Interaction of Anti-Phospholipid Antibodies With Late Endosomes of Human Endothelial Cells

Béatrix Galve-de Rochemonteix, Toshihide Kobayashi, Corinne Rosnoblet, Margaret Lindsay, Robert G. Parton, Guido Reber, Emmanuel de Maistre, Denis Wahl, Egbert K.O. Kruithof, Jean Gruenberg, Philippe de Moerloose

Abstract—Anti-phospholipid antibodies (APLAs) are associated with thrombosis and/or recurrent pregnancy loss. APLAs bind to anionic phospholipids directly or indirectly via a cofactor such as β2-glycoprotein I (β2GPI). The lipid target of APLA is not yet established. Recently, we observed that APLAs in vitro can bind lysobisphosphatidic acid (LBPA). The internal membranes of late endosomes are enriched in this phospholipid. The current study was undertaken to determine to what extent binding of APLA to LBPA is correlated with binding to cardiolipin and to β2GPI and to determine whether patient antibodies interact with late endosomes of human umbilical vein endothelial cells (HUVECs) and thus modify the intracellular trafficking of proteins. Binding of patient immunoglobulin G (n = 37) to LBPA was correlated significantly with binding to cardiolipin. Although LBPA binding was correlated to a lesser extent with β2GPI binding, we observed that β2GPI binds with high affinity to LBPA. Immunofluorescence studies showed that late endosomes of HUVECs contain LBPA. Patient but not control antibodies recognized late endosomes, but not cardiolipin-rich mitochondria, even when we used antibodies that were immunopurified on cardiolipin. Incubation of HUVECs with patient plasma samples immunoreactive toward LBPA resulted in an accumulation of the antibodies in late endosomes and led to a redistribution of the insulinlike growth factor 2/mannose-6-phosphate receptor from the Golgi apparatus to late endosomes. Our results suggest that LBPA is an important lipid target of APLA in HUVECs. These antibodies are internalized by the cells and accumulate in late endosomes. By modifying the intracellular trafficking of proteins, APLA could contribute to several of the proposed pathogenic mechanisms leading to the antiphospholipid syndrome. (Arterioscler Thromb Vasc Biol. 2000;20:563-574.)

Key Words: anti-phospholipid antibodies ▪ late endosomes ▪ lysobisphosphatidic acid ▪ β2-glycoprotein I ▪ human endothelial cells

The anti-phospholipid antibody (APLA) syndrome (APS) is a condition characterized by various clinical manifestations in combination with the presence of APLAs. It has been suggested that many of the APLAs bind indirectly to anionic phospholipids via phospholipid-binding proteins. Of these, β2-glycoprotein I (β2GPI) is the most intensively studied, but other phospholipid-binding proteins such as prothrombin, annexin V, protein C, thrombomodulin, proteoglycans, or other antigens on endothelial cells (ECs) or platelets have also been mentioned.1–4

The mechanisms of the prothrombotic state associated with the APS are poorly understood but may involve modifications of platelet functions and monocyte tissue factor expression.5–7 Moreover, several pathogenic mechanisms for APLA have been proposed that affect ECs.1,4,8–13 Taken together, these studies suggest that APLAs may, at least in part, exert their thrombogenic effect by inhibiting the activated protein C–thrombomodulin anticoagulant pathway on ECs, by activating ECs to become prothrombotic or by inducing apoptosis of ECs that leads to deendothelialization and exposure of the thrombogenic subendothelium. It remains to be established whether these proposed mechanisms are independent or reflect common underlying mechanisms.

Late endosomes reside on the pathway leading to degradation in the lysosomes and function as an important protein-sorting station between secretory and degradation pathways. A unique feature of late endosomes is that they contain a complex system of poorly characterized internal membranes in their lumen. Using baby hamster kidney (BHK) cells, we recently observed that these internal membranes contain large amounts of a unique anionic phospholipid, lysobisphosphatidic acid (LBPA), that is recognized by APLAs.14 Incubation of BHK cells with a monoclonal anti-LBPA antibody and with APLA from a...
few patients resulted in accumulation of the antibodies in late endosomes.

The current study was undertaken to investigate in more detail to what extent (direct or indirect) the binding of APLAs to intracellular LBPA may constitute a plausible mechanism for the thrombogenic effects of APLA. To this end, we studied (1) to what extent, in an ELISA assay, binding of APLA to LBPA is correlated with immunoreactivities toward cardiolipin and toward \( \beta_2 \) GPI; (2) whether LBPA is detectable in late endosomes of human ECs; and (3) whether antibodies from patients with APLA are internalized, retained in the late endosomes of EC, and interfere with the normal protein-sorting functions of endosomes in these cells. The results show that late endosomes of human ECs contain LBPA and that APLAs accumulate in late endosomes and modify intracellular protein trafficking.

**Methods**

**Subjects**

Plasma was obtained from 37 patients with APLAs who were referred to the Hemostasis units of the University Hospitals of Nancy, France, and Geneva, Switzerland. The presence of APLAs was defined by the presence of a lupus anticoagulant and/or a positive result in an anti-cardiolipin antibody assay. Patients were diagnosed as having primary APS (n = 16); APS secondary to systemic lupus erythematosus (SLE, n = 9); SLE or overlap syndrome without manifestations of APS (n = 5); neoplasia (n = 2); or psychosis treated with neuroleptics (n = 1). Moreover, in 4 patients lupus anticoagulant was discovered preoperatively; in these patients lupus anticoagulant activity remained strongly positive 1 year after the preoperative work-up. None of these 4 patients had clinical manifestations of APS. As a control group, blood was drawn from 15 healthy persons.

**Materials**

Unless stated otherwise, all biochemical reagents and chemicals used in this study were from Sigma Chemical Co or Fluka and of the highest grade available. The composition of the PBS used was 0.27 mol/L NaCl, 5.4 mmol/L KCl, 10 mmol/L \( \text{Na}_2 \text{HPO}_4 \), and 1.76 mmol/L \( \text{KH}_2 \text{PO}_4 \) at pH 7.4.

**Figure 1.** A, Molecular structures of LBPA and cardiolipin. Each glycerol residue of LBPA contains 1 fatty acid molecule. The position of fatty acid in glycerol is not determined. B, Comparison of purified LBPA with cardiolipin. LBPA, 2.3 \( \mu \)g, and 2 \( \mu \)g of cardiolipin were applied to a silica gel 60 high-performance thin-layer chromatography plate (Merck), and components migrated in a chloroform/methanol/32% ammonia (65:25:5, vol/vol/vol) solvent. Lipids were visualized after being charred with cupric acetate.

The following antibodies were used: monoclonal antibody (MoAb) 6C4 that binds LBPA\(^{14} \); affinity-purified rabbit polyclonal anti–C-terminal p23 antibody\(^{15} \); MoAb anti–human-\( \beta_2 \)GPI (9G1, a gift from Dr J. Arvieux, Grenoble, France)\(^{16} \); rabbit antibodies against the insulinlike growth factor 2/mannose-6-phosphate receptor (IGF2/M6PR, a gift from Dr B. Hoflack, Institut Pasteur, Lille, France); rabbit anti-human von Willebrand factor (vWF) antibodies (Dako); rhodamine-conjugated goat anti-mouse antibodies (Jackson); LBPA was purified from BHK lipid extracts by preparative thin-layer chromatography after silica gel and DEAE column chro-

**Figure 2.** Correlation between binding of IgG from patients with APLA or from controls to cardiolipin, LBPA, and \( \beta_2 \)GPI. IgG samples were purified from the plasma of healthy controls (n = 15) or patients with APLA (n = 37) and analyzed by ELISA on microtiter plates coated with LBPA, cardiolipin, or \( \beta_2 \)GPI. A, Comparison between control IgG and patient IgG in the 3 assays. The horizontal lines represent the upper limit of the normal range, which is defined as the mean \( + \) 2SD of the normal population. B, Correlation between LBPA and cardiolipin binding for patient IgG. C, Correlation between LBPA and \( \beta_2 \)GPI binding for patient IgG.
matography. The purity of LBPA was assessed by thin-layer chromatography and mass spectrometry. Bovine heart cardiolipin was from Fluka.

To determine the phospholipid composition of human umbilical vein ECs (HUVECs), cells were metabolically labeled for 20 hours with $^{32}$P$_{i}$. After extraction, phospholipids were separated by 2D chromatography. The first dimension was run with chloroform/methanol/25% ammonia (65:25:5, vol/vol/vol) and the second dimension with chloroform/acetone/methanol/acetic acid/water (50:20:10:12.5:5, vol/vol/vol/vol/vol). Radioactive lipids were detected by autoradiography and then quantified by using the Molecular Image System (Bio-Rad GS-363).

**Assays for APLAs and Anti-$\beta_2$GPI Antibodies**

The binding of APLAs to cardiolipin or to LBPA was assayed on ELISA plates coated with the respective phospholipids. Microwell plates (Nunc) were coated overnight with a solution (30 μL/well) of 25 μg/mL of either cardiolipin (Fluka) or LBPA in 98% ethanol and 2% chloroform. As controls for nonspecific binding of patient IgG to the ELISA plates, we used wells treated with ethanol alone. Thereafter the plates were blocked with 10% FCS (Life Technologies) in PBS for 2 hours at room temperature and washed 3 times with PBS. In some experiments, we used LBPA- or cardiolipin-coated plates that were blocked with 1% BSA (Fluka) by a 2-hour incubation. Plasma samples (100 μL, diluted 1:100) or IgG (100 μL) at the final concentration of 20 μg/mL were added in duplicate. The plates were then incubated for 2 hours at room temperature and washed 3 times with PBS. Alkaline phosphatase–conjugated goat anti-human IgG was added to each well and incubated for 90 minutes at room temperature. After 3 washes, the wells were incubated at 37°C with $p$-nitrophenyl phosphate (Sigma), and alkaline phosphatase activity was measured at 405 nm in a microplate reader (Molecular Devices). For each plasma or IgG, the binding on uncoated wells was subtracted from that on phospholipid-coated wells.

Maxisorb $\gamma$-irradiated ELISA plates (Nunc) were coated for 2 hours at room temperature with human $\beta_2$GPI (50 μg/mL), left overnight at 4°C, and washed before use. The coated plates were incubated for 1 hour at room temperature with plasma (diluted 1/50) or with IgG (20 μg/mL). After 3 washes in PBS–TWEEN 20 (0.01%), the plates were exposed to alkaline phosphatase–conjugated goat anti-human IgG and incubated for 90 minutes at room temperature. After 3 washes, the wells were incubated at 37°C with $p$-nitrophenyl phosphate (Sigma), and alkaline phosphatase activity was measured at 405 nm in a microplate reader. On each plate, dilutions of an APLA–positive control plasma were included. Sample blanks (uncoated wells) were included. All results are expressed in arbitrary units with respect to a standard curve made with the positive control.

**Figure 3.** Binding of $\beta_2$GPI to LBPA and to cardiolipin. Human $\beta_2$GPI was incubated on LBPA- (●) or cardiolipin (○)-coated plates. Binding of $\beta_2$GPI was quantified by using MoAbs to $\beta_2$GPI and horseradish peroxidase–labeled secondary antibodies. Results are expressed as the mean of the absorbance read at 490 nm ± SEM. Half-maximal binding was observed at an MoAb concentration of 2.1 nmol/L for LBPA binding and of 0.5 nmol/L for cardiolipin binding.

For the binding of $\beta_2$GPI to cardiolipin or LBPA, plates were coated with cardiolipin or LBPA as described above. Then the plates were treated with 3% BSA for 2 hours at room temperature and incubated for 2 hours with 5 μg/mL purified human $\beta_2$GPI (a gift from Dr J. Arvieux). The plates were washed 3 times with PBS and then incubated for 90 minutes with dilutions of an MoAb (9G1) to human $\beta_2$GPI. The plates were washed 3 times and incubated for 1 hour with horseradish peroxidase–conjugated goat anti-mouse IgG (Bio-Rad); after being washed, o-phenylenediamine dihydrochloride substrate (0.4 mg/mL) from Sigma was added for 30 minutes. The color development was blocked by addition of 3 mol/L HCl and the optical density read at 490 nm.

**Affinity Purification of Anti-Cardiolipin Antibodies**

A Nunc plate was coated with cardiolipin as described above. After a 2-hour incubation with 10% FCS, the plate was washed 3 times with PBS. Diluted plasmas (1:10) in PBS from 5 patients and 1 control or respective IgG (60 μg/mL) were adsorbed for 45 minutes on a cardiolipin-coated well. The nonsorbed material was recovered and reapplied to a second cardiolipin-coated well. PBS was added to prevent the wells from drying out. This manipulation was repeated 5 times. The wells were eluted with 100 μL of 0.1 mol/L

**Figure 4.** Presence of LBPA in late endosomes of HUVECs. A, Lipid analysis. HUVECs were incubated for 20 hours with $^{32}$P$_{i}$. Lipids were extracted and separated by 2D thin-layer chromatography. Lipids were detected by autoradiography and quantified by using the Molecular Imager System (Bio-Rad). B and C, Localization of LBPA in HUVECs by immunofluorescence analysis. Cells were processed for double labeling by using an MoAb (6C4) specific for LBPA (B) or rabbit anti-human vWF antibodies that specifically label Weibel-Palade bodies (C). Scale bar=10 μm.
glycine-HCl, pH 2.2, and immediately neutralized with 10 μL of 1 mol/L Tris, pH 11.0. The immunopurified material was analyzed for binding activity toward cardiolipin and LBPA and for binding to fixed and permeabilized HUVECs. As a control, we performed these binding assays with the material adsorbed to and eluted from plates coated with 10% FCS only.

**Cell Culture**

HUVECs were isolated from umbilical cords and grown at 37°C in a humidified atmosphere containing 5% CO₂. In brief, the umbilical vein was washed with Krebs-Ringer bicarbonate buffer (120 mmol/L NaCl, 4.75 mmol/L KCl, 1.2 mmol/L KH₂PO₄, 0.6 mmol/L MgSO₄, 1.2 mmol/L CaCl₂, 25 mmol/L NaHCO₃, and 25 mmol/L HEPES, pH
LBPA

vWF

control

Figure 6. Accumulation of an anti-LBPA MoAb (6C4) in HUVECs. Cells were incubated for 20 hours with the 6C4 antibody (A) or with control antibody (C). After incubation, the cells were fixed and permeabilized. Internalized anti-LBPA antibody was revealed with rhodamine-conjugated anti-mouse antibodies. The cells incubated with the anti-LBPA antibody were doubly labeled with antibodies to vWF (B). The 6C4 antibody was detected in perinuclear vesicular structures (A), but not in Weibel Palade bodies. No internalization of control antibodies was observed (C). Scale bar=10 µm.

Immunogold Labeling and Electron Microscopy

To determine the ultrastructural localization in HUVECs of the antigen that binds to the 6C4 MoAb, cryosections of fixed HUVECs were incubated with 6C4 or with plasma from a patient with APLA and prepared for immunogold labeling and electron microscopy as previously described. To determine the site of accumulation of 6C4 in HUVECs, the cells were incubated for 20 hours with 6C4 (20 µg/mL) and then fixed and prepared for immunoelectron microscopy.

Results

Comparison of Purified LBPA With Cardiolipin

LBPA is a structural isomer of phosphatidylglycerol, and its structure is related to that of cardiolipin (Figure 1A). To compare the immunoreactivity of purified LBPA and cardiolipin, it was important to establish that preparations were not cross-contaminated. With the use of a basic solvent system, the 2 phospholipids were clearly separated by thin-layer chromatography. As shown in Figure 1B, we did not detect a cardiolipin contaminant in our LBPA preparation. Commercially obtained cardiolipin also did not contain LBPA.

Correlation of the Binding of Patient IgG to LBPA With the Binding to Cardiolipin and β2GPI

We measured binding activity in plasma and in purified IgG from 37 patients and 15 controls on ELISA plates coated with either cardiolipin or LBPA. No immunoreactivity against cardiolipin or LBPA was detected. Three of these negative patients were asymptomatic and 4 had SLE. A highly significant correlation was observed between anticardiolipin and anti-LBPA activities ($R^2=0.869, P<0.001$; Figure 2B). Similar results were obtained when plasmas instead of purified IgGs were used ($R^2=0.761, P<0.001$; results not shown). We also analyzed and compared the binding of the patient IgGs to β2GPI and LBPA.

7.4) and incubated for 10 minutes with 1 mg/mL collagenase (CLS type 1, Worthington Biochemical). Cells were collected by flushing the vein with 50 mL of RPMI 1640 supplemented with 10% FCS (Life Technologies). The cells were then grown in RPMI 1640 containing 90 µg/mL heparin (Boehringer Ingelheim), 15 µg/mL EC growth supplement (Upstate Biotechnology), 10 mmol/L HEPES, 20°C, 4 minutes) or 4% freshly depolymerized HUVECs grown on glass coverslips were fixed with either methanol

Indirect Immunofluorescence Staining

HUVECs grown on glass coverslips were fixed with either methanol (−20°C, 4 minutes) or 4% freshly depolymerized p-formaldehyde in PBS, pH 7.4 (24°C, 20 minutes). p-Formaldehyde–fixed cells were washed with PBS, treated for 20 minutes with 0.27% NH4Cl–0.38% glycine in PBS, and permeabilized for 30 minutes with 0.05% saponin and 10% FCS in PBS in the presence of primary antibody. For single-label analyses, cells were incubated sequentially with the primary antibody and the fluorescent secondary antibody. For double-label analyses, cells were incubated sequentially with a mixture of primary antibodies and a mixture of fluorescent secondary antibodies. Antibodies were diluted in 10% FCS in PBS. All incubations were performed for 1 hour at room temperature. After being washed, coverslips were mounted in polyvinyl alcohol. Samples were analyzed with a confocal laser scanning microscope (LSM410 invert, Carl Zeiss Inc) equipped with argon and helium/neon laser fluorescence at 488 and 543 nm, respectively. Fluorescein and rhodamine signal were recorded sequentially (emission filters BP510-525 and LP590) by using ×63 or ×100 plan Apochromat oil-immersion objectives.

Negative control experiments were performed by omitting the primary antibodies or by using an irrelevant primary antibody of the same species and IgG subclass (for MoAbs). For double-label analyses, we verified that FITC fluorescence gave no signal in the rhodamine channel and conversely. We also confirmed that the fluorescent secondary antibodies did not cross-react with immunoglobulins from animal species other than the target species.

To determine whether APLAs accumulate in HUVECs, the cells were cultured for 20 hours in the presence of patient plasma (1:10) in PBS or purified IgG (20 µg/mL) obtained from 10 patients who were strongly positive in all tests for APLA. Thereafter the cells were fixed and permeabilized. Immunofluorescence analysis was performed as described above.
IgGs from 27 patients exhibited $\beta_2$GPI binding above the normal range. A significant correlation ($R^2 = 0.469, P < 0.001$) was obtained between the binding to LBPA and $\beta_2$GPI. This finding raised the question whether $\beta_2$GPI binds to LBPA. In a direct comparative binding study, we observed that $\beta_2$GPI indeed bound to LBPA with an affinity only 4-fold lower than that obtained for the binding of $\beta_2$GPI to cardiolipin (Figure 3).

To determine whether there were patients with IgGs that bound directly to LBPA or cardiolipin, we also performed experiments on LBPA- or cardiolipin-coated plates that were blocked with albumin, i.e., without serum-derived cofactor for binding. Four patients with primary APS and 3 with APS and SLE had a high level of direct binding to LBPA. Two of these patients (1 primary APS and 1 APS/SLE) also had a high level of direct binding to cardiolipin. Furthermore, 3 patients (1 with psychosis, 1 with SLE, and 1 with neoplasia) had a high level of binding to cardiolipin alone.

To determine whether antibodies that bind (directly or via a cofactor) to cardiolipin also bind to LBPA, from 5 patients we immunopurified antibodies on cardiolipin-coated plates. Before adsorption to cardiolipin, the activity on cardiolipin-coated plates was, on average, 2-fold higher than the activity on LBPA-coated plates. The binding of these cardiolipin

Figure 7. Analysis by immunoelectron microscopy of the localization of 6C4 accumulation in HUVECs. Cells were incubated for 20 hours with the 6C4 antibody and cryosectioned. Sections of 6C4-treated cells were labeled with rabbit anti-mouse IgG, followed by 10-nm protein A–gold particles to detect the internalized 6C4 antibody. The gold particles are associated with late endosomes (L). In some areas of the cell, parallel membrane arrays labeled with gold particles were detected (small arrows). g indicates Golgi; n, nucleus. Scale bar = 200 nm.
affinity–purified antibodies was tested on both cardiolipin- and LBPA-coated plates. All antibodies bound to LBPA. The ratio of binding on the 2 antigens was similar to that observed before purification (1.8-fold).

Presence of LBPA in Late Endosomes of Human ECs

To determine whether LBPA was present in HUVECs, cells were metabolically labeled with \[^{32}P\]Pi. Then the lipids were extracted and analyzed by thin-layer chromatography. As shown in Figure 4A, a lipid was revealed that comigrated with authentic LBPA. This lipid represented 0.7% of total phospholipids of HUVECs. The cellular localization of LBPA in HUVECs was visualized by indirect immunofluorescence and confocal microscopy with the LBPA-specific MoAb 6C4. The 6C4 antibody stained perinuclear vesicular structures with a morphology and cellular distribution that are characteristic of late endosomes (Figure 4B) and are distinct from the elongated, rod-shaped structures of Weibel-Palade bodies (Figure 4C). Analysis by immunoelectron microscopy on cryosections of HUVECs showed that the 6C4 antibodies labeled the internal membranes of late endosomes (Figure 5A), as shown at higher magnification (Figure 5B). No labeling was observed on other membranes such as Golgi membranes or nuclear membranes. Taken together, the results show that HUVECs contain LBPA and that the lipid is localized within the internal membrane system of late endosomes.

Accumulation of an Anti-LBPA MoAb in Late Endosomes of ECs

Because the late endosomes of HUVECs contain LBPA and late endosomes are accessible to antibody endocytosed from the medium, we tested whether the anti-LBPA antibody 6C4 could reach and then bind to its antigen on endocytosis. Cells were incubated for 20 hours with the 6C4 MoAb, fixed, and processed for double immunofluorescent labeling for the MoAb and vWF. We observed in all HUVECs an accumulation of 6C4 within structures that closely resembled those observed after direct labeling of fixed cells with 6C4 (see Figure 6A and 6B). In contrast, control antibodies did not accumulate intracellularly (Figure 6C). To better visualize the distribution of the internalized 6C4 antibody, cells were analyzed by electron microscopy. Immunogold labeling of cryosections showed that the antibody accumulated within the internal membranes of late endosomes and that this accumulation caused internal membranes to become more compact (Figure 7).

Binding of APLAs to and Accumulation in Late Endosomes

The observation that APLAs from patients bind LBPA led us to study whether these antibodies recognized the late endosomes of fixed and permeabilized HUVECs. In indirect immunofluorescence analysis, purified IgGs from a patient with APLAs recognized perinuclear vesicular structures on cardiolipin and used for double labeling with anti-LBPA. Note the colocalization of crude (C) and immunopurified (G) APLAs with LBPA (D, H) and the absence of colocalization with p23 (F). No staining was seen with control IgG (B). Scale bar=10 µm.
measuring IgG binding to LBPA, cardiolipin, and β2GPI. The clinical characteristics of these patients were primary APS (n=4) and APS combined with SLE (n=2). After analyzing the other 6 patients, we saw either no staining or high background fluorescence. The clinical characteristics of these patients were primary APS (n=1), APS combined with SLE (n=3), type II diabetes (n=1), and neoplasia (n=1).

The vesicular structures stained with APLAs colocalized with LBPA (Figures 8C and 8D). To exclude the possibility that the colocalization of patient antibodies and anti-LBPA MoAb was due to nonspecific binding of secondary antibodies to APLA antibodies, cells were doubly labeled with patient plasma and antibodies against p23, a Golgi membrane protein15 (Figures 8E and 8F). Clear segregation of fluorescent secondary antibodies indicates that the observed colocalization was not due to nonspecific absorption of secondary antibodies. These results indicate that APLAs from certain APS patients recognize late endosomes. Because IgGs from patients are heterogeneous, we also used IgGs from 2 patients and 1 control that were immunopurified on cardiolipin (see above). We also observed specific staining of late endosomes but not of mitochondria, which contain cardiolipin (Figures 8G and 8H). No staining was seen with the control IgG. Our ELISA results showed that anti-LBPA activity was copurified with anti-cardiolipin activity. These results confirm that anti-cardiolipin/-LBPA antibodies, but not other antibodies in patient IgGs, recognized late endosomes. Analysis by immunoelectron microscopy of cryosections of HUVECs showed that the APLA labeled the internal membranes of late endosomes (Figure 9).

As an obvious next step, HUVECs were then incubated for 20 hours at 37°C in the presence of plasma14 from patients with APLA in the medium and then analyzed by immunofluorescence. APLAs from all 10 analyzed patients

Figure 9. Intracellular localization of the antigen recognized by APLA in ultrathin frozen sections of HUVECs. Gold particles in the field are indicated by arrows and show the specific labeling on the internal membranes of late endosomes. Scale bar=200 nm.
were endocytosed by all HUVECs and then accumulated intracellularly, whereas we did not observe intracellular accumulation of control antibodies (Figures 10A and 10B). Colocalization of internalized antibodies with LBPA indicates that the antibodies accumulate in late endosomes.

**APLAs Induce a Late Endosome Sorting/Trafficking Defect**

One of the main functions of late endosomes is the sorting of the multifunctional receptor (IGF2/M6PR) for ligands bearing M6P, which include lysosomal enzymes and IGF2.20 IGF2/M6PR delivers newly synthesized lysosomal enzymes from the trans-Golgi network (TGN) to late endosomes and then recycles back to the TGN for reuse. At steady state, IGF2/M6PR localizes predominantly to the TGN in HUVECs. This perinuclear distribution of IGF2/M6PR was not affected when cells were treated with control plasma (Figure 11B). In contrast, incubation of HUVECs with plasma from a patient with APLA caused the IGF2/M6PR to redistribute to perinuclear vesicular structures (Figure 11A). These structures were identified as late endosomes by double labeling with the anti-LBPA MoAb (Figures 11C and 11D). Similar results were obtained after HUVEC treatment with each of the plasmas from 9 other patients with APLAs. Internalization of anti-LBPA MoAb also induced a redistribution of IGF2/M6PR from the TGN to late endosomes in HUVECs (Figures 11E and 11F).

**Discussion**

There is still much debate on the underlying pathogenic mechanisms associated with APLAs. Several recent studies have proposed that APLAs act on ECs and may compromise the normal antithrombotic function of ECs.1,4,8–13 These studies proposed, however, no obvious common mechanisms. Recently, we observed that antibodies from some patients with APLAs also recognized LBPA, an anionic phospholipid present in the late endosomes of BHK cells.14

In the current study, we have demonstrated that (1) there is a strong correlation between the binding activity in plasma from 37 APLA patients to LBPA and to cardiolipin and, to a lesser extent, to β2GPI; (2) β2GPI binds LBPA; (3) ECs contain LBPA in their late endosomes; (4) late endosomes of permeabilized ECs are recognized by APLAs, whereas mitochondria, which contain cardiolipin, are not; and (5) incubation of ECs with APLAs leads to accumulation of these antibodies in late endosomes and interference with their protein-sorting function. Our results therefore show that anti-LBPA antibodies are present in the plasma of patients with APLA, may interact with LBPA directly or via β2GPI, and are able to interact with and modify the cellular physiology of ECs.

We observed a highly significant correlation between cardiolipin and LBPA binding in the plasma or purified IgG of 37 patients with APLAs. A biochemical analysis of the 2 phospholipid preparations used in our study ruled out that the strong correlation was due to contamination of the LBPA preparation with cardiolipin or vice versa. Taken together, our results indicate that, in general, antibodies that bind directly or indirectly to cardiolipin also bind to LBPA. Moreover, some of the patient antibodies interacted with β2GPI (or other proteins) alone.
We observed that anti-cardiolipin antibodies also interacted with LBPA and that APLAs recognized late endosomes of HUVECs, which contain LBPA, but did not recognize mitochondria, which are rich in cardiolipin. One possibility for this observation is that cardiolipin is highly susceptible to oxidation. A recent study demonstrated that many APLAs bound to cardiolipin only after it had been oxidized. A recent study demonstrated that many APLAs bound to cardiolipin only after it had been oxidized. Inside ECs, cardiolipin is protected from oxidation, whereas in ELISA plates, cardiolipin is exposed to oxygen for a prolonged period. Alternative explanations that mitochondrial cardiolipin in fixed, permeabilized HUVECs is inaccessible to APLA or that cardiolipin had been washed away during fixation cannot be excluded at present.

Our findings raise the possibility that the correlation between anti-cardiolipin antibodies and the clinical manifestations of the APS reflects a pathogenic effect of the binding of APLAs to LBPA rather than to cardiolipin. The immunofluorescence and immunoelectron microscopy results showed that ECs contain LBPA in the internal membranes of late endosomes. On endocytosis, APLAs accumulated in late endosomes, containing LBPA, and then affected their sorting functions as shown by the redistribution of the marker protein IGF2/M6PR from the Golgi apparatus to late endosomes. The slow kinetics of internalization of APLAs suggests that fluid-phase endocytosis is a major route for internalization. However, we cannot exclude the possibility that LBPA recycles between late endosomes and plasma membranes and that the antibodies enter via a mechanism of receptor-mediated endocytosis.

Presently, the contribution of anionic, phospholipid-binding proteins such as β₂GPI, prothrombin, annexin V, or...
others to the accumulation of APLAs in late endosomes is unclear. Future work will be required to characterize precisely the interactions that may occur within the cell between APLA, LBPA, other anionic phospholipids, and anionic phospholipid-binding proteins. However, our data already indicate that at least some APLAs can bind LBPA directly, and our correlation studies show that anti-LBPA antibodies may be, at least in part, distinct from those reacting against anti-β2-GPI. One may thus speculate that the effects of APLAs on endosomal sorting functions are caused by multiple and complex interactions between APLAs and LBPA, β2-GPI, and/or LBPA-β2-GPI complexes. Alternatively, however, it is also possible that the observed APLA effects are caused by a competition of (some) APLA antibodies with β2-GPI for the same LBPA binding sites within late endosome internal membranes.

A wide variety of, at first sight, unrelated pathogenic mechanisms have been proposed by which APLAs affect ECs and induce a prothrombotic state.8,23,24 The incubation of HUVECs with APLAs induces an increase in monocyte adhesion, due to an increase in E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 expression at the EC surface.10,25 APLAs may also create an acquired protein C dysfunction, because anti-β2-GPI antibodies hampered the inactivation of factor Va by endogenous activated protein C,26 whereas lupus anticoagulant inhibited activated protein C anticoagulant activity but not thrombomodulin activity.13 Anti-prothrombin antibodies induced the recruitment of prothrombin on EC surfaces and thereby facilitated local thrombin generation.27 Annexin V–binding antibodies in sera from patients with lupus anticoagulant induced apoptosis in cultured ECs.12 Interestingly, removal of APLAs by incubation with phospholipid liposomes did not abolish the apoptosis-inducing activities or the binding to annexin V. Incubation of ECs with APLAs increased tissue factor expression and decreased annexin V expression at the cell surface and decreased the clotting time of recalcified plasma added to these cells.11,28 APLAs may bind to heparin-like glycosaminoglycans on ECs and thereby inhibit local antithrombin III activity.29 It remains to be established to what extent our finding that APLA interferes with intracellular protein trafficking contributes to 1 or more of the pathogenic mechanisms described above.

Our results indicate that APLAs can, as do other autoantibodies,30 enter into living cells. Until now, APLAs were thought to be directed to the outer membrane of ECs4 and to activate these cells.10,23,25,31 At the EC surface, β2-GPI or β2-GPI-phospholipid complexes were thought to be the main targets of APLAs, and it has been shown that both MoAbs and polyclonal anti-β2-GPI antibodies can upregulate adhesion molecule expression and interleukin-6 secretion after EC binding24,32 and induce adherence of monocytes to ECs.10,25 However, the binding of APLAs to the EC surface has never been clearly demonstrated and remains speculative in view of scarce evidence of the loss in membrane asymmetry.25 Our data indicate that APLAs enter ECs and bind to late endosomes but obviously do not exclude that APLAs first bind to the cell surface. For example, both β2-GPI and APLA could bind to the cell surface, enter the cell, and then accumulate in late endosomes.

Our data indicate that LBPA is an important lipid target of APLA, either direct or indirect, via a protein cofactor. Furthermore, they show that incubation of ECs with APLAs leads to accumulation of these antibodies in the late endosomes of ECs and a redistribution of the IGF2/M6PR from the Golgi apparatus to late endosomes.

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