Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 8606–8611, August 1996
Medical Sciences

A monoclonal IgG anticardiolipin antibody from a patient with the antiphospholipid syndrome is thrombogenic in mice

(β_2 glycoprotein 1/lupus anticoagulant activity/animal model)

TSAIWEI OLEE*†‡, SILVIA S. PIERANGELI‡§, HAROLD H. HANDLEY*, DZUNG T. LE¶, XIN WEI*, CHUNG-JENG LAI*, JULIE EN*, WILLIAM NOVOTNY*, E. NIGEL HARRIS§, VIRGIL L. WOODS, JR.*, AND POJEN P. CHEN*¶‖

*Department of Medicine and The Sam and Rose Stein Institute for Research on Aging, and ¶Department of Pathology, University of California at San Diego, La Jolla, CA, 92093; §Antiphospholipid Standardization Laboratory, Department of Medicine, University of Louisville, Louisville, KY, 40292; and ‖Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, 92037

Communicated by J. Edwin Seegmiller, University of California at San Diego, La Jolla, CA, May 1, 1996 (received for review August 2, 1995)

ABSTRACT **Antiphospholipid antibodies, including anticardiolipin antibodies (ACA), are strongly associated with recurrent thrombosis in patients with the antiphospholipid syndrome (APS). To date, reports about the binding specificities of ACA and their role(s) in causing and/or sustaining thrombosis in APS are conflicting and controversial. The plasmas of patients with APS, usually containing a mixture of autoantibodies, vary in binding specificity for different phospholipids/cofactors and vary in *in vitro* lupus anticoagulant activity. Although *in vivo* assays that allow assessment of the pathogenic procoagulant activity of patient autoantibodies have recently been developed, the complex nature of the mixed species prevented determination of the particular species responsible for *in vivo* thrombosis. We have generated two human IgG monoclonal ACA from an APS patient with recurrent thrombosis. Both bound to cardiolipin in the presence of 10% bovine serum, but not in its absence, and both were reactive against phosphatidic acid, but were nonreactive against purified human β-2 glycoprotein 1, DNA, heparan sulfate, or four other test antigens. Both monoclonal autoantibodies lacked lupus anticoagulant activity and did not inhibit prothrombinase activity. Remarkably, one of the monoclonal antibodies has thrombogenic properties when tested in an *in vivo* mouse model. This finding provides the first direct evidence that a particular antiphospholipid antibody specificity may contribute to *in vivo* thrombosis.**

Patients with systemic lupus erythematosus and recurrent thrombosis often have significant titers of a variety of anticardiolipin antibodies (ACA; particularly those of the IgG isotypes), as detected by solid phase immunoassay (1–5). Since most ACA also bind to a few other negatively charged phospholipids, such as phosphatidic acid (PA) or phosphatidylserine (PS), they are generally referred to as antiphospholipid antibodies (APA) (6). Consequently, the combined features of recurrent episodes of thrombosis and pregnancy loss in systemic lupus erythematosus patients with serum APA are termed the antiphospholipid syndrome (APS) (2–5). APS can be diagnosed in patients with no other identifiable autoimmune disorders as well and is referred to as "primary APS" (4–6). Paradoxically, many APS patients also have a serum factor that inhibits certain aspects of *in vitro* blood clotting and it is thus termed the "lupus anticoagulant" (LAC) (2–5, 7). Importantly, APS patients with LAC rarely have any bleeding problem.

There have been considerable differences in reports concerning the binding properties of APA and their role(s) in causing and/or sustaining thrombosis (8–10). Some studies showed that the binding of ACA to cardiolipin (CL) was markedly enhanced by a plasma protein, β_2 glycoprotein 1 (β_2GP1), suggesting that ACA reacted with phospholipid-β_2GP1 complexes (11–15). In contrast, other laboratories reported that these antibodies bound β_2GP1 alone (16–22). However, some laboratories failed to detect similar direct anti-β_2GP1 binding (15, 23). These conflicting data may reflect the heterogeneity of APA present in individual patient sera and/or different sets of these autoantibodies in clinically diverse patient populations in different studies. Moreover, the inconsistent findings suggest that thrombotic events in a patient may be caused by more than one kind of APA and autoantibody-mediated mechanism and that thrombosis may be accounted for by different kinds of APA in different patients (4, 24). In any case, the confounding data point to the critical need to obtain monoclonal IgG APA from seropositive APS patients with thrombosis and study each APA in terms of its *in vitro* binding properties, but much more importantly, study their thrombogenic potential in animals. Once a thrombogenic APA is identified, careful study of such monoclonal APA will allow delineation of the mechanisms by which APA produces thrombosis *in vivo* in humans.

It is well established that one of the criteria to define a pathogenic autoantibody is the ability of a suspected antibody to passively transfer the relevant clinical manifestation to a normal animal (25). Recently, murine models that demonstrate that passively administered APA can induce fetal loss and thrombosis have been established (26–30). Such *in vivo* models provide excellent tools for identifying pathogenic monoclonal APA. Here, we report the generation and characterization of two monoclonal IgG ACA from an APS patient with recurrent thrombosis and high titers of ACA. Both mAbs bound to CL in the presence of bovine serum, but not in its absence. The mAbs did not react with human β_2GP1 alone, nor with DNA, heparan sulfate, or four other test antigens, and lacked LAC activity as determined by a kaolin clotting time (KCT) test. Most important, one of the mAbs demonstrated thrombogenic properties in the *in vivo* mouse model. These findings provide strong evidence for the involvement of some ACA in the recurrent thrombosis of APS patients.

MATERIALS AND METHODS

Patient. Patient IS is a 19-year-old woman with primary APS. At age 16, she had spontaneous calf deep venous

Abbreviations: ACA, anticardiolipin antibodies; APA, antiphospholipid antibodies; APS, antiphospholipid syndrome; β_2GP1, β-2 glycoprotein 1; CL, cardiolipin; GPL, a unit equivalent to 1 μg/ml of an affinity-purified standard IgG; KCT, kaolin clotting time; LAC, lupus anticoagulant; NH, normal human; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PTT, partial thromboplastin time; ssDNA, single-stranded DNA; TBS, Tris-buffered saline.

†To whom reprint requests should be addressed at: Department of Medicine, 0663, University of California at San Diego, La Jolla, CA 92093-0663.
‡T.O. and S.S.P. contributed equally to this work.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

thrombosis and was treated for 3 months with heparin/coumadin. At age 18, she developed right-sided hemichorea associated with basal ganglia infarcts visible on magnetic resonance imaging and was treated with prednisone (10 mg per day) and low dose aspirin, which resulted in complete resolution of neurologic symptoms. Tests for serum ACA were performed in the clinical laboratory of the University of California at San Diego Medical Center Rheumatology Division. For the past 2 years, the patient has continuously had high titers of IgG ACA averaging about 780 GPL units. GPL is the unit of measurement for IgG ACA; one GPL unit is equivalent to 1 μg of affinity purified standard IgG ACA reference sample (31). In the standard anticardiolipin ELISA, we have the following ranges: <10 GPL, negative; 10–20 GPL, low positive; 20–80 GPL, medium positive; and >80 GPL, high positive.

Tests for LAC were performed in the Special Coagulation Laboratory of the University of California at San Diego Medical Center Hematology Clinical Laboratory, directed by Samuel Rapaport. One year before sample acquisition, the patient's prothrombin time was 13.3 sec (control value = 11.8 sec) and partial thromboplastin time (PTT) was 60.9 sec (versus the normal range of 26.0 ± 6 sec). The PTT remained prolonged after a 1:1 mix with normal plasma (43.8 sec). At the time of sample acquisition for the present study, the patient's prothrombin time was normal at 12.5 sec (the mean of normals was 11.7 sec), while the PTT was prolonged at 41.2 sec (versus the normal range of 26.4 ± 6 sec) and was 32.7 sec upon mixing at a 1:1 ratio with normal plasma (32). The LAC activity was confirmed with the dilute Russell viper venom test. The results showed a prolonged dilute Russell viper venom test at 63.9 sec (versus normal control at 32.5 sec), resulting in a ratio of 1.97 (a ratio of 1.2 or greater is considered positive) (33); the patient's dilute Russell viper venom test remained prolonged (ratio = 1.46) on a 1:1 mix with normal plasma. Levels of multiple phospholipid-dependent clotting factors assayed by an activated PTT technique were low: VIII, 86%; IX, 54%; XI, 40%; and XII, 42%. The measured levels increased upon dilution (1:5 and 1:10), characteristic of the LAC. Except for hypertension, mild proteinuria, and a marginal antinuclear antibody titer (1/40), the patient has never manifested rash, arthralgias, arthritis, organic brain syndrome, or serologies suggestive of lupus. The antithrombin activity (determined by chromogenic assay kit, Organon Technika–Cappel) and plasma levels of proteins C and S (determined by Laurell rocket immunoelectrophoresis) were normal. No mutation at position 1691 of factor V that renders the protein resistant to proteolytic inactivation by activated protein C was found. The blood sample for the present study was obtained when the patient was 2 months into a pregnancy, which culminated in the full-term delivery of a normal newborn. Medications at the time of blood donation consisted of prednisone (5 mg per day) and subcutaneous heparin.

Generation of Monoclonal IgG ACA-Secreting B Cell Lines. Peripheral blood mononuclear cells were isolated from patient IS and transformed with Epstein–Barr virus. The cells were resuspended in standard culture medium (RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 15 mM Hepes, pH 7.3) plus phytohemagglutinin (2 μg/ml; Sigma) and plated out at 10^4 cells per well in 96-well plates. The supernatants were screened for IgG ACA activity 10 days after plating. Cells from positive wells were subcloned twice at one cell per well to yield monoclonal cell lines.

Immunological Assays for IgG ACA. ACA was determined by ELISA as described in detail (31). Briefly, a microtiter plate was precoated with CL (50 μg/ml in ethanol; Sigma) or ethanol alone (as background control) and then blocked with 10% bovine serum in PBS. Test samples were diluted 1:1 with PBS containing 10% bovine serum and added to wells in duplicate. After a 1.5-hr incubation at room temperature, the plates were washed with PBS. Bound human IgG was detected with enzyme-labeled goat anti-human IgG and enzyme substrates. Binding to the ethanol-treated wells was subtracted from binding to CL-coated wells (31).

Subsequently, the mAbs were analyzed by ELISA for their reactivities with the following antigens: type VI collagen (Sigma), BSA, keyhole limpet hemocyanin, tetanus toxoid (Connaught Laboratories), and bovine thymus single-stranded DNA (ssDNA; Sigma). All antigens were used at 50 μg/ml to coat the ELISA plates. Detection of antibody activity against heparan sulfate was done by ELISA (34). The plates precoated with 150 μl of protamine chloride (0.5 mg/ml) were coated overnight at room temperature with 100 μl of heparan sulfate (25 μg/ml) and blocked with gelatin.

To determine the fine specificity of mAbs, mAbs were analyzed by ELISA for their binding to various phospholipids, including PA, PS, phosphatidylcholine (PC), and (PE; Sigma). The ELISA protocol was similar to that for CL, except that PA, PC, and PS were dissolved in a mixture of methanol/chloroform (4:1) (31). The isotypes of the mAbs were determined by ELISA with goat anti-human γ, κ, and λ. The subclasses of these two IgG mAbs were identified with murine mAbs against human IgG1, IgG2, IgG3, and IgG4 (Caltag, South San Francisco, CA) using ELISA.

To determine the β_2GP1-dependence of mAbs, IgG ACA were purified by protein G-affinity chromatography. These preparations were free of β_2GP1 contamination, as determined by an immunoblot assay with a rabbit anti-bovine β_2GP1 antiserum (data not shown). Purified mAb (at about 1 μg/ml) was then examined for binding to CL in the presence or absence of either bovine serum or purified β_2GP1. The monoclonal ACA B cell lines IS1 and IS2 were also examined for their binding to β_2GP1 by precoating wells with purified human β_2GP1 (20 μg/ml in PBS; kindly provided by R. Roubey, University of North Carolina at Chapel Hill) (22).

The LAC Test. The LAC activity of the isolated IgG ACA was determined by a modified KCT test as described (35). Briefly, 50 μl of monoclonal ACA (2 mg/ml) or IgG isolated from normal human sera (NH IgG; 6 mg/ml) in Tris-buffered saline (TBS) were each mixed with 50 μl of normal plasma, 50 μl of a 2% kaolin suspension (Sigma) was then added, and the mixture was incubated for 3 min at 37°C. The clotting reaction was then started by the addition of 100 μl of 0.03 M $CaCl_2$, and the clotting time was determined in a semiautomatic BBL fibrometer (Becton Dickinson). LAC activity was considered positive when the ratio of the clotting time of the test IgG to that of normal IgG exceeded 1.2 (33).

Prothrombinase Assay. Conversion of prothrombin to thrombin was quantitated by measuring the thrombin-dependent amidolysis of the synthetic thrombin substrate CBS 34.37 (H-D-CHG-But-Arg-pNA; American Bioproducts, Parsipanny, NJ). Ten mictoliters of the test mAb (2 mg/ml) or NH IgG (6 mg/ml) was mixed with 30 μl of human prothrombin (American Bioproducts) and 50 μl of the indicated phospholipid suspension; the mixture was incubated at 37°C for 15 min. Then, to each test mixture was added 10 μl a factor Xa and factor V solution (Enzyme Research Diagnostics, Indianapolis, IN) in TBS containing 0.5 mg/ml human serum albumin, and the mixture was incubated for 30 min. The final protein concentrations in the reaction mixture were 1.0 μM prothrombin, 0.1 nM factor Xa, and 0.2 nM factor V. After this second incubation, 12.5 μl of the reaction mixture was added to 450 μl of the CBS 34.37 substrate (at 0.2 mM in TBS containing 3 mM EDTA). Substrate amidolysis was measured by plotting increasing absorbance per minute at 405 nm with a Bio-Rad ELISA reader. The OD was recorded over a period of 1–10 min. The thrombin generated in the test samples was determined by comparison with a calibration curve constructed with known amounts of human thrombin. Previous studies with this assay system established the dependence of the observed

substrate amidolysis on thrombin generation. The rates of thrombin generation are linear during the 30-min incubation time. Thrombin activity under the aforementioned assay conditions is directly proportional to PS/PC vesicle concentrations between 0.2 and 10.0 μg/ml.

Purified human prothrombin displayed a single 68-kDa band on an SDS/10% polyacrylamide gel. Coagulation proteins were stored in aliquots at −70°C and thawed immediately before use in the assay. Inhibition of the prothrombinase reaction was calculated as [1 − (thrombin activity in a test sample/thrombin activity in NH IgG)] × 100% and was considered to be significant when percentage inhibition was ≥15%.

***In Vivo* Experiments.** The mouse model of thrombosis employed in this study has been described in detail (29). Briefly, CD1 mice (25–30 g, 4–8 weeks old, Charles River Breeding Laboratories) were injected i.p. with 500 μg of the indicated IgG preparations at time 0 and 48 hr later, resulting in a serum level in recipient mice of >50 GPL. At 72 hr, each animal was anesthetized, and the right femoral vein was exposed, resulting in a 1 cm segment of vein free for manipulation and observation. The vein was pinched with a pressure of 1500 g/mm^2 to introduce a standardized thrombogenic injury. Clot formation and dissolution in the transilluminated vein were monitored with a microscope equipped with a closed-circuit video system (including a color monitor and a recorder). Thrombus sizes (in square micrometers) were measured 1 min after each pinch by freezing the digitized image and tracing the outer margin of the thrombus; the times (in minutes) of formation (from appearance to maximum size) and disappearance (from maximum size to disappearance) of the thrombus were measured as well. The unpaired Student's *t* test was used to compare the means of thrombus sizes and times (formation, disappearance) among groups.

RESULTS

Generation of Monoclonal IgG ACA. Five million peripheral blood mononuclear cells from a primary APS patient (IS) with recurrent thrombosis and high titers of serum ACA were transformed with Epstein–Barr virus and were seeded at 10^4 cells per well. By day 10, growing transformed cells were found in all wells. Supernatants from 480 wells were analyzed for IgG ACA, and 14 wells were found to be positive. Cells from each positive well were split, and half of the cells were transferred to a new 96-well plate; after incubation for 4 days, the supernatants from the transferred wells were analyzed for ACA. Only 10 of the 14 initially positive wells remained positive. Cells from each confirmed positive well were subcloned at 100 cells per well.

Two weeks after this first subcloning, growing subcultures were found for all 10 positive clones. However, ACA activity was found in the subcultures of only 3 of the 10 previously positive wells. Thereafter, cells from one to three of the most strongly positive wells of each primary positive clone were subcloned at one cell per well. ACA-positive wells were found in two of the three primary positive clones. Thereafter, one positive well from each of these two remaining clones was cloned again twice at one cell per well to ensure monoclonality. The resultant monoclonal ACA B cell lines were designated ACA IS1 and ACA IS2. It should be noted that the ACA IS1 B cells grow rapidly and were subcloned easily at one cell per well. Consequently, the monoclonal ACA IS1 B cell line was obtained about 6 weeks after transformation, whereas the monoclonal IS2 B cell line was obtained about 10 weeks after transformation. These findings suggested that IS1 and IS2 were most likely derived from two distinct ACA-secreting B cells.

Immunological Characterization of the Two Monoclonal IgG ACA. Both mAbs were examined first for their binding specificity by ELISA with antigens including BSA, collagen, keyhole limpet hemocyanin, tetanus toxoid, ssDNA, and heparan sulfate. No significant binding to any of these antigens was observed (Fig. 1), indicating that both mAbs were specific for CL.

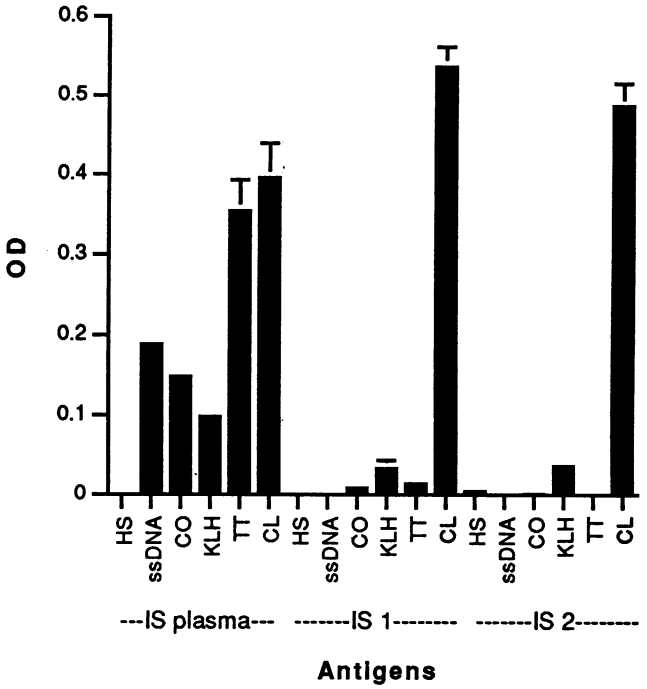

FIG. 1. Antigen specificity of ACA IS1 and IS2. Purified mAbs (at 5 μg/ml) and the patient IS plasma (at 1:100 dilution) were tested against various antigens (50 μg/ml), including CL, BSA, chicken ovalbumin (CO), keyhole limpet hemocyanin (KLH), ssDNA, tetanus toxoid (TT), and heparan sulphate (HS). The net OD readings (after subtracting the binding of the same antibody to wells coated with ethanol) with standard deviation are shown.

To determine the fine specificity of these two mAbs, the culture supernatants were analyzed against four other phospholipids, including PA, PC, PE, and PS, in the presence of bovine serum (containing bovine β_2GP1). As can be seen in Fig. 2, both mAbs bound to CL and PA, but not to PC, PE, or PS. Since both CL and PA are negatively charged phospholipids, the mAbs were analyzed for their binding to negatively charged double-stranded DNA, ssDNA, and heparan sulfate. No significant binding to either antigen was observed (data not

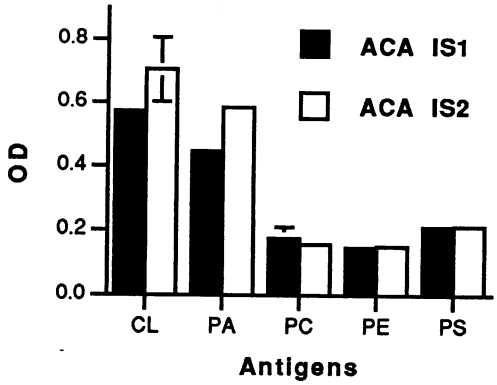

FIG. 2. Binding of ACA IS1 and IS2 (approximately 2 μg/ml) in culture supernatants (which contain bovine β_2GP1) to different phospholipids. The antigens are CL, PA, PC, PE, and PS. The mean net OD readings, after subtracting both the binding of the same antibody to wells coated with respective solvents (such as ethanol for CL) and the binding of a NH IgG to the corresponding antigens, with standard deviation are shown. Since the net OD of the upper limit of a 95% confidence level from analyses of 15 different NH IgG samples for all antigens is 0.2, any binding with a net OD ≤0.2 is considered not significant.

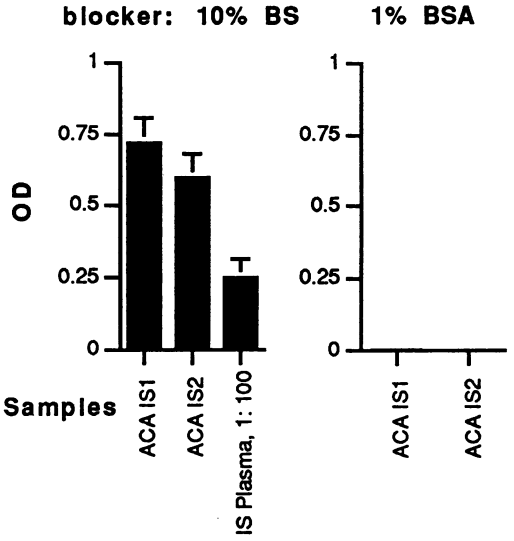

FIG. 3. Binding of affinity purified ACA IS1 and IS2 (at about 1 μg/ml) to CL in the presence of 10% bovine serum (BS) or 1% BSA. The net OD readings (after subtracting the binding of the same antibody to wells coated with ethanol) with standard deviation are shown.

shown). The combined data showed that both mAbs were specific for the phospholipids PA and CL. When the isotypes and subclasses of these two mAbs were tested, both ACA IS1 and IS2 were IgG1, κ.

To determine if cofactor was required in the binding of ACA IS1 and IS2 to CL, affinity purified mAbs free of β_2GP1 were compared for their binding to CL in the presence or absence of bovine serum. As shown in Fig. 3, both mAbs bound to CL in the presence of bovine serum but not in its absence. To further identify whether β_2GP1 was the cofactor required in the binding of both mAbs to CL, purified human β_2GP1 was used to block the CL-coated plate. No binding to wells containing β_2GP1 and CL was found for both mAbs (data not shown). These results suggest that the binding of ACA IS1 and IS2 to CL depends on other serum cofactors (11, 13, 36).

ACA IS1 and IS2 were tested for their binding to β_2GP1, according to the protocol of Roubey *et al.* (22). As can be seen

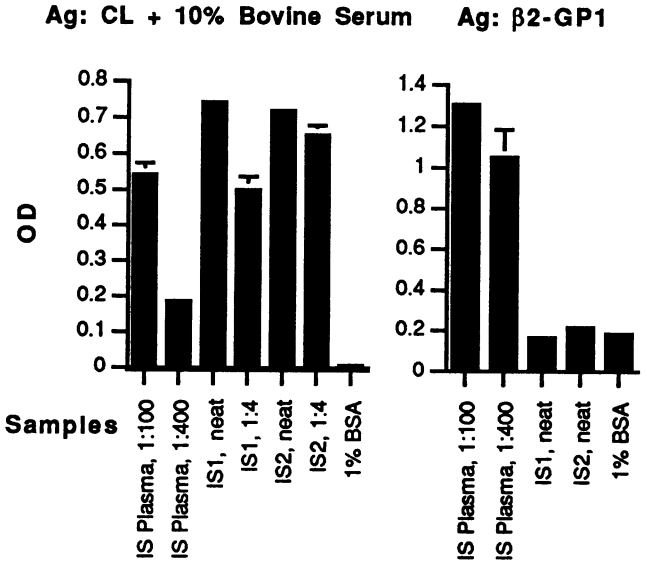

FIG. 4. Comparative binding of ACA IS1 and IS2 to CL plus bovine serum or to β_2GP1 alone. (*Left*) Wells were coated with CL and blocked with 10% bovine serum in PBS. (*Right*) Wells were coated with β_2GP1 and blocked with 0.5% BSA in borate-buffered saline. IS plasma is obtained from patient IS, from whom the mAbs were produced.

Table 1. ACA IS1 and ACA IS2* lack LAC activity

Test samples	Sample/NH plasma	LAC activity KCT(s)	LAC activity Ratio§
Control 1†	—	66 ± 7*	—
LAC control	—	133 ± 2	—
TBS‡	50 ul : 50 ul	82 ± 1	—
NH IgG‡	50 ul : 50 ul	78 ± 2	—
ACA IS1‡	50 ul : 50 ul	79 ± 6	1.01
ACA IS2‡	50 ul : 50 ul	78 ± 2	1.00

*The LAC activity of mAbs was determined by a modified KCT test (35). Each sample was measured three times, and the mean ± SD is given.
†NH plasma and a patient plasma with known LAC activity were provided in a commercial KCT test kit (Thromboscreen; Pacific Hemostasis, Ventura, CA).
‡The mAbs (2 mg/ml) and NH IgG (6 mg/ml) were in TBS.
§LAC activity is considered positive when the ratio of the clotting time of the testing IgG to that of NH IgG exceeds 1.2 (33).

in Fig. 4, we detected substantial anti-β_2GP1 antibodies in the serum of patient IS at 1:100 and 1:400 dilutions. However, under identical conditions, ACA IS1 and IS2 did not bind to β_2GP1. In contrast, the same mAb-containing supernatants (at both neat and 1:4 dilution) displayed significant binding to CL plus bovine serum.

***In Vitro* Physiological Properties of ACA IS1 and IS2.** The mAbs were then analyzed for LAC activity by a modified KCT assay (33, 35). As can be seen in Table 1, neither ACA IS1 nor ACA IS2 displayed LAC activity, as measured by the KCT. One of the proposed mechanisms for the LAC activity of APA

Table 2. Minimal inhibition of the prothrombinase complex by ACA IS1 and IS2*

Exp.	Liposome	Assay conditions	OD†	% inhibition‡
1	PS/PC	PL only	0.778	—
	20:80;	PL + NH IgG	0.727	0
	2 μg/ml	PL + APA IgG 1	0.455	38
		PL + APA IgG 2	0.132	82
		PL + ACA IS1	0.668	9
		PL + ACA IS2	0.656	10
		No PL	0.063	—
2	PS/PC	PL only	0.828	—
	20:80;	PL + NH IgG	0.727	0
	1 μg/ml	PL + APA IgG 1	0.331	65
		PL + APA IgG 2	0.092	98
		PL + ACA IS1	0.719	2
		PL + ACA IS2	0.771	0
		No PL	0.063	—
3	CL/PC	PL only	0.625	—
	20:80;	PL + NH IgG	0.658	0
	40 μg/ml	PL + APA IgG 2	0.367	45
		PL + ACA IS1	0.533	19
		PL + ACA IS2	0.556	16
		No PL	0.063	—

PL, phospholipid.
*The reactions were supported by phospholipid liposomes of the indicated compositions. There was neither plasma nor β_2GP1 in the reaction mixtures. Controls APA IgG 1 and APA IgG 2 are IgG prepared from APS plasma with medium and high antiprothrombinase activity, respectively. The concentrations of IgG were 6 mg/ml NH IgG, 5 mg/ml APA IgG 1 and APA IgG 2, and 2 mg/ml ACA IS1 and ACA IS2.
†The OD was recorded over a period of 1-10 min at 405 nm. The values are at the end point, which is after 10 min of color reaction.
‡Inhibition of the prothrombinase activity by test samples is considered to be significant when % inhibition is ≥15%. This value was determined by replicate testing of eight different NH IgG preparations on different days.

Table 3. The size and times of thrombus formation in mice injected with affinity-purified IgG ACA and monoclonal IgG ACA from APS patients

Experiment	IgG injected	No. of mice	Thrombus area, mean ± SD in μm^2	Thrombus time,* mean ± SD in min	
				Formation	Disappearance
1	ACA IS2	4	3400 ± 1079‡	2.6 ± 0.9‡	5.6 ± 0.5‡
	Neg Hu† mAb	4	354 ± 200	0.8 ± 0.3	3.0 ± 0.6
2	ACA IS2	4	2404.7 ± 1079.5‡	1.87 ± 0.25	4.5 ± 0.25‡
	H2†	4	606.25 ± 342.7	1.37 ± 0.25	3.25 ± 0.29

*Thrombus formation time was from the appearance of a clot to its maximum size, and disappearance time was from the maximum size to the disappearance of a clot.
†Controls used were two human monoclonal IgG with ACA activity (designated Neg Hu mAb and H2). H2 was a human monoclonal IgG that reacts with herpes simplex virus (38).
‡The difference between the ACA- and the Neg Hu mAb-treated mice is highly significant ($P \leq 0.02$).

is that the antibody binds to phospholipids in the prothrombinase complex and thus inhibits the generation of thrombin in the final common pathway of blood coagulation (6, 37). Accordingly, both mAbs were examined for their effects in inhibiting prothrombinase activity. Table 2 shows that the two present mAbs do not inhibit generation of thrombin in the presence of liposomes composed of PS and PC, and minimally inhibit thrombin generation in the presence of the liposomes composed of CL and PC. These data are consistent with our observation that both mAbs reacted strongly with CL but were unreactive with PC or PS.

Thrombogenic Properties of Monoclonal ACA IS2. An animal model was used to determine the thrombogenic potential of the two mAbs (29). An initial exploratory study indicated that passively administered IS2, but not IS1, was thrombogenic in the CD1 mouse when compared with polyclonal NH IgG (data not shown).

Subsequently, two more experiments were performed to confirm the thrombogenic potential of ACA IS2. In each experiment, groups of four mice were injected with either ACA IS2 or a control human monoclonal IgG without ACA activity. Five thrombi were induced per mouse; the five measurements for each variable in each mouse were averaged, and the results were used to calculate the mean ± SD for each group. As can be seen in Table 3, experiment 1, thrombus sizes were significantly larger (3400 ± 1079 μm^2) in mice injected with ACA IS2 compared with the thrombi in control mice (354 ± 200 μm^2). In addition, highly significant delays in thrombus formation and disappearance times were observed with ACA IS2 as compared with the non-ACA control. Similar results were shown in experiment 2 (Table 3), in which the thrombus size was significantly higher in mice with ACA IS2 injection (2404.7 ± 1079.5 μm^2 versus 606.25 ± 342.7 μm^2), and the thrombus formation and disappearance times were longer.

DISCUSSION

We generated two monoclonal IgG ACA by transforming peripheral blood mononuclear cells from a primary APS patient with recurrent thrombosis and high titers of serum ACA. This patient has normal protein C and protein S levels, and shows no mutation on position 1691 of factor V that renders the protein resistant to proteolytic inactivation by activated protein C. Therefore, the thrombosis events in this APS patient are not due to abnormal protein C, protein S, or factor V. The last finding is consistent with a recent report that factor V Leiden mutation is not common in APS patients (39).

Both mAbs were monospecific for phospholipids in the presence of bovine serum and both bound to negatively charged CL and PA, but not to PS, PC, PE, heparan sulfate, or DNA. Lack of DNA crossreactivity is usually seen with polyclonal APA antibodies (40–42). Neither ACA IS1 nor IS2 bound to purified β_2GP1 plus or minus CL, but their binding to CL depended on serum cofactors, a known characteristic of APA in APS patients (11, 13, 36). Based on a KCT assay, the mAbs did not possess any LAC activity. Hence, the mAbs described in this report have features similar to at least one subset of polyclonal APA from patients with the APS, in that they are specific for some negatively charged phospholipids, exhibit enhanced binding activity in the presence of sera, and lack LAC or prothrombinase inhibitory activity (11, 43–46).

During the course of our studies, Hasegawa *et al.* reported the generation of two monoclonal IgM ACA by transforming peripheral blood mononuclear cells from a 38-year-old woman with primary APS (47). Of these two mAbs, one displayed LAC activity. Importantly, Hasegawa *et al.* did not study the potential *in vivo* prothrombotic properties of these monoclonals. Taken together with our findings, the properties of these four monoclonal ACA from two APS patients clearly indicate that generation and characterization of monoclonal ACA from patients are required to define various properties of specific individual APA in APS. Such monoclonal reagents may contribute to identification of disease-relevant thrombogenic autoantibodies.

Though lacking LAC activity, ACA IS2 is thrombogenic in an *in vivo* animal model. This is the first evidence demonstrating that an APS patient-derived monoclonal ACA can have thrombogenic properties. Several candidate mechanisms have been proposed to explain the thrombogenic properties of APA in APS patients (for reviews, see refs. 4, 10, and 24). Certain APA may bind to β_2GP1 and/or its complexes with phospholipid and, thus, interfere with proposed anticoagulant functions of β_2GP1 and lead to a hypercoagulable state (11, 48, 49). However, a main problem with this hypothesis is that the individuals homozygous for the null allele of β_2GP1 are completely healthy (50). Alternatively, APA may interfere with antithrombin activity through binding to heparin-like molecules (51, 52). However, this mechanism may not explain the thrombogenic activity of ACA IS2 since ACA IS2 does not bind heparan sulfate. Some APA may react with phospholipids on endothelial cells and, thus, reduce their production of prostacyclin (which inhibits platelet aggregation) and/or interfere with the thrombomodulin-dependent activation of protein C (4, 36, 53). Since activated protein C proteolytically inactivates activated factors Va and VIIIa, the reduced activation of protein C may lead to a procoagulant effect and thrombotic events. In addition, APA may bind to platelets and promote platelet aggregation (38, 55–57).

The evidence from a single mAb does not establish that ACA in APS patients are thrombogenic, but it does support the hypothesis that this type of ACA in APS patients may be thrombogenic. The availability of the present patient-derived monoclonal thrombogenic ACA as well as the generation of additional monoclonal IgG APA will allow us to examine decisively all proposed mechanisms of APA-mediated thrombosis *in vitro* and *in vivo*. Moreover, the mAbs can be characterized at the molecular level, so that the structural bases of

various binding patterns and biological functions may be delineated.

We thank Dr. R. Roubey for kindly providing purified β_2GP1 and an anti-β_2GP1 serum sample, Dr. Chul-Soo Cho for advice on the data, Alex Okun for technical support, and Winnie Kwok and Jane Uhle for manuscript preparation. We gratefully acknowledge the assistance of the University of California at San Diego Institute of Biomedical Engineering Adhesion Receptor Structure/Function Core Facility, funded by a grant from the Whittaker Function. This research was supported by National Institutes of Health Grants AI32243, AR42506, CA58527, AG07996M, and HL30480.

1. Harris, E. N., Gharavi, A. E., Boey, M. L., Patel, B. M., Mackworth-Young, C. G., Loizou, S. & Hughes, G. R. (1983) *Lancet* **ii,** 1211–1214.
2. Lockshin, M. D. & Letendre, C. H. (1992) *Arthritis Rheum.* **35,** 1234–1237.
3. McNeil, H. P., Hunt, J. E. & Krilis, S. A. (1992) *Scand. J. Immunol.* **36,** 647–652.
4. Triplett, D. A. (1993) *Arch. Pathol. Lab. Med.* **117,** 78–88.
5. Hughes, G. R. V. (1993) *Lancet* **342,** 341–344.
6. Asherson, R. A. & Cervera, R. (1993) in *Dubois' Lupus Erythematosus*, eds. Wallace, D. J. & Hahn, B. H. (Lea & Febiger, Philadelphia), pp. 233–244.
7. Brandt, J. T., Triplett, D. A., Alving, B. & Scharrer, I. (1995) *Thromb. Haemostasis* **74,** 1185–1190.
8. Harris, E. N. & Pierangeli, S. S. (1994) *Springer Semin. Immunopathol.* **16,** 223–245.
9. Roubey, R. A. S. (1994) *Springer Semin. Immunopathol.* **16,** 211–222.
10. Roubey, R. A. S. (1994) *Blood* **84,** 2854–2867.
11. McNeil, H. P., Simpson, R. J., Chesterman, C. N. & Krilis, S. A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4120–4124.
12. Matsuura, E., Igarashi, Y., Fujimoto, M. & Ichikawa, K. (1990) *Lancet* **336,** 177–178.
13. Sammaritano, L. R., Lockshin, M. D. & Gharavi, A. E. (1992) *Lupus* **1,** 83–90.
14. Jones, J. V., James, H., Tan, M. H. & Mansour, M. (1992) *J. Rheumatol.* **19,** 1397–1402.
15. Pierangeli, S. S., Harris, E. N., Davis, S. A. & DeLorenzo, G. (1992) *Br. J. Haematol.* **82,** 565–570.
16. Galli, M., Comfurius, P., Maassen, C., Hemker, H. C., de Baets, M. H., van Breda-Vriesman, P. J. C., Barbui, T., Zwaal, R. F. A. & Bevers, E. M. (1990) *Lancet* **335,** 1544–1547.
17. Arvieux, J., Pouzol, P., Roussel, B., Jacob, M. C. & Colomb, M. G. (1992) *Br. J. Haematol.* **81,** 568–573.
18. Keeling, D. M., Wilson, A. J. G., Mackie, I., Machin, S. J. & Isenberg, D. A. (1992) *Br. J. Haematol.* **82,** 571–574.
19. Viard, J. P., Amoura, Z. & Bach, J. F. (1992) *Am. J. Med.* **93,** 181–186.
20. Matsuura, E., Igarashi, Y., Yasuda, T., Triplett, D. A. & Koike, T. (1994) *J. Exp. Med.* **179,** 457–462.
21. Ichikawa, K., Khamashta, M. A., Koike, T., Matsuura, E. & Hughes, G. R. V. (1994) *Arthritis Rheum.* **37,** 1453–1461.
22. Roubey, R. A. S., Eisenberg, R. A., Harper, M. F. & Winfield, J. B. (1995) *J. Immunol.* **154,** 954–960.
23. Gharavi, A. E., Harris, E. N., Sammaritano, L. R., Pierangeli, S. S. & Wen, J. (1993) *J. Lab. Clin. Med.* **122,** 426–431.
24. Santoro, S. A. (1994) *Blood* **83,** 2389–2391.
25. Bona, C. A. (1991) *Autoimmunity* **10,** 169–172.
26. Smith, H. R., Hansen, C. L., Rose, R. & Canoso, R. T. (1990) *J. Rheumatol.* **17,** 911–915.
27. Blank, M., Cohen, J., Toder, V. & Shoenfeld, Y. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3069–3073.
28. Bakimer, R., Fishman, P., Blank, M., Sredni, S., Djaldetti, M. & Shoenfeld, Y. (1992) *J. Clin. Invest.* **89,** 1558–1563.
29. Pierangeli, S. S., Barker, J. H., Stikovac, D., Ackerman, D., Anderson, G., Barquinero, J., Acland, R. & Harris, E. N. (1994) *Thromb. Haemostasis* **71,** 670–674.
30. Pierangeli, S. S., Liu, X., Barker, J. H., Anderson, G. & Harris, E. N. (1995) *Thromb. Haemostasis* **74,** 1361–1367.
31. Harris, E. N., Pierangeli, S. S. & Birch, D. J. (1994) *Am. J. Clin. Pathol.* **101,** 616–624.
32. Triplett, D. A. (1992) *Stroke* **23,** 11–14l.
33. Exner, T., Rickard, K. A. & Kronenberg, H. (1978) *Br. J. Haematol.* **40,** 143–151.
34. Faaber, P., Rijke, T. P. M., van de Putte, L. B. A., Capel, P. J. A. & Berden, J. H. M. (1986) *J. Clin. Invest.* **77,** 1824–1830.
35. Pierangeli, S. S., Harris, E. N., Gharavi, A. E., Goldsmith, G., Branch, D. W. & Dean, W. L. (1993) *Br. J. Haematol.* **85,** 124–132.
36. Oosting, J. D., Derksen, R. H. W. M., Bobbink, I. W. G., Hackeng, T. M., Bouma, B. N. & de Groot, P. G. (1993) *Blood* **81,** 2618–2625.
37. Thiagarajan, P., Shapiro, S. S. & De Marco, L. (1980) *J. Clin. Invest.* **66,** 397–405.
38. Huang, D.-F., Olee, T., Masuho, Y., Matsumoto, Y., Carson, D. A. & Chen, P. P. (1992) *J. Clin. Invest.* **90,** 2197–2208.
39. Dizon-Townson, D., Hutchison, C., Silver, R., Branch, D. W. & Ward, K. (1995) *Thromb. Haemostasis* **74,** 1029–1031.
40. Harris, E. N., Gharavi, A. E., Tincani, A., Chan, J. K., Englert, H., Mantelli, P., Allegro, F., Ballestrieri, G. & Hughes, G. R. (1985) *J. Clin. Lab. Immunol.* **17,** 155–162.
41. Eilat, D., Zlotnick, A. Y. & Fischel, R. (1986) *Clin. Exp. Immunol.* **65,** 269–275.
42. Smeenk, P. J. T., Luccassen, W. A. M. & Swaak, T. J. G. (1987) *Arthritis Rheum.* **30,** 607–613.
43. Galli, M., Comfurius, P., Barui, T., Zwaal, R. F. A. & Bevers, E. M. (1992) *Thromb. Haemostasis* **68,** 297–300.
44. Oosting, J. D., Derksen, R. H. W. M., Entjes, H. T. I., Bouma, B. N. & de Groot, P. G. (1992) *Thromb. Haemostasis* **67,** 499–502.
45. Shi, W., Chong, B. H., Hogg, P. J. & Chesterman, C. N. (1993) *Thromb. Haemostasis* **70,** 342–345.
46. Pierangeli, S. S., Goldsmith, G., Krnic, S. & Harris, E. N. (1994) *Infect. Immun.* **62,** 4081–4084.
47. Hasegawa, I., Suzuki, T., Ishii, S., Takakuwa, K. & Tanaka, K. (1994) *Thromb. Res.* **74,** 77–84.
48. Schousboe, I. (1985) *Blood* **66,** 1086–1091.
49. Mackworth-Young, C. (1990) *Immunol. Today* **11,** 60–65.
50. Alper, C. A. (1990) in *Hematology*, eds. Williams, W. J., Beutler, E., Erslev, A. J. & Lichtman, M. A. (McGraw-Hill, New York), pp. 1616–1627.
51. Chamley, L. W., McKay, E. J. & Pattison, N. S. (1993) *Thromb. Res.* **71,** 103–111.
52. Shibata, S., Harpel, P. C., Gharavi, A., Rand, J. & Fillit, H. (1994) *Blood* **83,** 2532–2540.
53. Malia, R. G., Kitchen, S., Greaves, M. & Preston, F. E. (1990) *Br. J. Haematol.* **76,** 101–107.
54. Galli, M., Cortelazzo, S., Viero, P., Finazzi, G., de Gaetano, G. & Barbui, T. (1988) *Eur. J. Haematol.* **41,** 88–94.
55. Hasselaar, P., Derksen, R. H. W. M., Blokzijl, L. & de Groot, P. G. (1990) *Thromb. Haemostasis* **63,** 169–173.
56. Marciniak, E. & Romond, E. H. (1989) *Blood* **74,** 2426–2432.
57. Campbell, A. L., Pierangeli, S. S., Wellhausen, S. & Harris, E. N. (1995) *Thromb. Haemostasis* **73,** 529–534.