Phospholipid Membranes Form Specific Nonbilayer Molecular Arrangements That Are Antigenic*

(Received for publication, April 7, 1999, and in revised form, June 19, 1999)

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Hexagonal phase (H₃)ₐ-prefering lipids such as phosphatidate, cardiolipin, and phosphatidylserine form nonbilayer molecular arrangements in lipid bilayers. While their presence in biological membranes has not been established, in vitro studies suggest that alterations in membrane properties modify their function. In this study, antiphospholipid monoclonal antibodies were developed against nonbilayer structures. One of the monoclonal antibodies identifies nonplanar surfaces in liposomes and in membranes of cultured cells. These results are the first evidence that natural membranes maintain a fragile balance between bilayer and nonbilayer lipid arrangements. Therefore, these antibodies can be used to evaluate the role of H₃ₐ-prefering lipids in the modulation of membrane activities. Our studies demonstrated that nonplanar surfaces are highly immunogenic. Although these structures are normally transient, their formation can be stabilized by temperature variations, drugs, antibodies, apolar peptides, and divalent cations. Our studies demonstrated that abnormal exposure of nonbilayer arrangements may induce autoimmune responses as found in the antiphospholipid syndrome.

The lamellar or bilayer phase is the most common molecular association adopted by phospholipids and glycolipids in the matrix of cell membranes. However, membrane lipids are in dynamic transition, 30–40% can adopt nonbilayer arrangements (1–5). In isolated form, some phospholipids form hexagonally packed cylinders of the hexagonal II (H₃ₐ) tubular phase. The H₃ₐ-prefering lipids can also form intermediate structures of the H₃ₐ phase in nonbilayer lipid bilayers of experimental model systems, such as liposomes (2–4). Electron microscopy studies demonstrate that these nonbilayer structures appear as protruberances on the surface of liposomes (2, 3, 6). These "rough" surfaces are transient and may represent a physical intermediate of natural membranes. Their formation can be triggered by temperature variations, drugs, antibodies, apolar peptides, and divalent cations (2, 3, 6, 7). Nonbilayer lipids may be involved in specific cellular activities, such as membrane fission-fusion processes (2–4, 6, 7), organization of tight junctions between mammalian cells (8), and the specific activation of several membrane enzymes (9). Nonbilayer structures have been shown in model systems by physical methods (2–4, 6, 7, 10–12); their presence in biological membranes has not been confirmed (10, 13), however, they appear to be essential for the viability of Escherichia coli cells (14, 15).

In this study, antiphospholipid monoclonal antibodies were developed against nonbilayer phospholipid arrangements produced on liposomes. These antibodies also identify nonbilayer structures in membranes of cultured cells. These results are the first evidence that nonbilayer lipid structures are present in cells and may be involved in the production of antiphospholipid autoantibodies which can participate in the development of the human antiphospholipid syndrome (16).

EXPERIMENTAL PROCEDURES

Preparation and Characterization of Liposomes—Liposomes prepared from specific lipids in TBS buffer (10 mM Tris-HCl, 1 mM NaCl, pH 7.4) (10) were characterized by freeze-fracture electron microscopy (6) and by their 31P nuclear magnetic resonance (NMR) spectra (6, 12).

Immunization Scheme to Obtain Polyclonal Antiphospholipid Antibodies against Nonbilayer Phospholipid Arrangements—Polyclonal antibodies were obtained as described previously (10) using egg yolk L-a-phosphatidylcholine/egg yolk L-a-phosphatidic acid (PC/PA) 2:1 (mole ratio) liposomes in 5 mM Mn²⁺ as antigen.

Preparation of Antiphospholipid Monoclonal Antibodies against Nonbilayer Phospholipid Arrangements (APmAb)—Hybridomas were obtained by fusing myeloma P3X63Ag8U.1 with spleen cells isolated from mice immunized with PC/PA liposomes in 5 mM Mn²⁺. Supernatants were screened for APmAb by a liposomal ELISA method and flow cytometry (10, 17). Individual monoclonal antibodies were obtained by limited dilution (17). Clones producing APmAb were expanded by in vitro culture and by inoculation in BALB/c mice to obtain ascites fluid. APmAb was detected by a liposomal ELISA method and flow cytometry.

Liposomal ELISA—ELISA wells were coated with liposomes (0.1 μmol/100 μl of TBS) alone or treated with 3 mM Ca²⁺ or 5 mM Mn²⁺. Wells blocked with 0.4% gelatin in TBS alone or containing the appropriate cation were incubated with inactivated immune mice sera or APmAb diluted in the correspondent TBS buffer. Peroxidase-conjugated goat anti-mouse polyclonal or anti-mouse IgM antibodies were used. Bound antibodies were detected by reaction with o-phenylenediamine solution.

Liposomal Flow Cytometric Analysis—Liposomes (0.1 μmol/100 μl of TBS) alone or treated with 3 mM Ca²⁺ or 5 mM Mn²⁺ were incubated at 4°C for 1 hour. Portions of 100 μl of liposome suspension were incubated in 96-well ELISA plates and washed three times with TBS. Bound antibodies were detected by reaction with o-phenylenediamine solution.

The abbreviations used are: H₃ₐ, hexagonal II; PC, L-a-phosphatidylcholine; PA, L-a-phosphatidic acid; APmAb, antiphospholipid monoclonal antibodies against nonbilayer phospholipid arrangements; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; FSC, forward scatter light; SSC, side scatter light; FL1, relative fluorescence; FRET, fluorescence resonance energy transfer assay; NBD, 1-acyl-2-[12(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl; CL, cardiolipin; PS, L-a-phosphatidylserine; PG, L-a-phosphatidyl-DL-glycerol; Chol, cholesterol; DPPC, dimyristoyl-L-a-phosphatidylcholine; DPPA, dipalmitoyl-L-a-phosphatidic acid.
37 °C for 1 h in the dark with FITC-H308-APAb at 1:10 dilution in TBS alone or containing 3 mM CaCl2 or 5 mM MnCl2. Liposomes were washed by sedimentation at 200,000 x g for 50 min at 18 °C with the appropriate TBS buffer. Flow cytometry was accomplished using a Becton Dickinson FACSCalibur Flow Cytometer. Ten-thousand liposomes were analyzed using CELLQuest software at FL1 compensation of 0.8% and a detector compensation threshold FSC-H of 52 V, with the detectors: FSC of 700, SSC of 401 V, and FL1 of 748 V. Results are reported as relative fluorescence (FL1) and relative forward (FSC) and side scatter (SSC) light histograms in logarithmic mode (18). In a similar way as was described for cells (18), the diffraction of the laser beam (FSC) is proportional to the liposome surface area and/or liposomal aggregation, while refraction plus reflection of the beam (SSC) are proportional to the complexity of liposomal bilayers (10). As a negative control an irrelevant monoclonal antibody of IgM isotype directed to a surface protein of *Trichinella spiralis* (Ts-mAb) was used (19).

**Detection of antiphospholipid antibodies by ELISA.** A. PC/PA 2:1 liposomes incubated with TBS buffer alone or containing 5 mM MnCl2 or 3 mM CaCl2 were used as antigens. Liposomes were tested with Ts-mAb, polyclonal antibodies from immune BALB/c mouse sera at 1:50 dilution, and H308 hybridoma supernatant containing APAb at 1:10 dilution. B. purified PC, PA, or CL was used as antigen. Lipids were tested with polyclonal antibodies from immune BALB/c mouse sera or H308 hybridoma supernatant containing APAb. Absorbance values were determined in triplicate and represent the means ± S.D. that did not modify the size of liposomes (Fig. 1A). Both cations also induced nonlamellar structures in liposomes containing the HII-preferring lipids bovine brain cardiolipin (CL) or bovine brain 1-α-phosphatidylinositol (PS), which were similar to those described for PC/PA liposomes. In contrast, PC and egg yolk 1-α-phosphatidylinositol-β-glycerol (PG)/cholesterol (Chol) 1:1 liposomes, without HII-preferring lipids, displayed smooth surfaces either alone or when they were incubated with the cations (data not shown). Liposomes treated with Ca2+ or Mn2+ remained nonaggregated, since it was not necessary to add EDTA to visualize them by freeze-fracture as individual entities (6). After the addition of Ca2+ (3 mM) to liposomes containing PA or CL, the 31P NMR spectrum became sharper than those without cations and had a slight shift to a lower field (Fig. 1B). Mn2+ induces the formation of nonbilayer structures. Mn2+ penetrates the vesicle exerting paramagnetic effects on phospholipids from both the outer and inner liposomal bilayers, which completely broadens the NMR spectra (6, 12). Similar changes in NMR spectra of all liposomes were obtained with 0.5 mM Ca2+ or Mn2+. PC liposomes did not show spectral changes when they were incubated with Ca2+ (Fig. 1B). However, in the presence of Mn2+ (3 mM) the spectrum signal intensity decreased. This change is due to the...
Mn\textsuperscript{2+} paramagnetic effect and was exerted only on lipids from outer liposomal bilayers (Fig. 1B); similar results were obtained with PG/Chol 1:1 liposomes. Freeze-fracture and NMR studies clearly showed the nonbilayer structures on liposomes. Both methods demonstrated that only liposomes containing HII-preferring lipids produce nonbilayer structures.

Antiphospholipid Antibody Specificity—Antibodies were evaluated by an ELISA method using PC/PA 2:1 liposomes as the antigen. Both polyclonal and hybridoma supernatant containing APmAb bind to nonbilayer structures. The highest binding was attained with APmAb clone H308 and Mn\textsuperscript{2+}-induced nonbilayer structures (Fig. 2A). Polyclonal antibodies, but not H308-APmAb, also recognized the smooth liposomes incubated with TBS buffer. The irrelevant monoclonal antibody Ts-mAb did not show reaction with smooth liposomes or bearing nonbilayer structures (Fig. 2A). Using this method, 42 hybridomas producing APmAb were selected. The isotype of all APmAb obtained was IgM as determined by ELISA. Since the routine method to clinically measure antiphospholipid autoantibodies is an ELISA assay that uses a coated plate with lipids dissolved in ethanol (22), the binding of H308-APmAb to purified PC, PA, or CL was evaluated. Our data showed that H308-APmAb did not interact with the nonliposomal lipid-coated plates. In contrast, polyclonal antibodies bind to these lipid-coated surfaces (Fig. 2B). These results strongly suggest that H308-APmAb interacts with nonplanar lipid surfaces.

The fact that H308-APmAb specifically recognizes nonbilayer structures on liposomes, an antigen similar to biological membranes, and not lipid-coated surfaces suggests that antibodies similar to H308-APmAb could be produced in human autoimmune disorders such as the antiphospholipid syndrome.

Binding of H308-APmAb to Nonbilayer Structures—H308-APmAb was affinity-purified directly from ascites by taking advantage of the property of H308-APmAb to bind to liposomes bearing Mn\textsuperscript{2+}-induced nonbilayer structures. All H308-APmAb bound to liposomes was specifically eluted by chelation of Mn\textsuperscript{2+} (Fig. 3C). A considerable portion of purified H308-APmAb was not glycosylated (Fig. 3C).

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Antigenic Phospholipid Membranes

**Fig. 3.** Flow cytometric and FRET analysis of the reactivity of H308-APmAb with nonbilayer structures on liposomes. A, liposomes containing the HII-preferring lipids PA or CL and those made from PC, DPPC, and DPPA were used as antigens. PC/PA 2:1, PC/CL 2:1, and PC liposomes alone or treated with 3 mM CaCl\textsubscript{2} or DPPC/PC/DPPA 1:2:0.8:1 liposomes treated with 5 mM BaCl\textsubscript{2} or 0.05 mM LaCl\textsubscript{3} were tested with affinity-purified H308-APmAb. Changes in liposomal fluorescence, bilayer complexity (SSC), and liposomal aggregation (FSC) due to the incubation of liposomes with the cations and/or FITC H308-APmAb or FITC Ts-mAb were evaluated. Controls represent both liposomes alone or incubated with Ca\textsuperscript{2+} or with Ca\textsuperscript{2+} plus FITC-Ts-mAb. One experiment representative of three is shown. B, fluorescent quenching was induced by binding of H308-APmAb to PC/NBD-PA or PC/NBD-PS 2:1 liposomes in 3 mM CaCl\textsubscript{2} or 5 mM MnCl\textsubscript{2}. Measurements were obtained using a 95% confidence level (p = 0.05). C, affinity purification of APmAb. Ascites fluid was mixed with PC/PA 2:1 liposomes in 5 mM MnCl\textsubscript{2}. SDS-polyacrylamide gel electrophoresis and immunoblot show purified APmAb. The IgM heavy chain (HC) is glycosylated (HC-glycos) and nonglycosylated (HC), and both forms were identified with peroxidase-labeled goat-anti-mouse IgM heavy chain. LC, light chain.

**Fig. 4.** Direct immunostaining of mammalian cells. HeLa cells were subjected to a direct immunofluorescence using Cy3-H308-APmAb (A) and Cy3-Ts-mAb (C). B and D show phase contrast of the same fields. Arrows show cells immunostaining.
after the immunoreaction, compared with the pattern of these structures on Ca\(^{2+}\)-treated liposomes (Figs. 3A, panels b and n). These profiles reflect the dynamic feature of nonbilayer arrangements. Liposomal aggregation (FSC), which can produce nonspecific increases in fluorescence, was not evident, since the FSC values, after the antibody binding, were similar to those of liposomes bearing nonbilayers structures without H308-APmAb (Figs. 3A, panels c and o). Liposomal fluorescence, SSC, and FSC values in the presence of affinity-purified Ts-mAb were similar to that of Ca\(^{2+}\)-treated liposomes, demonstrating that Ts-mAb does not bind vesicles. Analysis of liposomes containing PA incubated with Ca\(^{2+}\) and Ts-mAb is also shown in Fig. 3A, panels m to o. H308-APmAb also binds to liposomes bearing Ca\(^{2+}\)-induced nonlamellar structures formed by PS or CL. Analysis of liposomes containing CL is shown in Fig. 3A, panels d to f. This reaction, although positive, was clearly distinct from the reaction with liposomes containing PA (Fig. 3A, panels b and e). H308-APmAb did not show interaction with PC liposomes alone or incubated with Ca\(^{2+}\) (Fig. 3A, panel g). Furthermore, values of FSC and SSC did not show liposomal aggregation or the presence of nonbilayer structures (Fig. 3A, panels h and i). Similar results were obtained with PG/Chol 1:1 liposomes. In addition, similar binding of H308-APmAb was obtained when all liposomes were incubated with 0.5 mM Ca\(^{2+}\). These results support the fact that only liposomes containing nonbilayer structures have an immunoreaction with H308-APmAb.

An alternative conclusion is that the antibody recognizes the lipid-divalent cation complex and/or the resultant reduction in the liposomal charge in the presence of divalent cations. These possibilities were eliminated by the use of "rigid" liposomes. Liposomes made from dipalmitoyl-L-phosphatidylcholine (DPPC)/PC/dipalmitoyl-L-\(l\)-phosphatidate (DPPA) 1.2:0.8:1 or DPPC/PE/coli CL 1:2:0:8:1 have a higher content of saturated fatty acids in their DPPC, DPPA, or E. coli CL (23). Consequently the bilayers of these liposomes are more rigid than the bilayers of the liposomes containing similar lipids isolated from egg yolk or bovine heart such as those from Fig. 3A (panels a–f and m–o). Divalent (Ba\(^{2+}\) or Ca\(^{2+}\)) or trivalent (La\(^{3+}\)) ions can interact with rigid liposomes and modify their surface charge, but these interactions are not associated with the formation of nonbilayer structures as is shown for DPPC/PC/DPPA liposomes (Fig. 3A, panels k and l). These results were corroborated by NMR spectroscopy (data not shown). H308-APmAb did not show interaction with rigid liposomes incubated with Ba\(^{2+}\) or La\(^{3+}\) (Fig. 3A, panels j–l). These results confirm that the H308-APmAb recognition of the anionic lipid-metal ion complex is only associated with the transition of lipids to nonbilayer structures.

The binding of H308-APmAb was also analyzed by the quenching of NBD-chromophore induced by clustering of fluorescent NBD phospholipids upon H308-APmAb-membrane binding. Affinity-purified H308-APmAb bound to liposomes containing NBD-PA or NBD-PS, while Ts-mAb did not present any binding (Fig. 3B). These results corroborate that H308-APmAb specifically binds to Ca\(^{2+}\) or Mn\(^{2+}\)–induced nonbilayer structures.

Liposomal ELISA, flow cytometry, and FRET demonstrated that the specificity of H308-APmAb is against a nonbilayer structure formed by the HII–preferring anionic phospholipids PA, CL, or PS. These data suggest that the specificity of H308-APmAb is not against a chemical structure but against a specific lipid molecular arrangement.

**Immunolocalization of Nonbilayer Structures in Mammalian Cell Membranes**—In mammalian cells, negatively charged surfaces are formed by the exposure of anionic phospholipids on the outer leaflet of plasma membranes. In the present study, it has been demonstrated that the anionic phospholipids PA, CL, and PS form antigenic nonbilayer structures recognized by our APmAb. Thus, we analyzed whether nonbilayer structures are formed on the membranes of mammalian cells. Cell lines HeLa, C5337, C33, and MCF-7 were directly immunostained with a Cy3-conjugated H308-APmAb. Immunostaining of cells showed that H308-APmAb recognizes cellular membranes possible in apoptosis and necrosis (Fig. 4A). This finding indicates that nonbilayer phospholipid arrangements occur in these biological membranes. These findings support the studies that demonstrate a strong correlation between nonbilayer arrangements and the viability of E. coli (14, 15).

These results indicate that cells maintain a fragile balance between bilayer and nonbilayer lipid arrangements during the dynamic events of membrane function. The presence of these unique phospholipid arrangements suggests their involvement in membrane functions. Therefore, this monoclonal antibody can be used to evaluate the role of HII–preferring lipids in the modulation of physiological and pathological membrane activities. These unique antimonilayer antibodies could be different antibodies to those currently detected in clinical assays using lipid-coated ELISA plates, including anti-cardiolipin and anti-phosphatidylserine antibodies. It has been reported that lupus anticoagulant antibodies specifically recognize the HII tubular phase of phosphatidylethanolamine but not the bilayer arrangements of this lipid (13, 24). However, it is difficult to reconcile membrane features with extensive areas of the lipid HII phase. Instead, we propose that intermediate structures of the HII phase in lipid bilayers, such as those analyzed here, are more compatible with the structure and the modulation of physiological and pathological membrane activities. Since nonbilayer structures are immunogenic, their abnormal exposure may induce autoimmune responses as found in patients with primary antiphospholipid syndrome, systemic lupus erythematosus, stroke, and cancer.

**Acknowledgments**—We thank Dr. R. Mondragón and Dr. S. González from the Electron Microscopy Unit, Center for Research and Advanced Studies, México for electron microscopy studies.

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