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Lateral diffusion of specific antibodies bound to lipid monolayers on alkylated substrates

(fluorescent antibodies/fluorescence recovery after periodic pattern photobleaching/Langmuir–Blodgett films/immunology)

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ABSTRACT **We have measured the lateral mobility of fluoresceinated monoclonal IgG antibodies bound specifically to a spin label lipid hapten in phospholipid monolayers supported on alkylated silicon oxide surfaces. Dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine monolayers containing 5 mol% of the lipid hapten were transferred by conventional Langmuir–Blodgett techniques onto substrates alkylated with hydrocarbon chains containing 10, 16, and 18 carbon atoms. We show that the diffusion of the bound antibodies depends on their lateral density, the composition of the lipid monolayer, and the nature of lipid coupling to hydrocarbon chains on the alkylated substrate. Antibody diffusion coefficients at low antibody densities are within a factor of 2 of those displayed by the lipid hapten in the absence of the bound antibody. High antibody densities result in reduced antibody mobility, but the lateral diffusion of unbound lipids is unaffected.**

There is much current interest in the design of appropriate model systems to investigate the structure and function of cellular membranes. In recent work from our laboratory, a variety of model phospholipid membranes has been used in attempts to understand the molecular mechanisms of cell–cell communication in the immune system (1–3). The biochemical functions of some of these cells are triggered by interactions involving antibody molecules bound to suitable receptors on cell surfaces. For example, mast cells are triggered by the crosslinking of receptor-bound IgE, and macrophages can be triggered by the binding of IgG–antigen complexes to membrane-bound F_c receptors (4, 5).

The specific binding and subsequent interaction of antibodies bound to lipid haptens in model phospholipid membranes is representative of a general class of events involving ligands and receptors on cell surfaces. New and improved methods for reconstitution of receptors into artificial membranes have led to much progress in understanding membrane phenomena in the immune and nervous systems (6, 7). However, many fundamental questions remain unanswered due to the unavailability of suitable model systems.

Epifluorescence and total internal reflection spectroscopy have been used recently to study the interaction of cells of the immune system with planar phospholipid membranes (3, 8, 9). Investigations using optical microscopy are much more easily carried out on substrate-supported flat membranes than on vesicles and liposomes. Planar membranes containing histocompatibility antigens have been prepared by fusion of vesicles with solid glass substrates and employed in the study of antigen recognition by T cells (10–12). Planar monolayers and bilayers, transferred onto solid substrates from the air–water interface of a Langmuir trough, have been successfully employed as target membranes in experiments involving antibody-mediated binding of macrophages and basophils. Langmuir–Blodgett techniques afford a high degree of control over the composition and packing densities of molecules in these transferred layers (13–15). Furthermore, detailed structural information from adsorbed amphiphilic layers is becoming available with the aid of surface-sensitive techniques such as Fourier transform infrared and Raman spectroscopies (16, 17). Thus, a study of the physical properties of planar membranes with specifically bound antibodies could prove valuable in understanding the behavior of cellular membranes.

In previous work with antibodies bound to haptenated dimyristoyl phosphatidylcholine (Myr_2-PtdCho) bilayers "physisorbed" on silicon substrates, we found that (*i*) the binding of antibodies is highly specific to the presence of lipid hapten in the membrane, (*ii*) at low antibody densities, antibody diffusion coefficients are the same as those of the lipids, and (*iii*) at a given temperature, antibody diffusion coefficients decrease with increasing lateral antibody densities (18).

In the present study, we attempt to provide a systematic characterization of factors governing the lateral diffusion of antibodies specifically bound to monolayer membranes supported on alkylated substrates. This is motivated, in part, by the failure of preliminary efforts in our laboratory to consistently reproduce previously reported measurements of the lateral diffusion coefficients of monolayer-bound antibodies. Our principal objectives here are to (*i*) define necessary and sufficient conditions for the rapid long-range diffusion of antibodies bound to monolayer phospholipid membranes and (*ii*) find conditions under which such diffusion is affected by interactions between the bound antibodies. Our observations indicate that the mobility of the bound antibodies depends strongly on the coupling between hydrocarbon chains of the phospholipid monolayer and the alkylated surface. These results can be understood in terms of a previous description of interlayer coupling (15).

TECHNIQUES

Lipids. Dipalmitoyl phosphatidylcholine (Pam_2-PtdCho) and Myr_2-PtdCho were obtained from Sigma and used without further purification. Fluorescent lipid analogs *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-L-dipalmitoyl phosphatidyletha-

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Abbreviations: FRAP, fluorescence recovery after periodic pattern photobleaching; Myr_2-PtdCho, dimyristoyl phosphatidylcholine; Pam_2-PtdCho, dipalmitoyl phosphatidylcholine; NBD-Pam_2-PtdEtn, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-L-dipalmitoyl phosphatidylethanolamine; NBD-PtdCho, 1-palmitoyl-2-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino caproyl]phosphatidylcholine; SLLH, spin label lipid hapten; DTS, decyl trichlorosilane; HTS, hexadecyl trichlorosilane; OTS, octadecyl trichlorosilane.

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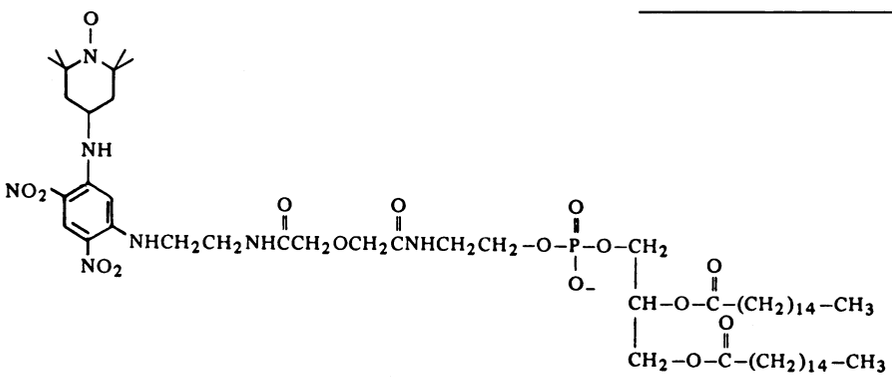
nolamine (NBD-Pam_2-PtdEtn) and 1-palmitoyl-2-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino caproyl]phosphatidylcholine (NBD-PtdCho) were from Avanti Polar Lipids.

The spin label lipid hapten (SLLH) used in these studies was synthesized as described (19) and has the following structure:



[^{14}C]Pam_2-PtdCho was from the Radiochemical Centre.

Antibodies. Monoclonal antibodies to the spin label hapten were isolated from a mouse hybridoma line developed in this laboratory (19) and were a generous gift from Tania Watts. Antibodies were labeled with fluorescein isothiocyanate according to the procedures described in ref. 20 to obtain fluorescein-to-protein ratios between 0.1 and 1.

Preparation of Monolayers at the Air–Water Interface. Lipid mixtures were freshly prepared from refrigerated stock solutions and spread as a chloroform solution at the air–water interface of a Langmuir trough equipped for epifluorescence microscopy (21). Surface pressures at the air–water interface were measured by monitoring the output voltage of a displacement transducer, model MDP.02, from Transducers & Systems, Inc. (North Branford, CT). The core of the transducer was suspended freely at the end of a specially designed linear spring from Stock Springs Ltd. (Dublin, CA). A square piece of no. 1 Whatman filter paper attached to the lower end of the core served as a Wilhelmy plate. Pressure–area isotherms were recorded during compression by using a stepping motor-based strip chart recorder. Water used in these studies was freshly deionized in a Millipore water-filtering system and stored in a quartz container. Monolayers were typically spread to densities corresponding to pressures of about 8 dynes/cm and compressed continuously at a rate of 0.05 Å^2/sec per molecule to a final precoating pressure of 30 dynes/cm.

Preparation of Derivatized Substrates. p-Type silicon wafers that were cut along the (111) plane and polished to "prime" finish on one side were purchased from the Aurel Co. (Sunnyvale, CA). A thermally grown oxide layer with a thickness of 8000 Å reduced surface quenching of fluorescence from probes in the transferred monolayer. Silicon oxide surfaces were derivatized with alkyl trichlorosilanes as described (22). Alkyl silanes with three different chain lengths were employed in this study: decyl trichlorosilane (DTS, C_{10}), hexadecyl trichlorosilane (HTS, C_{16}), and octadecyl trichlorosilane (OTS, C_{18}). All alkyl silanes were purchased from Petrarch (Levittown, PA).

Monolayer Transfer. Monolayers were transferred onto derivatized substrates by conventional Langmuir–Blodgett coating. Briefly, each substrate (area $\approx$ 5 cm^2) was lowered vertically through the monolayer at a speed of 2.5 mm/min, while an electronic feedback circuit held the pressure at the air–water interface constant to within $\pm$0.2 dynes/cm. The monolayer-coated substrates were collected at the bottom of the trough in a glass Petri dish. In all subsequent manipulations of the transferred monolayer, it was never exposed to air. Since it was necessary to exchange the aqueous phase in contact with the monolayer several times, the substrate was attached to a glass coverslip coated with strips of doubly adhesive tape purchased from 3M Co. The adhesive tape served as a spacer (150 μm thick) between the glass and monolayer surfaces, while capillary action maintained an aqueous solution in contact with the monolayer at all times. A schematic of the final sample assembly on the microscope stage is shown in Fig. 1.

After withdrawal of the sample assembly from the Langmuir trough, the water in contact with the monolayer was exchanged with P_i/NaCl by flowing an excess through the narrow space between the monolayer and the attached coverslip. The monolayer was subsequently incubated with 100 μl of a P_i/NaCl solution of the fluoresceinated antibody at concentrations of either 100 μg/ml or 10 μg/ml for a period of 5 min. Any unbound antibody was then washed away by an excess of P_i/NaCl. We found no significant dissociation of the bound antibody into the aqueous phase over a period of several hours.

Fluorescence Microscopy. Microscopic observations on the sample were made after placing it on the stage of a Zeiss photomicroscope III equipped for epifluorescence. Fluorescent emission from the bound antibodies was excited with the 488-nm line of an argon ion laser (Spectra-Physics, Mountain View, CA) and was detected by a cooled photomultiplier tube from RCA (Lancaster, PA). Measurements of lateral diffusion in the monolayer were made by monitoring fluorescence recovery after pattern photobleaching (FRAP) as described (23). Temperature variation was achieved by replacing the microscope stage with a thermoelectric stage, model CP 1-4-127-10L, from Melcor (Trenton, NJ). This was connected to a temperature controller, model DRC 84C, from Lakeshore Cryotronics (Westerville, OH). Absolute temperatures quoted in this manuscript were suitably calibrated with an external reference to an accuracy of 1°C. Temperatures were controlled to within 0.05°C.

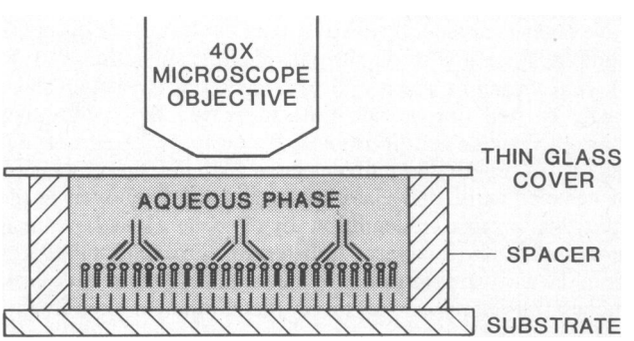


FIG. 1. Sample assembly for epifluorescence microscopy. In all experiments with antibodies, the aqueous phase used was phosphate-buffered saline (P_i/NaCl) at pH 7.4.

RESULTS AND DISCUSSION

We present a summary of our measurements of the diffusion coefficients and mobile fractions of the bound antibodies in Fig. 2. In samples that displayed rapid antibody diffusion (2–9 $\times 10^{-9}$ cm^2/sec), mobile fractions were either high (≈85%) or intermediate (≈60%) in magnitude. In those cases in which a very low fraction (<10%) of antibodies was mobile, diffusion coefficients were below the limit of detection of our apparatus (1×10^{-11} cm^2/sec). We make no distinction between high and intermediate mobile fractions for the purposes of the ensuing qualitative discussion.

Two principal conclusions emerge from our measurements. (*i*) Antibody mobility at low concentrations is nearly the same as that displayed by the lipid hapten in the absence of bound antibody. (*ii*) At higher densities of bound antibodies, their mobility is restricted and is lower than that of the lipid matrix, whose mobility remains unaffected. In what follows, we elaborate on our observations.

Low Antibody Concentrations. Transferred monolayers were incubated with antibodies at concentrations of 10 μg/ml according to the procedures described above. Antibodies bound to haptens in Myr_2-PtdCho monolayers on DTS- and HTS-derivatized substrates show rapid lateral diffusion at room temperature (25°C). On the other hand, antibodies bound to haptens in Myr_2-PtdCho monolayers on OTS-derivatized substrates show no detectable mobility at room temperature, even at the lowest antibody concentrations used. On heating the sample, a steep increase in the mobile fraction was observed at 38°C. This alteration in mobility was reversible upon cooling; the transition to a lower mobile fraction, however, occurred at slightly lower temperatures during the cooling cycle.

High Antibody Concentrations. Monolayers were incubated with fluoresceinated antibody at 100 μg/ml. For antibodies bound to haptens in Pam_2-PtdCho monolayers under these conditions, rapid diffusion was observed on DTS and HTS substrates only at temperatures greater than 31°C and 40°C, respectively. Antibodies bound to haptens in Myr_2-PtdCho membranes showed high mobility at room temperature on

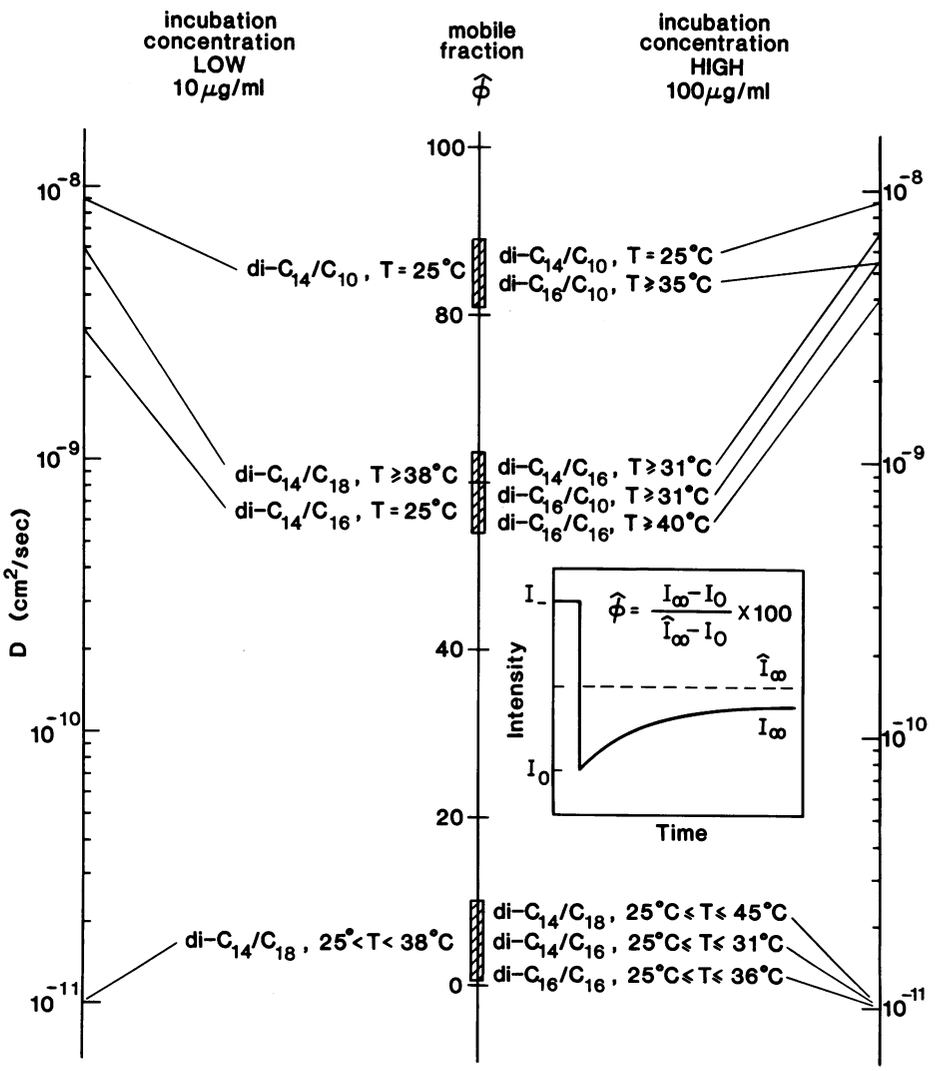


FIG. 2. Plot of lateral diffusion coefficients and mobile fractions of antibodies bound to SLLH in supported monolayers. All samples contained 5 mol% of SLLH and are identified here by the number of carbon atoms in the host lipid chains, the number of carbon atoms in the hydrocarbon chains of the alkylated substrate, and the temperature. Samples were incubated with high and low concentrations of fluorescent antibodies. Determinations of fluorescence intensities provided an approximate measure of monolayer-bound antibody densities. In the absence of accurate antibody adsorption isotherms, a direct correlation between incubation concentrations and bound densities cannot be assumed. Diffusion coefficients were extracted from single exponential fits of the time-dependent recovery of fluorescence intensities after pattern photobleaching. The *Inset* contains the definition of mobile fraction $\hat{\phi}$ in terms of I_-, the prebleach intensity, I_0, the intensity immediately after the bleach, I_∞, the fluorescence intensity at long times, and $\hat{I}_\infty$, the theoretical maximum for the recoverable intensity. Note that for Pam_2-PtdCho on DTS higher mobile fractions are found only for T ≥ 35°C.

DTS substrates and, for temperatures greater than 31°C, on HTS substrates. Changes in mobility were reversible on cooling and exhibited a hysteresis of 2–3° in the transition temperature. No significant mobility of antibodies was observed on OTS substrates even at 45°C. Measurements at higher temperatures were not attempted due to the possibility of protein denaturation. Lipid diffusion, as monitored by the lateral diffusion of the probe NBD-PtdCho, was found to be independent of antibody concentration.

It can be seen from Fig. 2 that at low antibody concentrations, antibodies on DTS and HTS substrates display diffusion coefficients similar to those of the lipids themselves. To verify if the mobility of the antibodies on OTS substrates reflected the mobility of the hapten in the absence of bound antibodies, we performed measurements of the lateral diffusion of the fluorescent probe NBD-Pam_2-PtdEtn in Myr_2-PtdCho monolayers supported on OTS substrates. The structural similarities of SLLH and NBD-Pam_2-PtdEtn suggest that their diffusivities (24) in Myr_2-PtdCho monolayers should be comparable. A sharp increase in the lateral diffusion coefficient and mobile fraction of NBD-Pam_2-PtdEtn was observed at 35°C in OTS-supported Myr_2-PtdCho monolayers stained with 5 mol% NBD-Pam_2-PtdEtn. Similar observations have been made for Pam_2-PtdCho and related phospholipids on OTS substrates and are consistent with an abrupt reduction of the interaction between NBD-Pam_2-PtdEtn and OTS hydrocarbon chains at 35°C (14, 24). It is possible that NBD-Pam_2-PtdEtn is phase-separated from Myr_2-PtdCho at temperatures between 25°C and 35°C. Since the monolayer was uniformly fluorescent, the size of such phase-separated regions must be below the limits set by optical resolution. The lower temperature observed for the onset of rapid lipid diffusion (35°C), as compared to rapid antibody diffusion (38°C), might either be due to correspondingly lower activation energies for lateral transport or to intraplanar interactions between antibodies.

Our results show that the factors governing the mobility of the bound antibodies are similar to those affecting lipid mobility. Furthermore, antibody diffusion coefficients are also sensitive to lateral antibody densities. In what follows, we briefly discuss some aspects of lipid diffusion in monolayers, followed by a consideration of factors that determine antibody densities on the monolayer. A detailed description of lipid diffusion is available elsewhere (24).

The diffusion of phospholipids in monolayers is known to depend on their lateral density and the temperature (25). Our current interest lies in accounting for the differences of lipid mobility in monolayers at different interfaces. In earlier experiments, we showed that the transfer onto DTS and HTS substrates of Pam_2-PtdCho monolayers in the coexistence region at the air–water interface resulted in a structural rearrangement of the monolayer. The transferred monolayers also displayed rapid diffusion at room temperature. On the other hand, Pam_2-PtdCho monolayers transferred onto OTS substrates showed no rearrangement and no detectable diffusion for temperatures below 35°C. The quality of the alkylated substrates used in these studies was monitored by measuring the contact angles of sessile drops of water on the surface. Well-alkylated OTS substrates showed contact angles of about 115° (26) and a hysteresis of 5–8° between the advancing and receding contact angles. We have observed rapid lipid diffusion at room temperature in Pam_2-PtdCho monolayers transferred onto OTS substrates that showed significantly lower contact angles than 115°. Lower contact angles are consistent with incomplete alkylation of the substrate. Our observations, therefore, suggest that at a given temperature, rapid lipid diffusion in monolayers is generally associated with shorter chain lengths and lower packing densities of the substrate-attached hydrocarbon chains in the alkylated substrate. Reduced antibody mobility at high antibody concentrations is independent of the nature of the substrate and could either be due to nonspecific aggregation or to the formation of oligomers. This does not preclude the possibility that under conditions in which long-range diffusion is limited by high antibody densities, "local" translational and rotational diffusivities remain high. Electron microscopic investigations may help in distinguishing between these possibilities. Two-dimensional crystallization of antibodies is known to occur under suitable conditions in some monolayer systems (27).

This brings us to a discussion of the density of the bound antibodies on the different substrates. For given incubation conditions, antibody densities depend on the concentration of the haptens available for antibody binding. This, in turn, is determined by (*i*) the hapten density in the transferred monolayer and (*ii*) the accessible fraction of haptens.

In preliminary experiments with [^{14}C]Pam_2-PtdCho, we find that under identical coating conditions, higher numbers of molecules are transferred onto OTS substrates as compared to DTS substrates. Under the same incubation conditions, we also observe higher densities of antibodies (as monitored by fluorescence intensities) bound to haptenated Myr_2-PtdCho monolayers on OTS than on DTS substrates.

In our earlier studies of antibody binding to Myr_2-PtdCho bilayers (18), it appeared from the very high hapten densities required for saturation binding that a large fraction of haptens was not accessible to the antibodies. The lipophilic nature of the haptenic headgroup suggests that a fraction of the haptens may bury their headgroups in the membrane; such insertion could be a function of the physical state of the membrane (28). An estimate of the average location of the haptenic headgroup in the monolayer may be obtained by monitoring the quenching of membrane-bound fluorescent probes by nitroxide spin labels. This phenomenon is well-characterized and is known to require close contact of the fluorophore with the free electron (29, 30). Therefore, quenching of chain-labeled fluorophores would arise only from those haptenic headgroups that are buried in the monolayer. In preliminary experiments with NBD-PtdCho and SLLH, we find far greater fluorescence quenching in fluid PtdCho monolayers than in Pam_2-PtdCho monolayers in the gel and fluid phases.

In summary, then, at a given temperature, the binding and lateral diffusion of antibodies bound to supported monolayers depend on (*i*) the lipid composition of the host membrane that contains the lipid hapten, (*ii*) the nature of coupling between the lipid hapten and the alkylated substrate, and (*iii*) the two-dimensional density of the bound antibodies. A schematic representation of monolayer-bound antibodies on the different substrates is provided in Fig. 3.

We end this discussion with a few specific comments:

(*i*) It is frequently assumed that mobility of fluorescent lipid probes in membranes reflects the self-diffusion coefficient of the host lipid matrix. Our experiments on the diffusion of NBD-Pam_2-PtdEtn in Myr_2-PtdCho monolayers on OTS substrates demonstrate that this assumption is not universally valid. Since antibody diffusion coefficients reflect the long-range mobility of the haptens themselves, we expect that in experiments with lipid haptens based on sufficiently short hydrocarbon chains, rapid hapten and antibody diffusion can be achieved in Myr_2-PtdCho monolayers on OTS substrates at room temperature.

(*ii*) Our results demonstrate that the mobility of substrate-attached hydrocarbon chains is not required for long-range antibody diffusion. Moreover, rapid lateral diffusion of the probe NBD-PtdCho is unaffected even under conditions in which high antibody densities inhibit antibody diffusion. These findings indicate that the interaction of the antibody molecules with the phospholipid monolayer, known to be highly specific, is confined to the headgroup region of the lipid hapten.

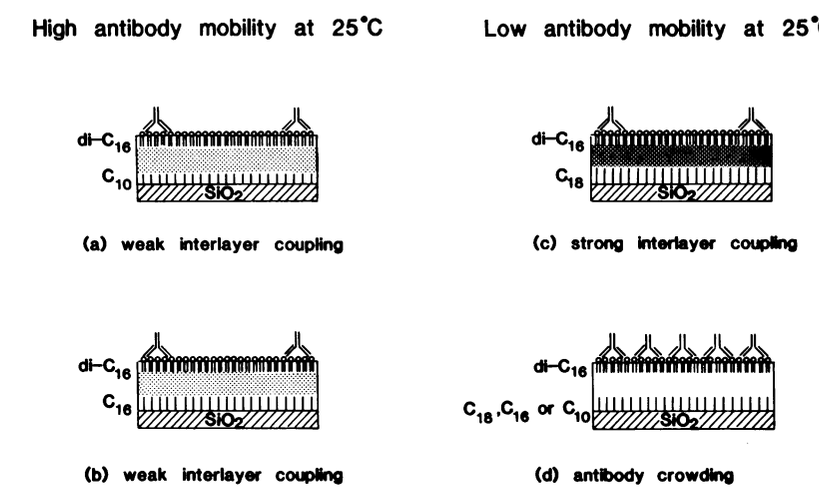


FIG. 3. Schematic representation of antibodies specifically bound to lipid haptens on silicon oxide substrates alkylated with DTS (C_{10}) (*a*), HTS (C_{16}) (*b*), and OTS (C_{18}) (*c*). Antibody crowding, as depicted in (*d*), leads to a reduction in the mobility.

(*iii*) The measured diffusion coefficients for hapten antibody complexes are as high as 9×10^{-9} cm^2/sec and, hence, within a factor of 2 of the lipid diffusion coefficients ($1–2 \times 10^{-8}$ cm^2/sec). Diffusion coefficients for antibodies in aqueous solution are known to be about 10^{-7} cm^2/sec (31). It is likely, therefore, that diffusion of the hapten–antibody complex is limited by lipid diffusion in the monolayer rather than the diffusion of antibodies in the aqueous phase. Antibodies bound to liposomes in aqueous suspension are known to diffuse at nearly the same rate as the lipids (32). One can show on theoretical grounds that if two diffusing monomers are linked one to another by a long linker contributing negligible viscous drag, then the diffusion coefficient of the dimer is one-half that of a monomer (unpublished calculations). Our antibody diffusion data are consistent with this idealized model, indicating that the bulk of the protein does not interact directly with the lipid molecules.

(*iv*) Our observations indicate that at low antibody densities, the mobility of antibodies is governed by the same factors that affect hapten mobility. It is known from independent studies that the affinity of this antibody for SLLH is high, with an equilibrium binding constant of 10^8 (19). Based on these facts, we rule out significant contributions to lateral antibody transport from antibody "walking" (32). It is possible, however, that at very high antibody densities, where long-range diffusion is slow, walking may compete as an effective mechanism for molecular transport.

CONCLUSIONS

The experiments discussed here demonstrate that the mobility of the lipid haptens is a necessary condition for the diffusion of monolayer-bound antibodies. Rapid antibody diffusion is always observed if the antibody densities are low and lipid hapten mobility is high. The mobility of the lipid hapten–antibody complex is hindered at higher antibody densities, but the diffusion coefficients of the host lipids remain unaffected. Our studies show that antibodies can diffuse freely on supported monolayers even though the chemisorbed substrate monolayer is immobile; these findings are relevant to considerations of lateral transport in asymmetric biological bilayer membranes. Model membranes of the type used in this study promise to be useful in understanding the molecular details of antibody-dependent triggering in cells of the immune system.

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