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Detection of bilayer phospholipid-binding antibodies using flow cytometry

V. ESCHWÈGE*†, I. LAUDE†, F. TOTI*, J.-L. PASQUALI* & J.-M. FREYSSINET*† *Institut d'Hématologie et d'Immunologie, Faculté de Médecine, Université Louis Pasteur, Strasbourg, and †Unité 143 INSERM, Hôpital de Bicêtre, Le Kremlin Bicêtre, France*

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SUMMARY

Antiphospholipid antibodies (APL) are usually detected using solid-phase immunoassays, where cardiolipin is the most common capture antigen. Phosholipids are believed to adopt a monolayer organization when coated onto polystyrene after evaporation of the solvent. However, bilayer phospholipids are probably those evidenced as microparticles or cell fragments circulating *in vivo* under various pathological circumstances. The surface density of monolayer phospholipids on polystyrene is six times lower than that of bilayer phospholipids. In order to assess the influence of phospholipid organization on the detection of APL, we prepared glass microspheres coated with bilayer phospholipids (cardiolipin, phosphatidylcholine, cholesterol). Such liposphere enabled us to study the binding of antibodies in 1:100 diluted plasma samples from patients with anti-cardiolipin antibodies of IgG isotype previously diagnosed by ELISA. Among the 39 plasma samples analysed by flow cytometry, 17 showed positive IgG binding to lipospheres. Only four additional samples became positive when adding 20 μg/ml apolipoprotein H. The specificity of the binding was demonstrated by complete reversibility with 1·4 μM annexin V and with a large excess of liposomes of the same composition. The absence of correlation between liposphere and ELISA results suggests that different subgroups of antibodies are detected depending on the method. The detection of APL using bilayer phospholipids is an original assay and may represent a more physiopathological approach to the specificity of APL.

Keywords antiphospholipid antibodies lipospheres apolipoprotein H (β_2 glycoprotein I)

INTRODUCTION

The presence of antiphospholipid antibodies (APL) is associated with an increased risk of thrombosis, thrombocytopenia and fetal loss, making their detection of prime importance. Phospholipid-binding antibodies form a highly heterogeneous family of immunoglobulins in which lupus-like anti-coagulants and anti-cardiolipin antibodies are the most common subgroups [1]. These antibodies are termed according to the method used for detection: lupus-like anticoagulants in phospholipid-dependent coagulation assays, and anticardiolipin antibodies in solid-phase immunoassays using cardiolipin as the capture antigen. In patients positive for lupus-like anti-coagulants or anticardiolipin antibodies, about 60% are positive for both. It seems reasonable to assume that anticardiolipin antibodies and lupus-like anticoagulants represent two overlapping populations [2]. The common feature is the affinity for anionic phospholipids, but several studies suggest that APL are directed to a protein–phospholipid complex antigen [3–6]. β_2 glycoprotein I, also referred to as apolipoprotein H (apo H), has been described as a necessary, sometimes essential, cofactor for detection of anticardiolipin antibodies [7]. Moreover, cofactor dependence could discriminate anticardiolipin antibodies detected in systemic lupus erythematosus (SLE) patients from those in patients with infectious diseases [8–10]. In the various as yet available solid-phase assays still under standardization evaluation, the cofactor is contributed by either 10% adult bovine serum or 10% fetal calf serum (FCS) used for saturation of non-specific binding sites and/or for sample dilution [11]. APL could recognize an epitope expressed following a conformational change occurring upon interaction of apo H with anionic phospholipids [12]. Under certain conditions, such a conformational change of apo H could be reproduced without phospholipids [13–15].

All the above results were obtained from solid-phase immunoassays on polystyrene microtitration plates. On this type of surface, phospholipids adopt a monolayer film rather than a bilayer membrane structure. The density of monolayer lipids is low, 3·5 nm^2 per phospholipid headgroup, compared with that currently measured in bilayers on glass microspheres ($\approx$ 0·6 nm^2 per phospholipid headgroup) [16]. Furthermore, the bilayer structure obtained on glass should allow a lateral diffusion of phospholipids which is prevented by hydrophobic interaction of monolayer phospholipids with polystyrene. The phospholipid organization

Correspondence: Valérie Eschwège, Unité 143 INSERM, Hôpital de Bicêtre, 94275 Le Kremlin Bicêtre, France.

on glass seems more likely to mimic the phospholipid counterpart of a complex antigen, as it could be expressed by activated cells, shed microparticles or apoptotic bodies under physiopathological conditions. The intrinsic antigenicity and cofactor-dependence of bilayer phospholipids may well be modified according to the mode of phospholipid presentation.

Here, we report the design of a novel flow cytometric assay for the detection of APL, where glass microspheres coated with bilayer phospholipids bearing a proportion of cardiolipin are used for solid-phase antigen presentation.

PATIENTS AND METHODS

Patients

Thirty-nine patients were studied; 25 had primary or secondary antiphospholipid syndrome, among whom 10 suffered from fetal wastage, and 11 from thrombosis, six had other clinical manifestations, and eight were asymptomatic. Six patients from the latter group were older than 65 years. All of them had APL of IgG isotype previously diagnosed by ELISA (Cardiolisa IgG; Biomedical Diagnostics, Marne-la-Vallée, France), titres ranging from 5 to over 150 GPL units. Thirty-three of these patients also had lupus-like anticoagulant. Fifteen healthy subjects without APL were included in the control group. Blood from 20 other healthy subjects without APL was used for the preparation of a control pool plasma.

Samples

Blood was collected from the different patients by venipuncture in citrated anti-coagulant (9 v blood/1 v anticoagulant). Platelet-poor plasma was prepared by two successive centifugation steps at 2000 ***g*** for 15 min at room temperature and then frozen at –70°C until assay. Aliquots of normal pool plasma were stored at –70°C and used as a control in each set of experiments.

Immunoglobulin purification

Plasma samples from two patients and from a normal subject were dialysed three times during 4 h at 4°C against 1 *l* of 25 mM Tris buffer adjusted at pH 8·8 and containing 35 mM NaCl and 1 mM EDTA. IgG was then purified on a DEAE Sepharose column (Pharmacia, Uppsala, Sweden). The flow-through eluate containing the fraction of interest was dialysed three times during 4 h at 4°C against 1 *l* of 50 mM Tris buffer adjusted at pH 7·5 and containing 120 mM NaCl and 2·7 mM KCl.

Cleaning of glass microspheres

Twenty milligrams of glass microspheres (1·6 μm diameter) (Duke Scientific Corp., Palo Alto, CA) were suspended in 3 ml Tris buffer saline (TBS) (Tris 50 mM/NaCl 120 mM/KCl 2·7 mM) and sonicated for 7 min to eliminate aggregates. Removal of very small particles was achieved after sedimentation at 80 ***g*** for 5 min. Beads in the pellet were resuspended in 3 ml TBS and the washing process was repeated twice. The pellet was finally resuspended in 2 ml TBS.

Microsphere coating

Cardiolipin, phosphatidylcholine and cholesterol (Sigma, St Louis, MO) were mixed at 2·5/10/4 molar proportions. Organic solvents were evaporated under nitrogen stream. The microsphere suspension prepared as described above was then added to the lipid pellet. Coating was achieved by five cycles of 2 min sonication/2 min rest, followed by 1 h incubation under slow stirring. The possible remaining uncoated lipids were removed by two washing steps with TBS. Final lipid concentration of the liposphere preparation was $\approx$ 240 μM as determined using L-3-phosphatidylcholine, 1,2-di[1–^{14}C]palmitoyl (Amersham, Aylesbury, UK) as a tracer. Lipospheres were stored at 4°C for less than 1 month.

Liposphere characterization

Lipospheres and control cleaned glass microspheres, diluted 1000-fold in TBS, were incubated with two different probes before flow cytometry analysis: (i) 1,1′-dihexyl-3,3,3′,3′-tetramethylindocarbocyanine iodide ($DiIC_6(3)$; Molecular Probes, Eugene, OR) 3 μM final concentration during 1 h; and (ii) annexin V purified according to Funakoshi *et al.* [17] and conjugated to FITC [18] (140 nM final concentration) for 10 min in the presence of 1 mM $CaCl_2$.

APL detection

Lipospheres 1000-fold diluted were incubated for 1 h with either 1:100 plasma dilutions or 0·1 mg/ml purified IgG in a final volume of 300 μl of TBS containing 0·1% (w/v) bovine albumin (TBS–bovine albumin). After three centrifugation/washing steps at 12 000 ***g*** for 15 s and replacing 200 μl liposphere supernatant by 200 μl TBS–bovine albumin, lipospheres were incubated for 15 min with FITC-conjugated $F(ab')_2$ fragment of goat anti-human IgG, Fcγ fragment-specific (Jackson ImmunoResearch Labs Inc., West Grove, PA) (1:150 final dilution). In order to eliminate excess $F(ab')_2$, lipospheres were washed twice as described above. Samples were analysed by using a Becton Dickinson (San Jose, CA) FACScan flow cytometer. The sheath fluid was FacsFlow (Becton Dickinson). Data acquisition and analysis were carried out using Lysis II software. The forward angle light scatter setting was E-01 with a threshold of 16. A total of 10 000 events per sample was analysed.

Influence of apo H

Purified human apo H (kindly provided by J. Amiral, Serbio, Gennevilliers, France) was added to the 1:100 dilution of plasma samples during incubation with the 1:1000 liposphere dilution, at a final concentration of 20 μg/ml, and for one patient at additional concentrations of 5, 10, 20 and 30 μg/ml.

Inhibition studies

Inhibition studies were performed for five of the samples found APL-positive with liposphères.

With annexin V. Purified annexin V was added to the plasma sample during incubation with the 1:1000 liposphere dilution, at a final concentration of 1·4 μM.

With liposomes. Liposomes containing cardiolipin, phosphatidycholine and cholesterol, or phosphatidylserine, phosphatidylcholine and cholesterol at molar ratios of 2·5/10/4, and 5/7·5/4, respectively, were prepared by the dialysis method [19]. Final lipid concentration was $\approx$ 2·5 mM as determined using ^{14}C-phosphatidylcholine as a tracer. Plasma samples were incubated during 1 h with liposomes and liposphères. Final dilutions were 1:100 for plasma, 1:30 for liposomes and 1:1000 for liposphères. Ratio between liposome and liposphere lipid concentrations was 350.

RESULTS

Liposphere characterization

A significant shift of fluorescence intensity was observed for

liposphere compared with cleaned control glass microspheres in the presence of $DiIC_6(3)$, or FITC-conjugated annexin V (Fig. 1).

APL detection with liposphere

The fluorescence intensity obtained with the control pool in 20 consecutive experiments was 2 ± 0·5 (mean ± s.d.) arbitrary fluorescence units. Hence, 3 was chosen as the positive threshold value. All samples from the control group gave values below three arbitrary fluorescence units. The detection of APL with liposphere without added cofactor, i.e. using only bovine albumin for sample dilutions, was positive in 17 samples out of 39 (Fig. 2). Titres ranged from 3 to 100 arbitrary fluorescence units. All samples previously diagnosed above or equal to 50 GPL units were found positive in the present assay as well as six of those below this value (Fig. 2), but no correlation could be observed with respect to titres. All the positive plasma samples were controlled with cleaned microspheres instead of cardiolipin-coated liposphere, and the corresponding fluorescence intensities consistently remained below 3. All the samples found positive also had lupus-like anticoagulant, but no correlation could be found between the liposphere assay and either assay used to detect lupus-like anticoagulants. Regarding clinical status, neither the titre nor the positive response in this assay was able to discriminate asymptomatic from other groups of patients. Among the 17 patients showing reactivity with liposphere, five had recurrent fetal loss and six suffered from thrombosis. Compared with the five cases of fetal loss and the five cases of thrombosis in the group of 22 remaining patients, there is no significant difference. However, in the group of eight asymptomatic patients, only one was detected as positive.

IgG fractions gave positive results comparable to those obtained with the corresponding whole plasma (Fig. 3).

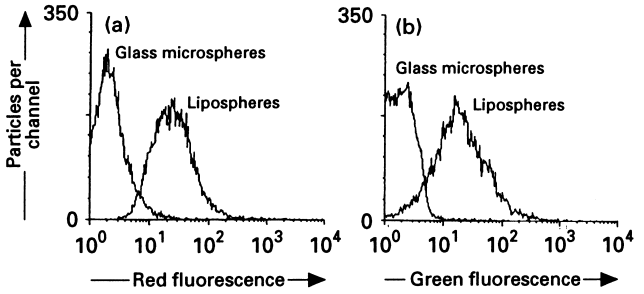

Fig. 1. Fluorescence histograms of lipospheres and glass microspheres after incubation with (a) 1,1′-dihexyl-3,3,3′,3′-tetramethylindocarbocyanine iodide ($DiIC_6(3)$; 3 μM) and (b) FITC-conjugated annexin V (140 nM) in the presence of 1 mM $CaCl_2$.

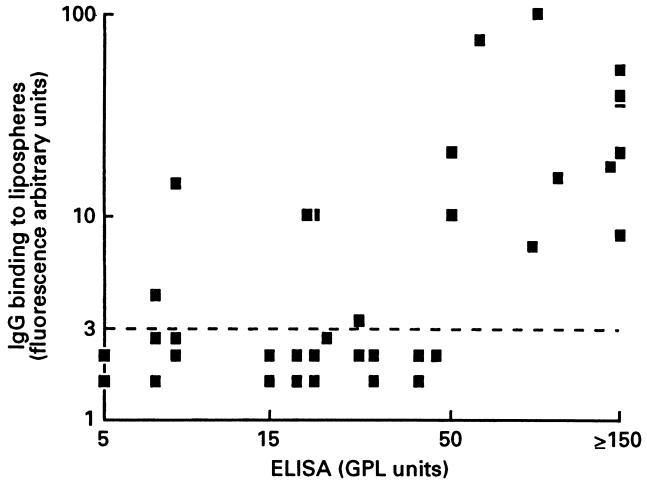

Fig. 2. Comparison of antiphospholipid antibody (APL)-IgG binding to lipospheres (fluorescence arbitrary units) with the titres (GPL units) measured by ELISA for each patient's sample.

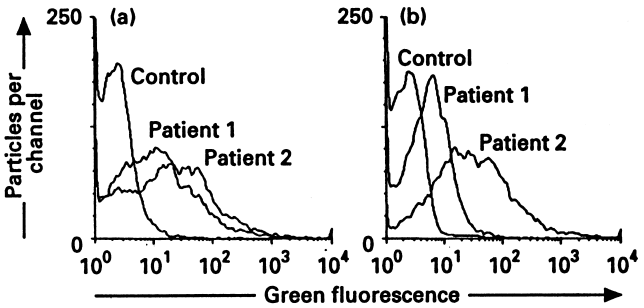

Fig. 3. Fluorescence histograms showing the binding of antiphospholipid antibody (APL)-IgG to lipospheres in diluted purified IgG fractions (0·1 mg/ml) from two patients (a), and in corresponding plasma dilutions (1:100) (b). Control histograms were obtained with the same dilution of purified IgG fraction and plasma from a sample of the control group.

Importance of apo H

After addition of 20 μg/ml of apo H to all dilutions of plasma samples, 21 out of 44 samples were positive. Only four additional samples, with respect to the 17 previous ones, became positive, and four samples already positive without addition of apo H displayed enhanced IgG binding. The results obtained in one of the samples becoming positive in the presence of different concentrations of apo H are shown in Fig. 4.

Inhibition studies

In five patient samples, APL interaction with the lipospheres was completely inhibited by 1·4 μM annexin V or by liposomes of the same lipid composition, and to various extents by liposomes of different lipid composition (Table 1).

DISCUSSION

This study demonstrates the feasibility of the detection of APL using lamellar phospholipids coated onto glass microspheres. The phospholipid organization of lipospheres can be assessed using

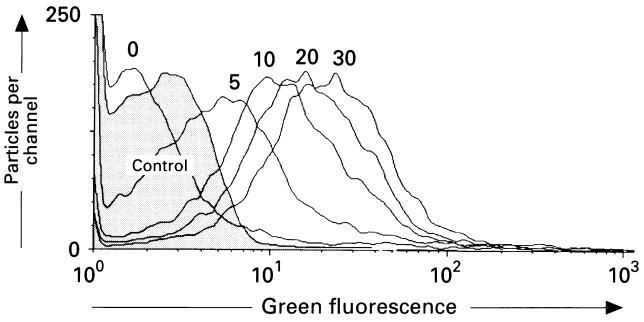

Fig. 4. Fluorescence histograms showing the effect of apo H at different concentrations (0, 5, 10, 20 and 30 μg/ml) on the binding of antiphospholipid antibody (APL)-IgG to lipospheres in the diluted plasma (1:100) from one patient.

Table 1. Inhibition of the interaction of antiphospholipid antibodies (APL) with lipospheres observed in the presence of annexin V (1·4 μM) or liposomes containing cardiolipin, phosphatidylcholine and cholesterol (CL/PC/Chol) (2·5/10/4 molar ratio) or phosphatidylserine, phosphatidylcholine and cholesterol (PS/PC/Chol) (5/7·5/4 molar ratio)

	Inhibition (%)		
Patient	Annexin V	CL/PC/Chol	PS/PC/Chol
1	100	91	53
2	100	100	40
3	100	100	5
4	100	70	13
5	100	85	0

Ratio between lipospheres and liposomes lipid concentration was 350. The decreases of fluorescence intensity due to the presence of either inhibitor are expressed as per cent of fluorescence intensity measured in its absence.

fluorescent membrane probe $DilC_6(3)$ which is incorporated into bilayers. Furthermore, the binding of FITC-conjugated annexin V testifies to the presence of negatively charged phospholipids at the liposphere outer surface. Cardiolipin was the anionic phospholipid coated onto the microspheres in order to compare with results obtained by ELISA. The absence of any signal in the absence of lipids on glass microspheres, and the total inhibition of binding of APL in the presence of annexin V or an excess of liposomes of the same composition, demonstrate the specificity of the interaction with the lipospheres. There is a clear discrepancy between the results obtained with lipospheres and by ELISA. On the other hand, Stewart *et al.* [20] observed a true correlation between ELISA results and flow cytometry detection using polystyrene microspheres. This emphasizes the importance of the chemical composition of the solid phase used for the presentation of the phospholipid antigen.

Although cardiolipin is commonly used as the capture antigen, it is usually accepted that anti-cardiolipin antibodies cross-react with all anionic phospholipids [21]. In the present study, the various degrees of inhibition of binding observed in the presence of phosphatidylserine liposomes suggest that the APL detected do not fully cross-react with phosphatidylserine. The difference of phospholipid presentation in the two systems could explain the absence of correlation of the corresponding results.

The difference of phospholipid presentation could also be responsible for the discrepancies in apo H-dependence. The cardiolipin/phosphatidylcholine/cholesterol (2·5/10/4 molar ratio) mixture has already been used for affinity-purification of APL in order to demonstrate the importance of a protein cofactor in ELISA [3,4]. Although the method for purification of the whole IgG fraction cannot rule out the presence of apo H, it seems that the two purified APL are able to bind to this model membrane in the absence of added protein cofactor. With the 15 other samples, it is striking to observe that binding occurs with the only apo H contained in the 1:100 diluted plasma samples compared with that in the 10% animal serum necessary in ELISA. These results suggest that different subgroups of antibodies are detected, depending on the method.

The positive signal observed with four additional samples only in the presence of exogeneous apo H could be due to a cofactor effect of apo H on APL, or to the presence of additional antibodies directed to apo H. The actual methods of detection of APL by ELISA may not reflect a single subgroup of APL, but rather a mixture of anti-apo H antibodies and APL directed to monolayer phospholipids, showing or not apo H-dependence. Except in SLE, the significance of APL is still controversial with respect to clinical consequences. Many patients exhibiting APL are asymptomatic. High levels of APL IgG have been reported to be more often associated with thrombotic disorders, nevertheless a better definition of the APL subgroup associated with thrombosis could help to define a genuine marker of thrombotic risk. Such APL could occur as a response to excess of anionic phospholipid exposure, microparticle shedding and apoptosis in stimulated cells, or under situations where the reticuloendothelial clearance of activated cells and fragments is impaired. The resulting circulating phospholipids most probably adopt membrane organization, hence corresponding APL are expected to be directed to a lamellar conformation identical to that of the phospholipid counterpart of lipospheres.

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