Anti-cardiolipin antibody from a patient with antiphospholipid syndrome (APS) recognizes only an epitope expressed by cardiolipin/β_2-glycoprotein-I (β_2GPI) complex and induces APS

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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 (β_2GPI) 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/β_2GPI, while not reacting with a complex composed by another phospholipid (phosphatidylserine/β_2GPI), as confirmed by direct binding and competition assays. Immunization of naive mice with this unique affinity purified anti-cardiolipin antibody resulted in the induction of experimental APS (thrombocytopenia, prolonged coagulation timed and fetal resorptions). The anti-cardiolipin/β_2GPI injected mice developed high titers of mouse anti-cardiolipin/β_2GPI 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.

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Introduction

The 'Hughes Syndrome' or anti-phospholipid syndrome (APS) is characterized by the presence of anti-cardiolipin antibody (aCL), β_2GPI (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 (β_2GPI) molecule (3-7). β_2GPI (50KD), a member of the complement control protein (CCP) family, is the target antigen for many of the autoimmune anti-β_2GPI antibodies, and is composed of 5 respective short consensus repeats (2,8). β_2GPI 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-β_2GPI 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-phosphatidylserine antibodies. In the current study we report for the first time that antiphospholipid antibodies (derived from a patient with monoclonal gammopathy) bind cardiolipin/β_2GPI complex, neither phosphatidylserine/β_2GPI nor β_2GPI 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 erythematous, 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 acenocumarol 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 anti-human 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-GPI, cardiolipin-lyposomes/β_2GPI (CL/β_2GPI), phosphatidylserine-lyposomes/β_2GPI (PS/β_2GPI), 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 β_2GPI (10 μg/ml). In order to detect direct binding to β_2-GPI, irradiated ELISA plates were coated with β_2GPI (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 by 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, β_2GPI, CL/β_2GPI, or PS/β_2GPI, 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 10 μg/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 $CaCl_2$ 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/β_2GPI, and not with cardiolipin, β_2GPI, PS/β_2GPI, 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/β_2GPI (0.834 ± 0.112 and 0.799 ± 0.083 for IgG and IgM, respectively; $P < 0.001$, when compared to the binding to PS/β_2GPI). No binding was detected to CL, β_2GPI alone or to a zwitterionic charged phospholipid - phosphatidylcholine. This observation was confirmed by competition studies (Fig. 3 a,b). CL/β_2GPI (10 μg/ml) inhibited the binding of aCL IgM in 62% while at the same concentration CL/β_2GPI as competitor inhibited the binding of human aCL IgG in 49%. Both IgM and IgG aCL were maximally inhibited

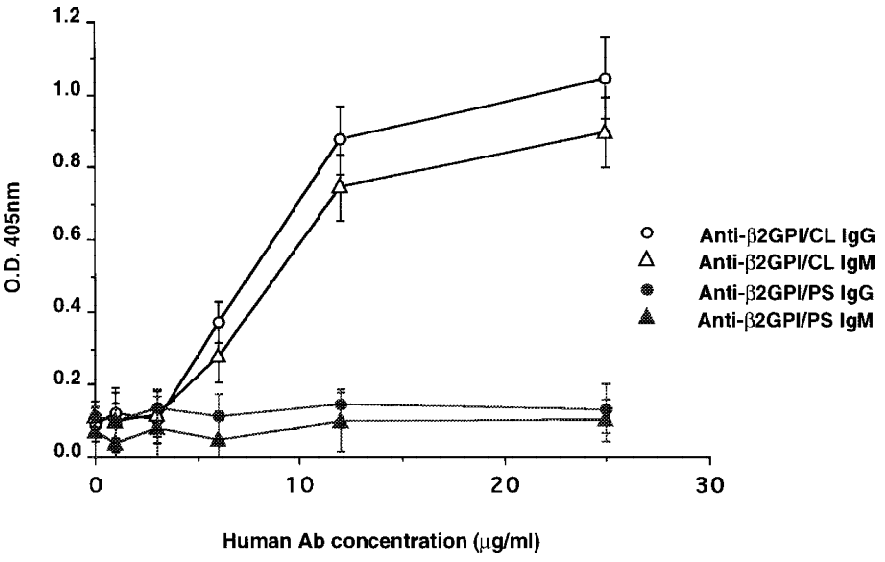

Fig. 1. A dose-response curve for the binding activity of aCL purified from a patient with APS.

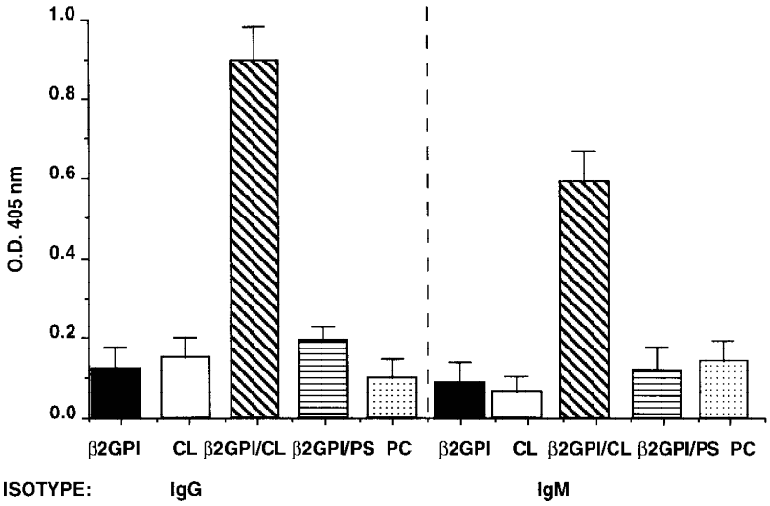

Fig. 2. Binding characteristics of affinity purified aCL: IgG and IgM human aCL derived from a patient with APS and monoclonal gammopathy, were tested for binding to βGPI (full bars), cardiolipin (empty bars), CL/βGPI complex (diagonal dashed bars), PS/βGPI complex (ladder bars), and phosphatidylcholine (dotted bars).

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

Immunization	aCL/β2GPI IgM (n = 20)	aCL/β2GPI IgG (n = 20)	H IgM (n = 20)	H IgG (n = 20)
APTT (sec)	53 ± 3	65 ± 4	32 ± 2	33 ± 4
Platelet count (cells/mm^3 x 10^{-3})	697 ± 131	564 ± 113	1089 ± 129	1711 ± 124
Resorption (%)	43 ± 2	38 ± 3	7 ± 2	8 ± 2

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 = $\frac{\text{\% fetal loss = live fetuses - resorped fetuses x 100}}{\text{live fetuses}}$

at 100 μg/ml CL/β_2GPI (89%, 92%, respectively. Thus, the studied aCL recognizes a target epitope expressed by CL/β_2GPI 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/β_2GPI) 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-

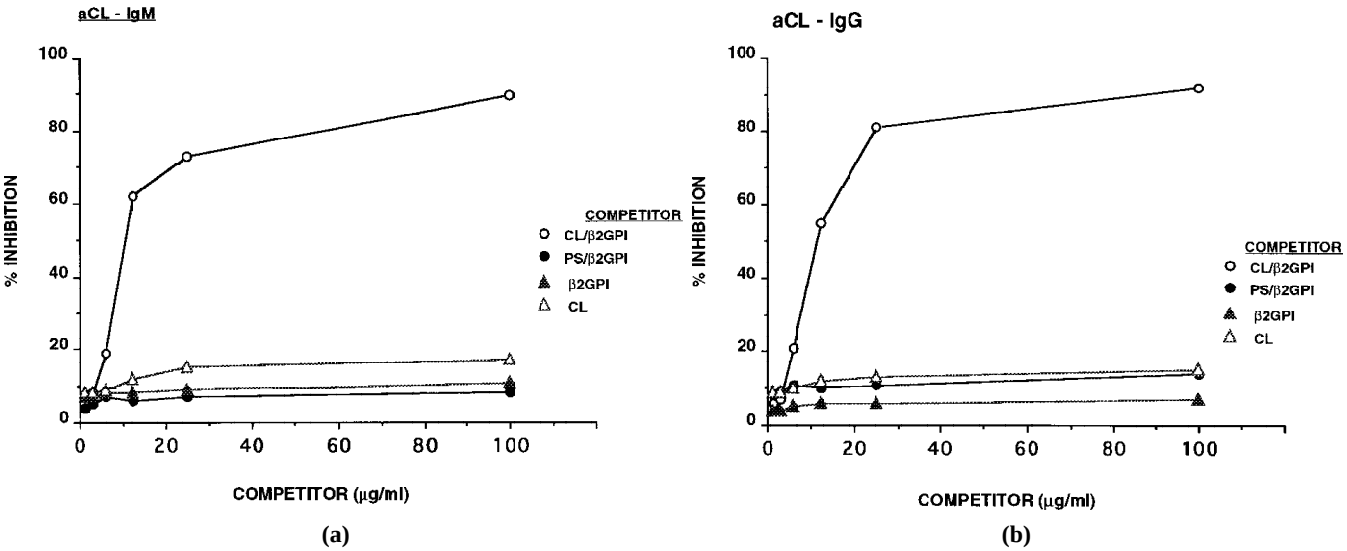

Fig. 3. Inhibition of aCL binding to cardiolipin by CL/βGPI complexes. (**a**) Inhibition of affinity purified human IgM aCL from a patient with monoclonal gammopathy by different concentrations of CL/βGPI complex. (**b**) Inhibition of affinity purified human IgG aCL from a patient with monoclonal gammopathy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

Table II. aCL binding of sera from mice immunized with human aCL derived from a patient with APS and monoclonal gammopathy

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

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

tical to those of the patient's aCL, i.e. they reacted only with CL/β_2GPI, and not with PS/β_2GPI, β_2GPI, CL, PS, or PC alone (Table II, Fig. 4 a,b).

Discussion

Herein we describe a unique human affinity purified aCL from a patient with monoclonal gammopathy. This antibody reacted only with cardiolipin attached to β_2GPI, and not with PS/β_2GPI complex, cardiolipin, β_2GPI alone, or a zwitterionic phospholipid such as phosphatidylcholine. Since inhibition studies confirmed the binding of autoantibodies only when both cardiolipin and β_2GPI were present, it is natural to conclude that the cryptic epitope against which the patient's antiphospholipid antibodies are directed may be either: 1) A new epitope of β_2GPI which is expressed only when β_2GPI is attached to cardiolipin, and not to other negatively charged phospholipids such as phosphatidylserine; or (2) an epitope formed only by the CL/β_2GPI complex. Concerning the first possibility, it has been generally proven that when β_2GPI interacts with negatively charged phospholipids, the complex exposes new cryptic epitopes (5-7). However, the possibility that the CL/β_2GPI complex and PS/β_2GPI might expose different epitopes of β_2GPI is not documented in the literature. It is widely accepted that aCL, which binds the complex of β_2GPI attached to negatively charged phospholipids, actually binds the β_2GPI molecule (3-7, 28, 29) [although there are alternative explanations as well (30)]. Possible locations of the target epitope for antiphospholipid binding on β_2GPI have been described on either: domains I-IV (31); domain I (32); domains III or IV (33); or domain V of β_2GPI (34). Therefore, we postulate that it is likely to relate the specific binding of our aCL only to CL/β_2GPI complex, but not to PS/β_2GPI complex, to the formation of a new epitope by both cardiolipin and β_2GPI.

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 anti-autoantibodies (anti-Id = Ab2), and after 1 to 2 months anti-anti-autoantibodies (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/β_2GPI but not PS/β_2GPI was found to be pathogenic *in vivo*. Mice immunized with this unique

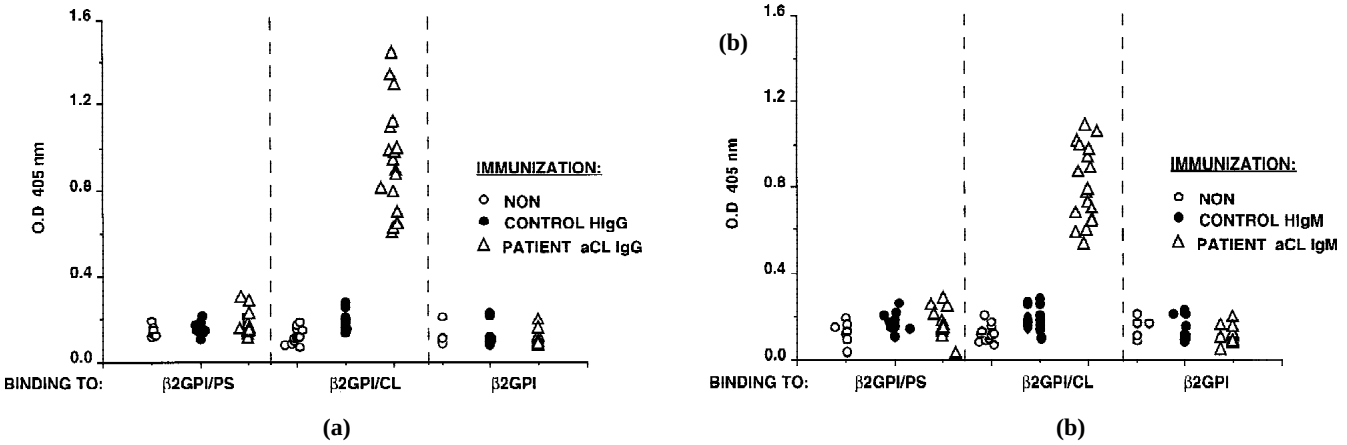

Fig. 4. Binding of mouse aCL to CL/β_2GPI complex. The sera of mice immunized with affinity purified human aCL IgG (**a**) or IgM (**b**) were tested separately for each mouse for binding to CL/βGPI complex PS/βGPI complex and direct binding to βGPI, at dilution of 1:400. Direct binding to cardiolipin ranged between 0.089 OD at 405 nm to 0.321 OD at 405 nm for all groups of mice.

antibody as either IgG or IgM developed experimental APS. Since the mice developed Ab3 which also binds only the CL/β_2GPI complex, this model in addition provides evidence for the pathogenicity of the antibodies drawn from this patient with monoclonal gammopathy.

In conclusion, the fact that anti-cardiolipin antibodies can bind an additional new target epitope expressed by the complex CL/β_2GPI, 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'.

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