Dns to aggregate lipid vesicles containing 1 mol % DLPE was clearly dependent on the amount of cholesterol incorporated into the lipid vesicles (Figure 3). Vesicles prepared by sonication which contained no cholesterol were not precipitated by bivalent anti-Dns IgG antibody. As the mole percent of cholesterol was increased from 0 to 40, the precipitation efficiency also increased. The precipitation curves for vesicles containing 40 and 50 mol % cholesterol were essentially identical. Two controls (not shown) were run in conjunction with the precipitation analysis. Hybridomas specific for Dnp, of the IgG1 and IgM isotypes, were unable to agglutinate vesicles containing 1 mol % DLPE at the same concentration of vesicles over the same range of protein concentration. Similarly, the anti-Dns hybridoma was unable to precipitate vesicles which did not include the DLPE probe. Thus, the immunological specificity of the assay was verified.

The spectroscopic analysis of the dependence of antibody binding on cholesterol content is shown in the comparison of the kinetics of the binding of rabbit heterologous anti-Dns IgG and the monoclonal murine anti-Dns IgG as a function of varying DMPC:cholesterol ratios in the vesicle preparation (Figure 4). Murine anti-Dns was unable to bind the Dns probe when the molar percentage of cholesterol was 15% or less. In contrast, the heterogeneous rabbit anti-Dns was able

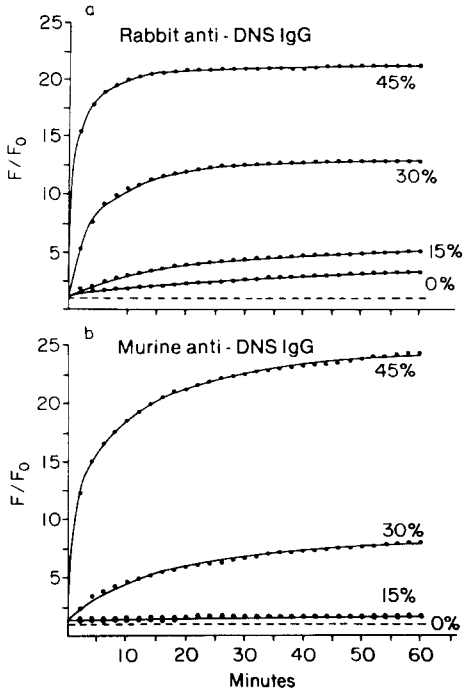

FIGURE 4: Kinetic analysis of the binding of anti-Dns IgG to 0.1% DLPE associated with dimyristoylphosphatidylcholine lipid vesicles prepared by sonication. The mole percent of cholesterol for each vesicle preparation is indicated. Excitation was at 280 nm with emission at 480 nm. The fluorescent emission was corrected for the signal due to protein and light scattered by the lipid vesicles. (a) Rabbit anti-Dns IgG at 3×10^{-7} M active sites and 3×10^{-7} M available DLPE (assuming 70% in the outer leaflet). (b) Murine monoclonal anti-Dns IgG at 3×10^{-7} M active sites and 3×10^{-7} M available DLPE.

to bind the liposomal-associated Dns even in the absence of a cholesterol membrane component. The molar percent of cholesterol in the vesicle preparations affected both the rate and the extent of protein binding for both rabbit and murine antibody preparations. As previously mentioned, the emission spectra indicate that the Dns fluorophore was not found exclusively in the aqueous compartment but rather must spend some time associated with the vesicle surface. It was not possible, however, from our steady-state fluorescence data to determine the relative distribution of Dns found in the aqueous compartment vs. that which was membrane associated. Since the murine anti-Dns IgG was unable to interact with the Dns fluorophore unless the molar percent of cholesterol exceeded 15, it appears that in the absence of cholesterol the liposomal-associated Dns is sequestered at the vesicle surface. There is, however, a subpopulation of heterogeneous rabbit anti-Dns which appears to be able to surmount this potential energy barrier to protein–ligand interactions even in the absence of a cholesterol component. For both rabbit and murine anti-Dns antibodies, the increased mole percent of cholesterol in the vesicle preparations increased the accessibility of the Dns group. This effect is most likely due to perturbations of the topography of the polar head-group region.

Evidence for such a perturbation comes from X-ray diffraction data (Levine, 1972) and ^{31}P NMR spectroscopic data (Yeagle et al., 1977) on phosphorylcholine vesicles which demonstrate an increased separation of phospholipid head groups with increasing concentrations of cholesterol. The increase in phosphorus order parameters (McLaughlin et al., 1975) and the increase in bilayer surface hydration (Newman & Huang, 1975) indicate an increase in the freedom of motion of the phosphorylcholine moiety. Further insight into the molecular basis of the increased accessibility of the lipid ligand,

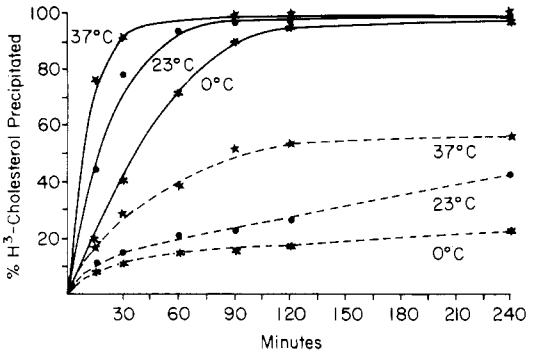

FIGURE 5: Kinetics of precipitation of DMPC/cholesterol (7:3) lipid vesicles, prepared by sonication, with murine anti-Dns IgG were shown to be dependent upon temperature as well as antigenic density. The precipitation was performed at 0, 23, or 37 °C. Each point is the average of duplicates. The mole percent of DLPE was either 0.1 (---) or 1.0 (—). The concentration of Dns in the outer leaflet (assuming 70%) was 7.3×10^{-8} M.

associated with enhanced rotational and lateral mobility, was provided by a study of the dynamic properties of mixtures of cholesterol and dimyristoylphosphatidylcholine containing a spin-labeled probe (Rubenstein et al., 1980). Finally, our finding of enhanced accessibility of the ligand to antibody with increased cholesterol content parallels the observations of Brûlet & McConnell (1977) involving the binding of anti-nitroxide IgG antibody to a nitroxide conjugate of PE incorporated in the liposomal membrane.

Effect of Antigenic Density upon Antibody Binding. The effect of varying the antigen density upon the ability of monoclonal murine anti-Dns to aggregate lipid vesicles prepared by sonication was studied with DMPC vesicles containing 30% cholesterol and 1.0, 0.1, and 0.01 mol % DLPE. If an average diameter of 300 Å is assumed for sonicated vesicles, with a bilayer 50 Å thick (Pagano & Weinstein, 1978), then 1.0% DLPE represents approximately 47 Dns's per vesicle, 0.1% DLPE 4.7 Dns's per vesicle, and 0.01% DLPE 0.5 Dns per vesicle. It was found that the precipitability of the sonicated vesicles by bivalent antibody was increased for vesicles which contained a greater number of antigenic determinants per vesicle, as is also evident from Figure 5. In addition, when the points on these curves corresponding to the same bulk concentration of available ligand are compared, the vesicles with a higher antigenic density were more extensively agglutinated.

An analysis of the kinetics of vesicle agglutination by bivalent antibody was performed at different temperatures and antigenic densities. Figure 5 shows the kinetics of vesicle agglutination at 0, 23, and 37 °C when the concentration of bulk vesicle associated Dns is constant, but in which the antigen density is either 1.0% or 0.1%. The rate of vesicle agglutination was clearly accelerated by increasing the temperature from 0 to 37 °C. When the rate of vesicle agglutination was monitored for vesicles with 1.0% DLPE at a Dns concentration 10-fold greater than that used in Figure 5, greater than 95% of the vesicles were precipitated within 15 min at the three temperatures tested (data not shown).

Since lipid vesicles prepared by sonication generally range in size from 200 to 500 Å in diameter (Pagano & Weinstein, 1978), we considered the possibility that intervesicle binding by bivalent antibody was favored over intravesicle binding because of the high degree of vesicle curvature relative to the dimensions of the antibody molecule. Vesicles were prepared, therefore, by the solvent evaporation method (Deamer & Bangham, 1976; Schieren et al., 1978). This method leads to the formation of unilamellar vesicles in which the diameter

Table I: Association Constants and Heterogeneity Indexes for Binding of Dns Ligands

antibody[a]	ligand	K_a[b]	a[c]
IgG	Dns-Lys	1.58×10^8	0.97
$F(ab')_2$	Dns-Lys	1.00×10^8	1.01
Fab'	Dns-Lys	0.63×10^8	0.98
Fab'	DLPE in DMPC:chol[d] (7:3) lipid vesicles	1.12×10^6	1.04

[a] Murine anti-Dns antibody preparations. [b] K_a was calculated by using the Sips distribution function. [c] Index of heterogeneity. [d] chol is cholesterol.

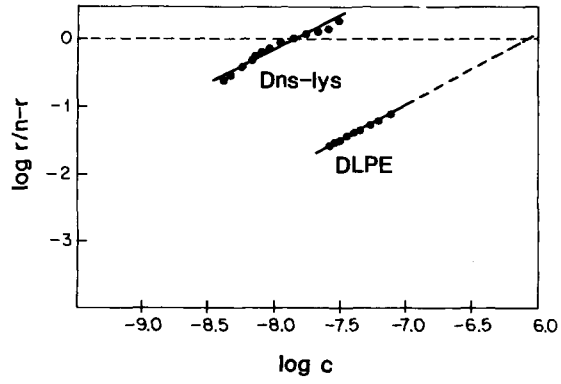

FIGURE 6: Comparison of the binding of Fab' from murine anti-Dns to Dns-lysine and DLPE associated with lipid vesicles. Both Sips plots were generated by titrating anti-Dns Fab' into 3.8×10^{-8} M Dns-lysine or 0.1% DLPE in DMPC/cholesterol (7:3) vesicles with 9.7×10^{-8} M DLPE available for binding (assuming 70% on the outer leaflet). For Dns-lysine, the correlation coefficient of linear regression analysis was 0.98. For DLPE in vesicles, $F_\infty/F_0 = 18.0$, and the correlation coefficient of linear regression analysis was 0.99. Here c represents the concentration of free ligand, r the number of ligands bound per Fab', and n (=1) the number of binding sites per Fab'.

of the vesicles ranges from 1000 to 4000 Å. The kinetics of vesicle agglutination prepared by the solvent evaporation procedure indicated that, analogous to the sonicated vesicles, the larger vesicles were extensively aggregated by bivalent antibody. The rate of agglutination was slower, however, for the larger vesicles than for the vesicles prepared by sonication. The rate of agglutination of large vesicles was also found to be dependent upon the antigenic density as well as the temperature (data not shown).

Monovalent and Bivalent Binding of Antibody to DLPE Small Unilamellar Vesicles. A comparison was made of the affinities of murine anti-Dns Fab' for Dns-lysine and DLPE (Table I). In the latter case, the vesicles contained DMPC:cholesterol in the ratio of 7:3 and 0.1 mol % DLPE. The Sips plots, shown in Figure 6, reveal a 50–100-fold difference in the intrinsic association constants for the anti-Dns antibody and the Dns-Lys presented in the two different forms. This result provides further evidence for the affinity of the Dns groups for the vesicle surface. It is important to note that 60% of the ligand assumed to be present in the outer leaflet (70% of the total) was bound to Fab' in the titration without, however, representing the maximum accessible ligand. The low values of r in the titration (Figure 6) reflect the disparity between the bulk concentrations of DLPE and Fab'.

The value of the association constant for the interaction of Fab' and ligand at the liposome surface depends upon the validity of several assumptions. In order to obtain a value for the total ligand concentration, we have assumed 70% of the DLPE is located in the outer leaflet of the lipid vesicle. This figure is based on an average diameter for a small unimellar vesicle of approximately 300 Å. Further, we assume that the

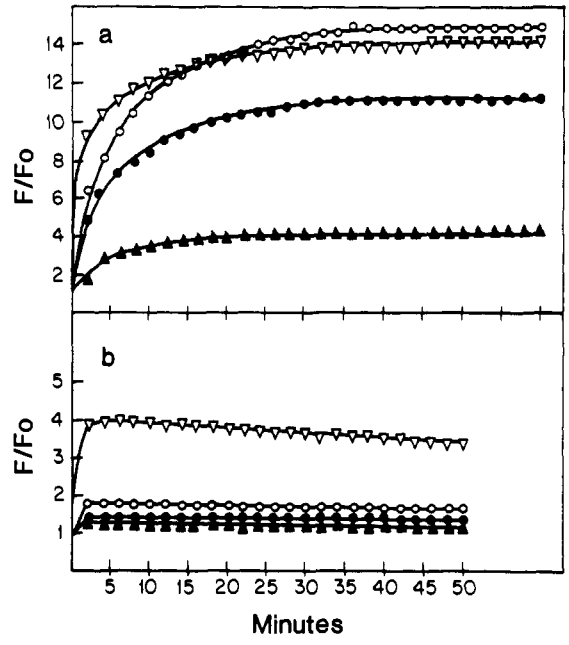

FIGURE 7: Kinetics of the interaction between murine anti-Dns $F(ab')_2$ (a) and murine anti-Dns Fab' (b) and 0.1% DLPE in DMPC/cholesterol (7:3) lipid vesicles prepared by sonication. Excitation was at 280 nm and emission at 480 nm with 10- and 20-nm slits, respectively. DLPE available for binding was 1.5×10^{-7} M (assuming 70% in outer leaflet). The concentration of active anti-Dns binding sites was (▲) 0.39×10^{-7}, (●) 0.98×10^{-7}, (○) 1.24×10^{-7}, and (▽) 9.8×10^{-7} M.

Dns groups in the outer leaflet are equivalent with respect to complex formation with antibody. Therefore, the value for F_∞/F_0 represents the ratio of fluorescence for Dns in the outer leaflet in a bound state or free state.

In an attempt to assess independently the concentration of available ligand at the liposome surface, we have compared the fluorescence signals for excess anti-Dns and DLPE-containing vesicles in the presence and absence of sodium cholate. We found approximately a 2-fold increase in the emission signal. This result indicates that at least 50% of the total DLPE is available for binding and is consistent with the value of 70% employed in our calculation of the association constant.

A kinetic spectroscopic analysis was carried out to characterize the interaction of $F(ab')_2$ and Fab' with DMPC/cholesterol (7:3) vesicles containing 0.1 mol % DLPE. As shown in Figure 7 for several concentrations of protein, the bivalent fragment exhibited a more complex behavior. With Fab', the equilibrium was established too rapidly to measure after addition of the protein to the vesicle solution. With $F(ab')_2$, the kinetic experiments demonstrate that two distinguishable interactions take place with bivalent antibody. The first is relatively rapid and consists of the monovalent attachment of the antibody to a vesicle. The second, and slower, process involves the subsequent cross-linking of vesicles by interaction of the unoccupied antibody site with another Dns group. Since F/F_0 is a measure of the fraction of ligand bound and larger values are attained at the same concentration of antibody sites with bivalent antibody compared to monovalent antibody, it is evident that bivalence leads to more extended complex formation.

Further evidence for a second, slower step in the bivalent binding process is presented in Figure 8 where a comparison of the binding of murine anti-Dns $F(ab')_2$ to DLPE associated with liposomes was monitored over a 10-fold range of liposome concentration. At the higher concentrations of liposomes, equilibrium was reached within about 10 min. With lower

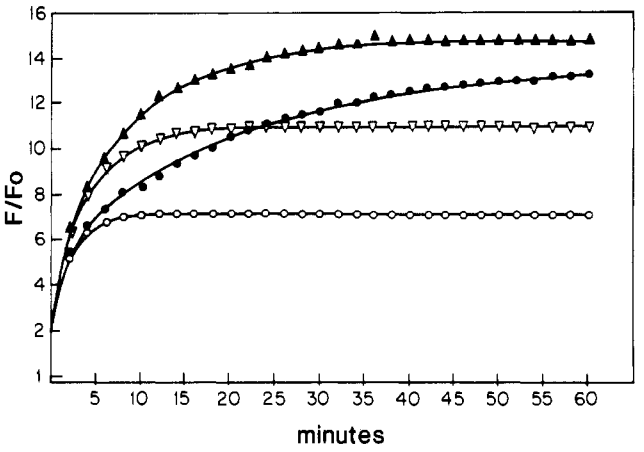

FIGURE 8: Kinetics of the interaction between murine anti-Dns $F(ab')_2$ and 0.1% DLPE in DMPC/cholesterol (7:3) lipid vesicles, prepared by sonication, as a function of the liposome concentration. Excitation was at 280 nm and emission at 480 nm with 10- and 20-nm slits, respectively. The concentration of anti-Dns binding sites was 1.24×10^{-7} M with (○) 6.12×10^{-7}, (▽) 3.06×10^{-7}, (▲) 1.52×10^{-7}, and (●) 0.61×10^{-7} M available DLPE (assuming 70% in the outer leaflet).

liposomal concentrations, the biphasic character of the binding process became more pronounced. The dependence of the slower binding step upon the concentration of antibody and vesicles indicates the process was an intervesicle one, rather than intravesicle. The thermodynamic advantage is probably a consequence of the formation of circular complexes consisting, for example, of two vesicles and two antibody molecules (Schumaker et al., 1980).

In conclusion, our kinetic analysis of precipitation and resonance energy transfer indicates that the binding of bivalent antibody to liposomal membrane ligands is primarily associated with the cross-linking of vesicles rather than intravesicle binding. Our studies indicate that bivalent binding is a two-step process. The first step is rapid and involves the interaction of a combining site with antigen, analogous to antibody–hapten interactions. The slower, second step involves the formation, and possibly the rearrangement, of intervesicle complexes. Our findings parallel other studies on the interaction of monoclonal antibodies directed against cell membrane antigens. In the studies with allo antigens of murine lymphoblasts (Hackett & Askonas, 1981) and membrane antigens of rat thymocytes (Mason & Williams, 1980), monogamous bivalent attachment did not play a major role in the thermodynamic properties of the process and in its kinetic features. These observations are in constrast to earlier findings where monogamous bivalent interactions were important in virus neutralization experiments (Hornick & Karush, 1972). The relative contribution of intermembrane vs. intramembrane binding modes for bivalent antibody may depend upon several factors, such as (1) the concentration of reactants which influence the rates of collision, (2) the antigenic density and rates of lateral diffusion of the antigen within the plane of the membrane, and (3) the degree of segmental flexibility and relative orientation of the antibody combining sites which characterize an antibody of a given isotype.

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