Incorporation of Acylated Antibody into Planar Lipid Multilayers: Characterization and Cell Binding†

Leaf Huang

Department of Biochemistry, University of Tennessee, Knoxville, Tennessee 37996-0840
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ABSTRACT: Multiple (up to 14) layers of lipid were deposited onto an alkylated glass surface by dialysis of egg phosphatidylcholine (PC) and deoxycholate mixed micelles in the presence of alkylated glass coverslips. The amount of lipid associated with the coverslips was measured by using radioactive PC. It was found that the number of PC molecules in the multilayer increased with increasing initial lipid concentration in the dialysis mixture. Inclusion of cholesterol resulted in a significant increase in the amount of total lipid deposited in the multilayer. However, the PC/cholesterol ratio was up to 2-fold higher in the multilayers than in the liposomes present in the same dialysis bag. In addition, mouse monoclonal anti-H2K^k antibody which had previously been derivatized with palmitic acid could be readily incorporated into the lipid multilayer during dialysis. Measurements of lateral mobility with the fluorescence recovery after photobleaching technique on fluorescently labeled lipid or antibody in the multilayer showed that the lipid molecules diffused rapidly while the antibodies were essentially immobile. Lymphoma cells such as RDM4 cells expressing surface H2K^k glycoproteins could rapidly bind to the antibody-containing multilayers. The binding was blocked by free antibody or by goat anti-mouse immunoglobulin G, indicating the immunospecificity of the binding. Cell binding to the multilayer also exhibited a threshold dependence on the antibody density of the multilayer. A lower threshold was found for cells expressing a higher surface density of H2K^k. This system may be useful for model studies of cellular recognition.

For studies of specific cell interactions, model lipid membranes which are large, planar, and optically clear are highly desirable, since a number of biophysical measurements such as the evanescent radiation and the fluorescence recovery after photobleaching (FRAP)[1] can be conveniently done on cells as well as on the model membranes. von Tscharner & McConnell (1981) have developed a technique to prepare lipid monolayers coating an alkylated glass surface. In this method, a lipid monolayer is first spread at the air/water interface in a Langmuir trough. Glass coverslips or slides which have been alkylated to become hydrophobic are then pushed through the interface and thereby pick up a lipid monolayer on their surfaces. It has been shown that the physical properties of the monolayer closely resemble those of a bilayer (von Tscharner & McConnell, 1981). Furthermore, haptenated lipids can be incorporated into the monolayer, and specific interactions of the antibody-treated rat basophil leukemia cells with the monolayer have been observed (Weis et al., 1982). These model studies have revealed new information concerning the distribution and redistribution of cell surface macromolecules during cellular recognition.

Although the monolayers on a glass surface are very useful in many instances, their applications are limited mainly to the use of lipid. Incorporation of membrane proteins into the monolayer has been difficult and inefficient, due mostly to the problem of spreading protein molecules at the air/water interface. Furthermore, incorporation of transmembrane proteins such as the major histocompatibility antigen into the monolayer involves a proteolytic removal of the cytoplasmic portion of the molecule (Nakanishi et al., 1983) prior to insertion. Therefore, we decided to develop new methods with which intact membrane proteins could be easily incorporated into planar lipid membranes coating a glass surface. We report here that this can be done by a simple dialysis procedure. To illustrate the utility of the method, we have used an acylated monoclonal antibody and studied the cell binding. An abstract of this work has been published (Huang & McConnell, 1983).

Materials and Methods

Materials. Egg phosphatidylcholine (PC) and cholesterol (chol) were purchased from Sigma. NBD-PE was obtained from Avanti. Anti-H2K^k was a mouse monoclonal antibody purified from the culture supernatant of a hybridoma (11-4.1) and radioiodinated as described previously (Huang et al., 1980). Monoclonal P3–IgG produced by P3 myeloma cells was similarly purified and used as control IgG. Palmitoyl-IgG was prepared by the reaction of IgG with the *N*-hydroxysuccinimide ester of palmitic acid at a molar ratio of 1/10 followed by purification with Sephadex G-75 column chromatography (Huang et al., 1982). Under these conditions, three to four palmitoyl chains covalently bound per IgG molecule (Huang et al., 1982) are obtained. [^{3}H]PC was obtained as described (Huang & Pagano, 1975), and [^{14}C]-cholesterol was purchased from Amersham.

Fluorescence Labeling of Palmitoyl Antibody. NBD-labeled ^{125}I-palmitoyl antibody (3.4×10^{14} cpm/mol) was prepared by adding a 100-fold molar excess of NBD-Cl to 2.7 nmol of palmitoyl antibody in 100 μL of phosphate-buffered saline (PBS) containing 0.3% deoxycholate (DOC). After a

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[1] Abbreviations: PC, phosphatidylcholine; IgG, immunoglobulin G; FRAP, fluorescence recovery after photobleaching; chol, cholesterol; PBS, phosphate-buffered saline; DOC, deoxycholate; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole.

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4-h incubation at room temperature in the dark, the reaction mixture was chromatographed on a Sephadex G-75 column which was equilibrated and eluted with PBS containing 0.15% DOC. Labeled antibody in the void volume fractions was identified by counting ^{125}I radioactivity and then pooled and stored at 4 °C in the dark. NBD-labeled palmitoyl antibody had absorption maxima at 390 and 480 nm, indicating that both Tyr and Lys residues were labeled with NBD (Cantly & Hammes, 1975).

Alkylation of Glass Coverslips. Round glass coverslips (1.8 cm in diameter, no. 2 thickness, VWR) were washed in diluted 7× detergent (Linbro) by alternate boiling and sonication for 30 min followed by thorough rinsing with running deionized water overnight. They were further rinsed with boiling distilled water, sonicated, and finally dried at 150 °C for 1 h. Alkylation was carried out in a bath of *n*-hexadecane/CCl_4/$CHCl_3$ (80/12/8 v/v) containing approximately 0.5% (v/v) octadecyltrichlorosilane (Petrarch Systems) under low heat. After overnight incubation, the alkylated coverslips were washed 5 times with $CHCl_3$ and once with EtOH before being dried at 110 °C for 1 h. Hydrophobicity was tested by dropping water onto the coverslip from about 5 cm above the coverslip. Only those coverslips that allowed water droplets to quickly bounce off were used for this study.

Coating Alkylated Coverslips with Lipid Multilayers. In standard preparations, 10 mol of lipids with or without palmitoyl antibody suspended in 1 mL of PBS containing 0.5% DOC was added to a 23-mm dialysis bag (Spectrapor 2) enclosing up to six alkylated coverslips. Air bubbles were carefully squeezed away before the bag was closed. This mixture was dialyzed against PBS containing 0.02% NaN_3 at room temperature for 3 days with at least five changes of buffer of 2 L each. The dialysis bag was opened, and the liposome suspension was carefully removed with a Pasteur pipet without letting air come in contact with the coverslips. Coverslips were washed 3 times with about 2 mL of PBS each while still in the bag. They were then removed from the bag and washed 3 times with 300 mL of PBS each time. During the entire procedure, coverslips were never exposed to the air. The liposome suspensions could be reused for the coating of more alkylated coverslips after addition of an appropriate amount of deoxycholate.

Cells. Mouse lymphoma RDM4 cells were grown in RPMI-1640 medium supplemented with 5% horse serum. YAC, CH1, EL4, and P815 lymphoma cells were similarly grown and were generously provided by Thomas Frey. They were washed 3 times with ice-cold cell binding buffer (Hank's balanced salt solution containing 0.2% bovine serum albumin and 10 mM Hepes, pH 7.4), enumerated, and resuspended before use at (2–5) × 10^6 cells/mL in the same buffer.

Cell Binding to Lipid Multilayer. Coverslips coated with lipid multilayers were mounted under PBS solution onto a glass slide with the help of two strips of double adhesive tape. The slide was then taken out of PBS solution and dried of excess water. The lipid multilayer, now located at the underside of the coverslip, was washed with about 200 μL of cell binding buffer. For fluorescence photobleaching experiments, the lipids and antibody on the top surface of the coverslip in contact with the air were washed away by a few drops of EtOH and water. Fifty microliters of cell suspension was added from the edge of the coverslip into the space between the coverslip and the slide. The slide was immediately turned upside down and incubated at room temperature for various periods of time to allow cell binding to the lipid multilayer. The slide was then turned right side up to allow unbound cells to fall off the multilayer. After 5–10 min, the bound and unbound cells were counted in the same microscope field at different focal depths. At least 100 cells were counted in each of 5 different areas of the multilayer.

Table I: Lipid Coating Efficiency of Alkylated Coverslips as a Function of Lipid Concentration[a]

concn (mM)	no. of lipid molecules on coverslip (×10^{-15})
10	4.26 ± 0.52
1	2.56 ± 0.28
0.1	1.77 ± 0.33
0.01	0.76 ± 0.16

[a] [^{3}H]PC (1.5 × 10^5 to 2.7 × 10^8 cpm/μmol) was used at the concentrations indicated.

Measurement of Lateral Diffusion. The lateral motion of the NBD-labeled lipid or antibody incorporated in the lipid multilayer was measured by the FRAP technique. Coverslips coated with lipid multilayers were thoroughly washed with PBS and mounted on a glass slide as described above. A pattern bleaching method was used (Smith & McConnell, 1978; Petty et al., 1980). Bleaching times of the order of 20 ms were used with an argon laser power density of about 10 kW/cm^2 at 488 nm. The bleaching grid was approximately 30 m in diameter. All measurements were done at 23 °C.

RESULTS

Molecular Composition of the Lipid Multilayer. After extensive dialysis, phospholipids were found to associate with the alkylated glass coverslips. When 1% NBD-PE was included in the lipid mixture, the layers on the coverslip could be clearly seen with a fluorescence microscope. The fluorescence was quite homogeneous with occasional scratches on the multilayer. No large-scale defects or adhered liposomes were observed. The amount of phospholipid on the coverslip was measured by using [^{3}H]PC. Table I shows the result of an experiment in which the concentration of PC was varied. As can be seen, the number of PC molecules on the coverslip was on the order of 10^{15} molecules per coverslip. The more concentrated the lipid solution in the dialysis mixture, the more lipids were found on the coverslip. Since the diameter of the coverslip was 1.8 cm, one continuous monolayer of PC covering both sides of the coverslip would require 0.85 × 10^{15} molecules, assuming that each PC molecule occupies a cross-sectional area of 60 Å^2 (Wolff et al., 1971). Therefore, the number of lipid layers on the coverslip ranged from 0.9 to 5, depending on the lipid concentration in the dialysis mixture. No lipid coating was observed if nonalkylated coverslips were used. Furthermore, the coating of the alkylated glass required the initial presence of DOC. No multilayer was formed if the alkylated coverslip was dialyzed for 3 days in the presence of the detergent-free liposomes prepared by dialysis or sonication of PC.

The number of lipid molecules in the multilayer also depended on the presence of cholesterol. In an experiment in which both [^{3}H]PC and [^{14}C]cholesterol were used, increasing cholesterol content in the dialysis mixture resulted in an increase in the total number of the lipid molecules, PC and cholesterol, on the coverslip (Figure 1). However, the increase for PC was greater than that for cholesterol. For example, if the dialysis mixture contained 50% cholesterol, there were approximately 3-fold more total lipids (about 14 lipid layers) found on the coverslip as compared to a dialysis mixture containing no cholesterol. However, of the lipids associated with the coverslip, only about 34% was cholesterol; i.e., the multilayers were more enriched with PC with respect to the

Table II: Incorporation of Palmitoyl Antibody into Lipid Multilayers[a]

initial antibody/lipid ratio	coverslip: no. of lipid molecules ($\times 10^{-15}$)	coverslip: no. of antibody molecules	coverslip: antibody/lipid ratio
0	8.43 ± 1.32	0	0
2.03×10^{-6}	7.19 ± 0.38	$(5.89 \pm 0.50) \times 10^{11}$	8.19×10^{-5}
1.45×10^{-5}	8.40 ± 0.08	$(2.26 \pm 0.12) \times 10^{12}$	2.68×10^{-4}
1.32×10^{-4}	7.39 ± 0.35	$(1.13 \pm 0.10) \times 10^{13}$	1.53×10^{-3}

[a] Total ^{3}H-lipid (PC/chol = 3/1; 8.9×10^5 cpm/μmol) concentration was 10 mM. ^{125}I-Palmitoyl antibody was added at different amounts.

lipids in the bulk. The multilayers in this case were still homogeneous when viewed with a fluorescence microscope if 1% NBD-PE was included in the lipid mixture.

Incorporation of Palmitoyl Antibody into the Multilayer. Unlabeled or NBD-labeled palmitoyl antibody could be incorporated into the multilayer. As can be seen in Table II, the extent of antibody incorporation was directly proportional to the amount of antibody in the initial dialysis mixture. The amount of lipid molecules associated with the coverslip was not significantly altered. Furthermore, the multilayers were about 11–40-fold enriched with antibody compared to the initial dialysis mixture, depending on the initial antibody/lipid ratio. Since less than 0.1% of the total initial lipids were associated with the coverslip, the multilayers were also greatly enriched with antibody with respect to the bilayer liposomes in the dialysis bag. The incorporated antibody molecules were homogeneously distributed in the multilayer as revealed by the uniform fluorescence of the multilayer when NBD-labeled palmitoyl antibody was used. Nonacylated antibody could not be incorporated into the multilayers.

Lateral Diffusion of Lipid and Antibody in the Multilayer. The lateral motion of the lipid and the antibody molecules in the multilayer was examined by the FRAP technique. The diffusion coefficients of the NBD-labeled lipid and antibody are listed in Table III. Lipids in the multilayers containing PC alone (including 1% NBD-PE as a probe) diffused rapidly with a coefficient of 2.8×10^{-8} cm^2/s at 23 °C, indicating that the lipid in the multilayer was in a fluid state (von Tscharner & McConnell, 1981). Since the PC used was from the egg yolk which contained unsaturated acyl chains, this result suggests that the lipids in the multilayer were arranged in a structure similar to that of the lipid bilayer. Inclusion of cholesterol or antibody in the multilayer only slightly decreased the lateral mobility of the lipids. When the mobility of the antibody was examined by using NBD-labeled palmitoyl antibody, the lateral mobility was surprisingly low. The bleached lines on the multilayer were clearly visible even after 15 min at 23 °C. The diffusion coefficient could only be estimated

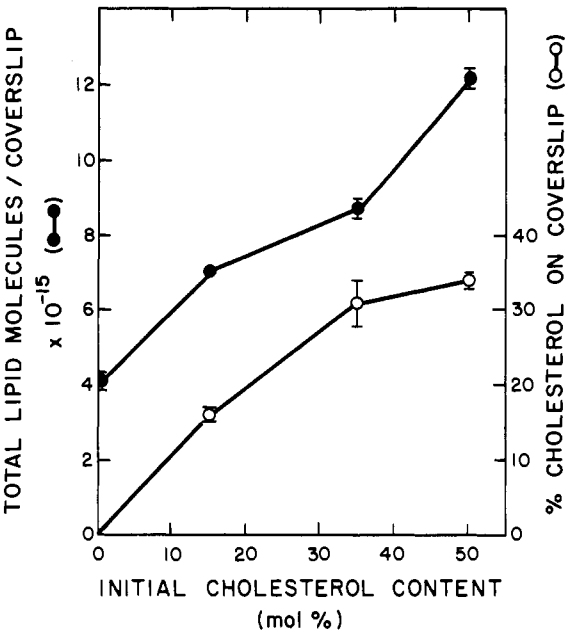

FIGURE 1: Composition of the lipid multilayer containing cholesterol. Multilayers were prepared by using various amounts of [^{3}H]PC [1.2–2.0) $\times 10^6$ cpm/μmol] and [^{14}C]cholesterol [(1.1–3.6) $\times 10^6$ cpm/μmol] at a total lipid concentration of 10 mM in the dialysis mixture. The total number of lipid molecules including PC and cholesterol (●) and also the percent cholesterol in the multilayer (○) were measured as a function of the initial cholesterol content in the dialysis mixture.

to be less than 1×10^{-11} cm^2/s. The same results were obtained when the multilayer contained cholesterol or when the multilayer contained 10-fold less antibody. Therefore, while the lipids in the multilayer diffused rapidly over a long distance (several micrometers in these experiments), the antibodies were essentially immobile.

Cell Binding to Multilayers Containing Antibody. Various mouse lymphoma cells were tested for binding to the multilayer. When multilayers prepared with an initial antibody/lipid ratio of 10^{-4} were used, different types of lymphoma cells bound to the multilayers at different rates (Figure 2). Binding of RDM4 cells was most rapid; maximal binding was achieved as soon as the cells touched the multilayer. The short lag time (about 45 s) was the time it took the cells to travel the narrow gap between the coverslip and the glass slide. Other cell types also bound to the multilayer, but with slower rates. The rate of cell binding apparently correlated with the amount of the H2K^k antigen on the cell surface, which was directly measured by using free ^{125}I-anti-H2K^k (Table IV). Cells belonging to the k or a haplotypes such as the RDM4, YAC, and CH1 cells contain a higher number of H2K^k molecules on their surface and bound to the multilayer rapidly. EL4 and P815 cells

Table III: Lateral Diffusion Coefficient of Lipid and Protein in the Multilayer[a]

fluorescent molecule	lipid	protein[c]	D (cm^2/s)	% recovery[d]
NBD-PE[b]	PC	none	$(2.8 \pm 1.2) \times 10^{-8}$	~35
NBD-PE[b]	PC/chol (3/1)	none	$(8.2 \pm 6.7) \times 10^{-9}$	~40
NBD-PE[b]	PC/chol (3/1)	palmitoyl anti-H2K^k	$(5.8 \pm 5.1) \times 10^{-9}$	~35
NBD-pal-anti-H2K^k[f]	PC	palmitoyl anti-H2K^k	$<1 \times 10^{-11}$	
NBD-pal-anti-H2K^k	PC/chol (3/1)	palmitoyl anti-H2K^k	$<1 \times 10^{-11}$	
NBD-pal-anti-H2K^k	PC/chol (3/1)	palmitoyl[e] anti-H2K^k	$<1 \times 10^{-11}$	

[a] All measurements were done at 23 °C. [b] One percent NBD-PE was included in the initial dialysis mixture. [c] All multilayers containing protein were prepared with an initial antibody/lipid ratio of 10^{-4} except in *e*. [d] Maximum recovery of the bleached fluorescence is 50%, due to the geometry of the pattern bleaching (Smith & McConnell, 1979). [e] Multilayer was prepared with an initial antibody/lipid ratio of 10^{-5}. [f] pal is palmitoyl.

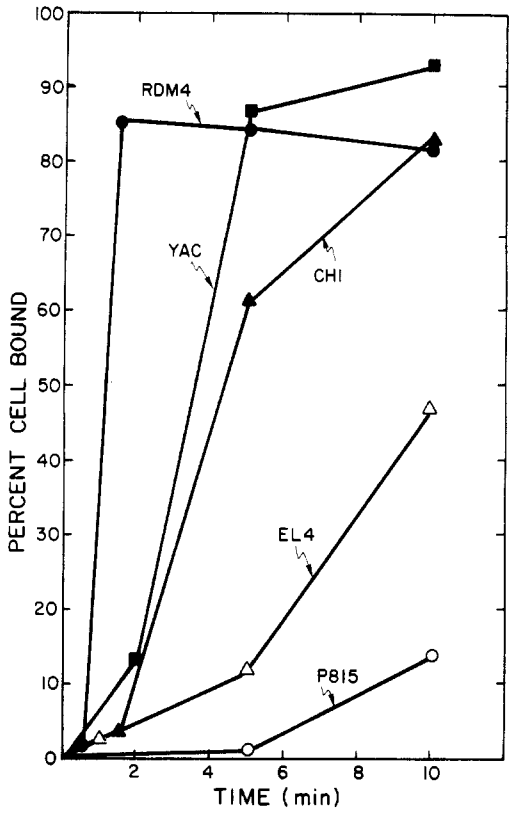

FIGURE 2: Kinetics of cell binding to multilayers. Multilayers were prepared with 10 mM lipid of PC/chol (3:1) and a palmitoyl-anti-H2K^k lipid ratio of 10^{-4}. RDM4 (●), YAC (■), CH1 (▲), EL4 (△), and P815 (○) cells were allowed to bind to the multilayer for different periods of time at room temperature.

Table IV: Amount of Surface H2K^k Molecules on Lymphoma Cells[a]

cell	haplotype	no. of H2K^k molecules/cell
RDM4	k	6.9×10^4
YAC	a	nd[b]
CH1	a	2.88×10^4
EL4	b	5.4×10^2
P815	d	0

[a] Measured by binding 10 μg/mL free ^{125}I-anti-H2K^k to 2×10^6 cells for 3 h at 4 °C. Nonspecific binding was measured by including 1 mg/mL unlabeled anti-H2K^k in the incubation mixture. Nonspecific binding has been subtracted from the total binding to obtain specific binding. [b] Not determined.

contain little or no measurable H2K^k molecules, and they bound to the multilayer only with very slow rates.

Immunospecificity of RDM4 Cell Binding to Multilayers. In order to examine whether the rapid (1.5-min incubation) binding of RDM4 cells to the multilayer was mediated by antibody/antigen complex formation, we did the experiment described in Table V. Two types of multilayer were used: those prepared with an initial antibody/lipid ratio of 10^{-4} and those containing no antibody. As can be seen from the table, only background binding (approximately 10–20%) was observed with multilayers containing a control P3–IgG or no antibody. The binding to the anti-H2K^k-containing multilayers could be completely blocked by pretreating either the cells with free anti-H2K^k (but not with the normal mouse IgG) or the multilayers with free goat anti-mouse IgG (but not with the normal goat IgG). This result clearly indicates that the rapid binding of RDM4 cells to the multilayer was immunospecific; i.e., it was mediated by immune complex formation.

The specific binding of RDM4 cells to the antibody-containing multilayers was very strong. If the bound cells were

Table V: Immunospecificity of RDM4 Cell Binding to Multilayers[a] Containing Palmitoyl-anti-H2K^k

pretreatment of multilayer[b]	pretreatment of cells[c]	% cell binding[d] for an initial antibody/lipid ratio of 10^{-4}	% cell binding[d] for an initial antibody/lipid ratio of 0
none	none	85	12
goat × mouse IgG	none	12	12
normal goat IgG	none	85	14
none	free anti-H2K^k	18	16
none	free normal mouse IgG	90	11
none	none	18[e]	12

[a] Multilayers were prepared with PC/chol (3/1). Total lipid concentration was 10 mM. [b] Pretreatment was with 1 mg/mL for 1 h at room temperature. [c] Pretreatment was with 0.2 mg/mL for 2 h at 4 °C. [d] Cell binding to multilayers was for 1.5 min at room temperature. [e] Palmitoyl-P3–IgG was incorporated into the multilayer.

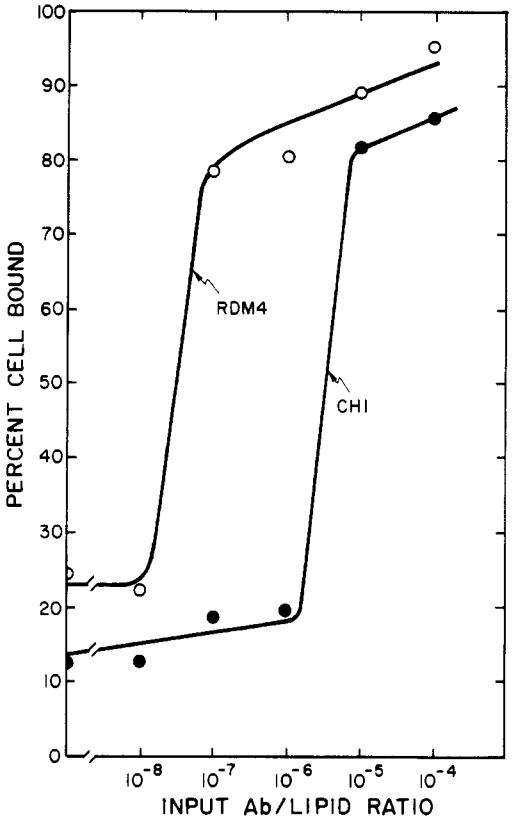

FIGURE 3: Dependence of cell binding on the antibody density of the multilayer. Multilayers were prepared with 10 mM lipid of PC/chol (3:1) and various amounts of palmitoyl-anti-H2K^k. RDM4 (○) and CH1 (●) cells were allowed to bind to the multilayers for 5 min at room temperature.

sonicated for 30 s in a bath sonicator, pieces of plasma membrane left on the multilayer could be seen with the cytoplasm and nuclei of the cells removed by the sonication.

The nonimmunospecific binding of RDM4 cells, i.e., binding to the multilayers containing no antibody, was a very slow process. It took about 10–20 min at room temperature to reach 50% cell binding. Such binding could not be inhibited by free anti-H2K^k or by the goat anti-mouse IgG (data not shown).

Dependence of Cell Binding on Antibody Density. A series of multilayers were prepared with different initial or input antibody/lipid ratios. RDM4 and CH1 cells, both k haplotype, were allowed to bind to the multilayers for 5 min at room temperature. As can be seen in Figure 3, the binding of both

cell types exhibited threshold dependence on the antibody density of the multilayer. Above the threshold density, maximal cell binding (80–100%) was readily obtained. Below the threshold, only background binding was observed. The threshold antibody density for the RDM4 cells was approximately 2 orders of magnitude lower than that for the CH1 cells.

Discussion

Phospholipids and cholesterol can be deposited on the surface of an alkylated glass by a simple procedure of dialysis. These lipids are apparently organized in a continuous multilayer which is quite homogeneous and free of large-scale structural defects as revealed by fluorescence microscopy. This morphological feature suggests that the lipid multilayer is a continuous structure, at least within the resolution of light microscopy. This notion is also supported by the observation that the lipids in the multilayer can diffuse rapidly over a distance of a few micrometers as indicated by the result of the FRAP measurements. At present, it is not known how these large-scale continuous multilayers are generated. One possiblity is that they are assembled by a process of nucleation similar to that of crystal growth. In the presence of deoxycholate, the hydrophobic surface of the alkylated glass might be coated with a mixture of lipid and detergent molecules in a rapid equilibrium with the mixed micelles remaining in the bulk solution. As the detergent is removed from the glass surface by dialysis, lipids are left behind and organized into lamellar structures. This hypothesis is supported by the fact that the number of lipid layers on the coverslip is directly related to the lipid concentration in the initial dialysis mixture. The lipids in the multilayer are probably not in a rapid equilibrium with those in the liposomes, since no multilayer can be deposited on the alkylated coverslip when dialyzed in the presence of detergent-free liposomes. This may be due to the extremely low aqueous solublity of the egg PC.

One interesting observation is that the acylated, but not the nonacylated, IgG can be readily incorporated into the multilayer. Previous analyses have shown that the palmitoyl-IgG contains three to four palmitoyl chains per IgG molecule (Huang et al., 1982) and further that most of the palmitoyl chains are located in the F_c portion of the molecule (L. Huang and A. S. Wilhite, unpublished results). These derivatized antibody molecules are uniquely suited for incorporation into lipid membranes such as the liposome (Huang et al., 1980, 1982; Shen et al., 1982). While it is not clear why the multilayer is relatively enriched with the palmitoyl antibody, the distribution of the antibody in the multilayer appears to be uniform. Furthermore, at least some of the incorporated antibody molecules are located on the surface of the multilayer, since the antigen-bearing cells such as RDM4 cells can rapidly bind to them. The observation that the majority of the incorporated antibody is laterally immobile is somewhat surprising. The immobility is probably not due to the overcrowding of the antibody as a result of the high degree of antibody enrichment in the multilayer, since antibody is still immobile in multilayers containing 10-fold less antibody. The immobility of the antibody is also not an intrinsic property of the antibody. The same palmitoyl antibody is relatively mobile ($D = 1.87 \times 10^{-8}$ cm^2/s at room temperature) when incorporated into multilamellar liposomes of the same lipid composition (L. Huang and R. M. Weis, unpublished result). Probably the multilayers on the glass slides are closely opposed to one another, and this may lead to the majority of the protein being tightly constrained by neighboring bilayer interactions. Another possibility is that the palmitoyl chains of the antibody are simultaneously inserted into the neighboring lipid bilayers, causing a reduced rate of diffusion. Furthermore, the acylated antibody could be trapped between the lipid layers in a form of lipid/protein aggregates or micelles which would not be free to diffuse laterally.

Antigen-bearing cells can rapidly bind to multilayers containing the appropriate antibody. The rate of binding is apparently a function of the antigen content of the cell. This result indicates that a sufficient number of antigen/antibody bonds can be formed in a short period of time when a high density of antigen is present on the cell surface. For those cells expressing a lower density of the surface antigen, perhaps stable binding can be achieved only after the antigen molecules laterally diffuse into the region of contact with the multilayer. Assuming that the lateral diffusion coefficient of the $H2K^k$ glycoprotein on the cell surface is that of a typical transmembrane protein, i.e., $D = 10^{-9}$–10^{-10} cm^2/s at room temperature (Peters, 1981), it would take a few minutes for the antigen molecules to diffuse into the contact area and establish stable cell binding. This is consistent with the time course of cell binding shown in Figure 2. This model does not take into account the complex cell surface geometry (e.g., microvilli) and the repulsive forces between the cell surface and the multilayer. Although the model is undoubtedly an oversimplified one, it is consistent with the observation that the immunospecific cell binding exhibits a threshold dependence upon the antibody density on the multilayer. Stable cell binding can occur only if the attractive force provided by a sufficient number of antigen/antibody bonds exceeds the repulsive forces existing between the cell and the multilayer. It is not difficult to imagine that the RDM4 cells with a higher antigen density[2] can better establish a sufficient number of antigen/antibody bonds with the multilayers containing a lower antibody density than that required for stable binding of CH1 cells. Thus, the threshold antibody density for the RDM4 cells is significantly lower than that for the CH1 cells. Similar threshold phenomena have been observed by Schnaar et al. (1978) and by Weigel et al. (1979) on the binding of hepatocytes to slab gels containing various densities of specific monosaccharide residues. The underlying thermodynamic principles have been thoroughly discussed by Dembo & Bell (1984).

In summary, we have developed a simple procedure to incorporate antibody into a large, planar, and optically clear model membrane. Although the incorporated antibody is laterally immobile, a sufficient number of functionally intact antibody is expressed on the surface of the multilayer for specific cell binding. Preliminary results indicate that other membrane proteins such as the $H2K^k$ (M. Nakanishi, personal communication) and the Herpes Simplex virus glycoproteins (L. Huang and S. Painter, unpublished observation) can also be incorporated into the multilayers. It remains to be seen whether these proteins are also laterally immobile as the acylated antibody studied here. Since the system is a convenient one for biophysical measurements as well as for cell binding, it may be very useful for model studies of cellular recognition.

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[2] RDM4 cells contained 2.4-fold more $H2K^k$ antigen molecules per cell than the CH1 cells (Table IV). Since the RDM4 cells were approximately half the size of the CH1 cells, the actual antigen density of the RDM4 cells was approximately 10-fold higher than CH1 cells.

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Registry No. Chol, 57-88-5; NBD-Cl, 10199-89-0; palmitic acid, 57-10-3.

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Hemichrome Binding to Band 3: Nucleation of Heinz Bodies on the Erythrocyte Membrane†

Stephen M. Waugh and Philip S. Low*

Department of Chemistry, Purdue University, West Lafayette, Indiana 47907
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ABSTRACT: Hemichromes, the precursors of red cell Heinz bodies, were prepared by treatment of native hemoglobin with phenylhydrazine, and their interaction with the cytoplasmic surface of the human erythrocyte membrane was studied. Binding of hemichromes to leaky red cell ghosts was found to be biphasic, exhibiting both high-affinity and low-affinity sites. The high-affinity sites were shown to be located on the cytoplasmic domain of band 3, since (i) glyceraldehyde-3-phosphate dehydrogenase, a known ligand of band 3, competes with the hemichromes for their binding sites, (ii) removal of the cytoplasmic domain of band 3 by proteolytic cleavage causes loss of the high-affinity sites, and (iii) the isolated cytoplasmic domain of band 3 interacts tightly with hemichromes, rapidly forming a pH-dependent, water-insoluble copolymer upon mixing in aqueous solution. Since the copolymer of hemichromes with the cytoplasmic domain of band 3 was readily isolatable, a partial characterization of its properties was conducted. The copolymer was shown to be of defined stoichiometry, containing ~2.5 hemichrome tetramers (or ~5 hemichrome dimers) per band 3 dimer, regardless of the ratio of hemichrome:band 3 in the initial reaction solution. The copolymer was found to be of macroscopic dimensions, generating particles which could be easily visualized without use of a microscope. The coprecipitation was also highly selective for hemichromes, since, in mixed solutions with native hemoglobin, only hemichrome was observed in the isolated pellet. Furthermore, no precipitate was ever observed upon mixing the cytoplasmic domain of band 3 with oxyhemoglobin, deoxyhemoglobin, (carbonmonoxy)hemoglobin, or methemoglobin. The affinity of the cytoplasmic domain of band 3 was likely much higher for hemichromes than for native hemoglobin, since a 20-fold molar excess of hemoglobin was required to reduce copolymerization by 50%. We suggest that the copolymerization of band 3 and hemichromes in vivo can explain the aggregation of Heinz bodies on the erythrocyte membrane and the resulting hemolysis observed in numerous hemoglobinopathies.

The interaction of native hemoglobin (Hb) with the human erythrocyte membrane has recently received considerable attention. Two classes of binding sites have been identified, and one of these, the higher affinity class, has been located on the cytoplasmic domain of the major erythrocyte protein, band 3 (Shaklai et al., 1977a,b; Salhany et al., 1980; Sayare & Fikiet, 1981). This interaction has been shown to be electrostatic in nature with the affinity of Hb for the membrane increasing as pH and ionic strength decrease (Shaklai et al., 1977a,b; Fung, 1981; Fischer et al., 1975). A similar pH dependence of Hb binding to the membrane has also been reported for intact cells (Eisinger et al., 1982). The isolated cytoplasmic domain of band 3 has also been shown to bind two molecules of hemoglobin, supporting the identification of band 3 as a major membrane attachment site for soluble

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