Anti-cardiolipin antibody from a patient with antiphospholipid syndrome (APS) recognizes only an epitope expressed by cardiolipin/β2-glycoprotein-I (β2GPI) complex and induces APS

Y. Levy1, Y. Sherer1, A. Mathieu2, A. Cauli2, G. Passiu2, G. Sanna2, Z. Janackovic1, M. Blank1, Y. Shoenfeld1

1Department of Medicine ‘B’ and Research Unit of Autoimmune Diseases, Sheba Medical Center, Tel-Hashomer, and Sackler Faculty of Medicine, Tel-Aviv University, Israel; 2Cagliari University, Institute of Clinical Medicine, Cattedra of Rheumatology, Cagliari, Italy.

Abstract

Objective
As the antiphospholipid syndrome (APS) is characterized by antibodies which bind negatively charged phospholipids either directly or mainly through different target epitopes located on the beta-2-glycoprotein-I (β2GPI) molecule, the aim of this study is to describe an additional target epitope for anti-cardiolipin binding.

Methods
The binding characteristics of affinity purified anti-cardiolipin antibodies from a patient with monoclonal gammopathy associated with clinically overt APS were studied; inhibition studies were also carried out. These antibodies were used for the active induction of experimental APS.

Results
The affinity purified anti-cardiolipin antibodies were found to bind a target epitope created by the complex of cardiolipin/β2GPI, while not reacting with a complex composed by another phospholipid (phosphatidylserine/β2GPI), as confirmed by direct binding and competition assays. Immunization of naïve mice with this unique affinity purified anti-cardiolipin antibody resulted in the induction of experimental APS (thrombocytopenia, prolonged coagulation time and fetal resorptions). The anti-cardiolipin/β2GPI injected mice developed high titers of mouse anti-cardiolipin/β2GPI antibodies with the same binding characteristics as the human antibody which was used for disease induction.

Conclusion
APS is a unique syndrome that is characterized by a diversity of pathogenic anti-phospholipid antibodies which may explain the diversity of clinical manifestations reported in patients.

Key words
Anticardiolipin antibody, antiphospholipid syndrome, beta-2-glycoprotein I, monoclonal gammopathy, thromboembolism.
Introduction
The ‘Hughes Syndrome’ or anti-phospholipid syndrome (APS) is characterized by the presence of anti-cardiolipin antibody (aCL), β₂GPI (beta-2-glycoprotein-I)-dependent antibodies and/or lupus anticoagulant associated with thromboembolic phenomena, thrombocytopenia and recurrent fetal loss (1-3). It is generally accepted that aCL purified from patients with primary or secondary APS consist of several populations of aCL: antibodies that bind directly to negatively charged phospholipids (3,4), and antibodies that bind anionic phospholipid through (2-glycoprotein-I (β₂GPI) molecule (3-7). β₂GPI (50KD), a member of the complement control protein (CCP) family, is the target antigen for many of the autoimmune anti-β₂GPI antibodies, and is composed of 5 respective short consensus repeats (2,8). β₂GPI exhibits several properties in vitro which define it as an anticoagulant (e.g. inhibition of prothrombinase activity, ADP-induced platelet aggregation, platelet factor IX production) (9, 10). It binds negatively charged phospholipids through a lysine rich locus (Cys281-Cys288) located in the fifth domain (11). It has been postulated that anti-β₂GPI antibodies exert a direct pathogenic effect by interfering with homeostatic reactions occurring on the surface of platelets or vascular endothelial cells (12-17). In previous studies we (18-22) and others (23-26) have shown the ability to induce experimental APS in naive mice, following passive transfer (18,21,26) or active immunization (19, 20, 22, 24, 25) with human and mouse monoclonal and polyclonal aCL or anti-phosphatidyserine antibodies. In the current study we report for the first time that antiphospholipid antibodies (derived from a patient with monoclonal gammopathy) bind cardiolipin/β₂GPI complex, neither phosphatidyserine/β₂GPI nor β₂GPI or cardiolipin alone. Furthermore, these antibodies are able to induce experimental APS in naive mice.

Materials and methods
Clinical details of the patient
An 81-year-old man had a 6-month history of non-healing skin ulcers at his extremities and arthralgia. The lesions were erythematosus, purple, macular with central ulceration and profoundly tender to the touch. A few days later he developed deep vein thrombosis of his right arm. Laboratory abnormalities included prolonged activated partial thromboplastin time (aPTT) (40 s), elevated IgM aCL (145 MPL; reference value < 10 MPL), and the presence of an IgM monoclonal gammopathy (κ light chain) and urine Bence Jones protein (urine κ light chain was 12 mg/dl; normal levels < 1.8 mg/dl). Lupus anti-coagulant, antinuclear antibodies, and antibodies to dsDNA, extractable nuclear antigens and neutrophil cytoplasmic antigens were either absent or within the normal range, as were circulating immune complexes. Pathologic examination of a biopsy taken from a skin lesion demonstrated vasculitis and the presence of amorphous, eosinophilic and PAS positive material within the vessel lumen. Immunohistochemical staining of the thrombotic lumen were strongly positive for the κ light chain and negative for the γ light chain.

The patient was treated with heparin 7500 units tid and iv cyclophosphamide 500 mg. Nonetheless, while receiving this therapy a second deep vein thrombosis (this time of the right leg) was diagnosed. Currently, the patient is receiving oral acenecumarol 3 mg/day, cyclophosphamide 50 mg/day for 15 days/month, and prednisone 10 mg every other day; only seldomly does he need a modification of his treatment regimen due to the recurrence of vasculitic skin lesions.

Affinity purification of the patient’s aCL
The aCL were affinity purified according to a method previously described (27). Briefly, 2 ml of cardiolipin (10 mg) were evaporated on the wall of U-shaped glass tube. The tube was vortexed for 5 min with 2 ml of Tris base-buffered saline (TBS) for the preparation of cardiolipin lyposomes. The patient’s serum (1:2 v/v suspension with cardiolipin lyposomes) was incubated overnight with rotation. The suspension was washed twice for 15 min at 30,000 g, and thereafter eluted by adding 1 ml of 1M KI followed by 1 hour shaking. Finally, the immunoglobulin fraction was separated in the presence of chloroform. The puri-
fied aCL was separated into IgG and IgM fractions employing protein G and antihuman IgM CNBr activated sepharose columns (Pharmacia). The purity of the immunoglobulin protein was checked on SDS-PAGE gels and the presence of cardiolipin was assayed on TLC plates and was found to be negative.

**Binding characteristics of affinity purified aCL from the patient’s sera and APS mice**

aCL activity in the sera of the patient or the immunized mice was detected by ELISA as follows: 96-well ELISA plates (Nunc, Roskilde, Denmark) were coated with either cardiolipin-lyposomes, (2-βGPI, cardiolipin-lyposomes/β2GPI (CL/β2GPI), phosphatidylserine-lyposomes/β2GPI (PS/β2GPI), or phosphatidylcholine (PC) (Sigma) at a concentration of 50 µg/ml in ethanol or ethanol/chloroform. The plates coated with phospholipids (CL, PS, PC) were evaporated at 4°C, blocked with 5% gelatin and incubated with or without β2GPI (10 µg/ml). In order to detect direct binding to β2GPI, irradiated ELISA plates were coated with β2GPI (10 µg/ml) in PBS and blocked with 5% gelatin. Different concentrations of the affinity purified aCL or immunized mice sera, were incubated for 2 hrs at room temperature. Bound antibodies were probed using goat anti-human or anti-mouse immunoglobulin (IgG or IgM) conjugated to alkaline phosphatase (Jackson) and p-nitrophenyl phosphate substrate. Color was read in a Titertek ELISA reader at 405 nm.

**Inhibition of aCL binding**

Affinity purified IgG or IgM aCL, at dilutions that gave 50% of maximal binding to cardiolipin were preincubated with different concentrations of either cardiolipin, β2GPI, CL/β2GPI, or PS/β2GPI, in order to confirm the specific binding. Following incubation at 4°C, the remaining activity was tested by ELISA as detailed above. The percentage of inhibition was calculated as follows: % Inhibition = [OD control - OD with inhibitor x 100] / OD control

**Induction of experimental APS in naive mice**

BALB/c mice (8-10 week old females) were purchased from the Sackler Faculty of Medicine, Tel-Aviv University. The induction of experimental APS was performed by intradermal immunization of 10µg/mouse of affinity purified aCL, as previously described (16, 19, 20, 22). As a control we used IgG and IgM purified from the same patient lacking the aCL fraction (these antibodies were checked on SDS-PAGE gels for purity). The studied immunoglobulins were emulsified in complete Freund’s adjuvant (CFA, Difco, Detroit, MI) and injected intradermally. Three weeks later a booster injection in PBS was given. The following clinical manifestations were studied. The presence of lupus anticoagulant was evaluated by the prolongation of aPTT, in a mixing test, by adding one volume of plasma (whole blood mixed with Na-citrate 0.13 moll/l, in a 9:1 ratio) to one volume of cephalin and incubating for 2 min at 37°C. Another volume of 0.02 M CaCl2 was added, and the clotting time was recorded in seconds. The results were confirmed by measuring the kaolin clotting time. The mice were bled from their retroorbital plexus for cell counts and serological studies. Platelet counts from individual blood samples were quantified in diluted blood using a single optical cytometer (Coulter Counter HC Plus Cell Control, Miles, Tarrytown NY). The immunized mice were mated 2 months after boost administration. The number of resorped embryos were recorded and the resorption rate (%R) was calculated as follows: %R = [(Number of live fetuses - Number of resorped fetuses) / Number of live fetuses] x 100.

**Statistical analysis**

Statistical analysis was done using the Students’ t-test. P value < 0.05 was considered as statistical significant.

**Results**

**Binding characteristics of the patient’s aCL**

Autoantibody detection and inhibition studies demonstrated the unique binding characteristics of the patient’s IgG and IgM aCL: they reacted only with CL/β2GPI and not with cardiolipin, β2GPI, PS/β2GPI, or phosphatidylcholine. A dose-response curve for the binding activity of the antibody is presented in Figure 1. As demonstrated in Figure 2, the affinity purified human aCL IgG and IgM bound only the complex CL/β2GPI (0.834 ± 0.112 and 0.799 ± 0.083 for IgG and IgM, respectively; P < 0.001, when compared to the binding to PS/β2GPI). No binding was detected to CL, β2GPI alone or to a zwitterionic charged phospholipid - phosphatidylcholine. This observation was confirmed by competition studies (Fig. 3 a,b). CL/β2GPI (10 µg/ml) inhibited the binding of aCL IgM in 62% while at the same concentration CL/β2GPI as competitor inhibited the binding of human aCL IgG in 49%. Both IgM and IgG aCL were maximally inhibited.

![Fig. 1. A dose-response curve for the binding activity of aCL purified from a patient with APS.](image-url)
Fig. 2. Binding characteristics of affinity purified aCL: IgG and IgM human aCL derived from a patient with APS and monoclonal gammopathy, were tested for binding to βGPI (full bars), cardiolipin (empty bars), CL/βGPI complex (diagonal dashed bars), PS/βGPI complex (ladder bars), and phosphatidylcholine (dotted bars).

(a) (b)

Fig. 3. Inhibition of aCL binding to cardiolipin by CL/β2GPI complexes. (a) Inhibition of affinity purified human IgG aCL from a patient with monoclonal gammopathy by different concentrations of CL/β2GPI complex. (b) Inhibition of affinity purified human IgG aCL from a patient with monoclonal gammopathy by different concentrations of CL/βGPI complex.

Table I. Experimental antiphospholipid syndrome in the mice immunized with human IgG and IgM aCL derived from a patient with APS and monoclonal gammopathy.

<table>
<thead>
<tr>
<th>Immunization</th>
<th>aCL/β2GPI</th>
<th>aCL/β2GPI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td></td>
<td>(n = 20)</td>
<td>(n = 20)</td>
</tr>
<tr>
<td>APTT (sec)</td>
<td>53 ± 3</td>
<td>65 ± 4</td>
</tr>
<tr>
<td>Platelet count (cells/mm$^3$ x 10$^{-3}$)</td>
<td>697 ± 131</td>
<td>564 ± 113</td>
</tr>
<tr>
<td>Resorption (%)</td>
<td>43 ± 2</td>
<td>38 ± 3</td>
</tr>
</tbody>
</table>

The sera were tested 2 months after immunization. The values are expressed as the mean ± SD OD at 405 nm. $P < 0.001$ in the comparisons between mice immunized with aCL and controls for all 3 parameters tested.
aPTT = Activated partial thromboplastin time; H: human.
% resorption = % fetal loss = live fetuses - resorped fetuses x 100 / live fetuses

at 100 µg/ml CL/β2GPI (89%, 92%, respectively. Thus, the studied aCL recognizes a target epitope expressed by CL/β2GPI complex. In addition, it was found that the paraprotein do not participate in the aCL activity.

Induction of APS in mice by active immunization

BALB/c mice were immunized intradermally with either IgG or IgM aCL purified from the serum of the patient with monoclonal gammopathy and APS. Two control groups were immunized with either control IgG or IgM (purified from the same patient). Blood samples were drawn 2 months after immunization to determine the serological and clinical findings. The mice immunized with either IgG or IgM aCL developed the clinical features of APS (Table I): they had prolonged aPTT, lower platelet counts, and a higher resorption index than the control mice that were immunized with human IgG or IgM (38 ± 3% and 43 ± 2% versus 8 ± 2% and 7 ± 2%, respectively).

Sustained high titers of mouse aCL (anti-CL/β2GPI) were detected in the sera of both groups of mice immunized with either human IgG or IgM aCL; no significant aCL binding was found in the sera of mice immunized with control IgG or IgM (P < 0.05). Moreover, the binding characteristics of both groups of mice subjected to IgG or IgM aCL were iden-
Table II. aCL binding of sera from mice immunized with human aCL derived from a patient with APS and monoclonal gammapathy

<table>
<thead>
<tr>
<th>Antibody to:</th>
<th>aCL/β2GPI</th>
<th>aCL/β2GPI</th>
<th>Human IgM</th>
<th>Human IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM (n = 20)</td>
<td>IgG (n = 20)</td>
<td>Human IgM (n = 20)</td>
<td>Human IgG (n = 20)</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>0.126 ± 0.127</td>
<td>0.193 ± 0.103</td>
<td>0.067 ± 0.033</td>
<td>0.084 ± 0.045</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>0.132 ± 0.067</td>
<td>0.164 ± 0.108</td>
<td>0.099 ± 0.047</td>
<td>0.111 ± 0.087</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>0.121 ± 0.065</td>
<td>0.142 ± 0.079</td>
<td>0.102 ± 0.062</td>
<td>0.119 ± 0.075</td>
</tr>
<tr>
<td>β2-glycoprotein-I (β2GPI)</td>
<td>0.141 ± 0.094</td>
<td>0.139 ± 0.064</td>
<td>0.087 ± 0.053</td>
<td>0.137 ± 0.042</td>
</tr>
<tr>
<td>Cardiolipin/β2GPI</td>
<td>0.967 ± 0.088</td>
<td>0.821 ± 0.103</td>
<td>0.077 ± 0.034</td>
<td>0.081 ± 0.039</td>
</tr>
<tr>
<td>Phosphatidylserine/β2GPI</td>
<td>0.141 ± 0.098</td>
<td>0.137 ± 0.078</td>
<td>0.091 ± 0.047</td>
<td>0.122 ± 0.083</td>
</tr>
</tbody>
</table>

The sera were tested at dilution of 1:200. Data are presented as mean ± SD of 2 different experiments.

Discussion

Herein we describe a unique human affinity purified aCL from a patient with monoclonal gammapathy. This antibody reacted only with CL/β2GPI, and not with PS/β2GPI, β2GPI, CL, PS, or PC alone (Table II, Fig. 4 a,b).

The in vivo pathogenicity of this unique affinity purified aCL was confirmed by testing its ability to induce experimental APS. We (16, 19, 20, 22, 35-39) have previously shown that it is possible to induce autoimmune conditions by dysregulation of the idiotypic network through active immunization with pathogenic autoantibody (i.e. idiotype). Upon stimulation with the autoantibody carrying a specific idiotype (Ab1), naive mice develop auto-idiotypic antibodies (anti-Id = Ab2), and after 1 to 2 months anti-anti-idiotypic antibodies (anti-anti-Id = Ab3) that may have similar binding specificities to the Ab1 that was used for immunization. Coincidental with the expression of Ab3, the immunized mice often develop an overt autoimmune condition that resembles the human disorder from which the inducing Ab1 was obtained, e.g. systemic lupus erythematosus in mice immunized with anti-DNA (36, 37), APS in mice immunized with aCL (16, 19, 20, 22), vasculitis in mice immunized with anti-endothelial antibodies (38) and heparin induced thrombocytopenia following immunization with anti-PF4/heparin upon exposure to heparin (39). The aCL which recognizes CL/β2GPI but not PS/β2GPI was found to be pathogenic in vivo. Mice immunized with this unique
antibody as either IgG or IgM developed experimental APS. Since the mice developed Ab3 which also binds only the CL/β2GPI complex, this model in addition provides evidence for the pathogenicity of the antibodies derived from this patient with monoclonal gammapathy. In conclusion, the fact that anti-cardiolipin antibodies can bind an additional new target epitope expressed by the complex CL/β2GPI, supports the concept of the diversity of the aCL populations, which is expressed in the various clinical manifestations seen in patients with APS. Our data contributes to the verification of the syndrome nature of the ‘Hughes syndrome’.

Acknowledgement

We would like to thank Mrs. Ludmila Rachlin for excellent laboratory assistance.

References