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ASSOCIATION OF AUTOANTIBODIES AGAINST THE PHOSPHATIDYLSERINE–PROTHROMBIN COMPLEX WITH MANIFESTATIONS OF THE ANTIPHOSPHOLIPID SYNDROME AND WITH THE PRESENCE OF LUPUS ANTICOAGULANT

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Objective. **To clarify the association of autoantibodies against prothrombin with the clinical manifestations of the antiphospholipid syndrome (APS) and with the presence of lupus anticoagulant (LAC).**

Methods. **We examined 265 patients who visited our autoimmune disease clinic. IgG and IgM antiprothrombin antibodies were tested by enzyme-linked immunosorbent assay (ELISA) as either antiphosphatidylserine–prothrombin complex (aPS/PT) antibodies or as antibodies against prothrombin coated on irradiated ELISA plates (as antigen) (aPT). IgG, IgM, and IgA anticardiolipin (aCL) antibodies and their β_2-glycoprotein I (β_2GPI) dependency were also evaluated by ELISA. LAC was tested by 3 different methods.**

Results. **The presence of aPS/PT, but not of aPT, significantly correlated with the clinical manifestations of APS (odds ratio [OR] 4.39, 95% confidence interval [95% CI] 2.06–9.38), and aPS/PT antibodies were as specific as β_2GPI-dependent aCL for APS (93.1% for both). IgG aPS/PT strongly correlated with the presence of LAC as detected using the dilute Russell viper venom time test (OR 38.2, 95% CI 13.4–109.1).**

Conclusion. **Antiprothrombin antibodies are heterogeneous and their clinical relevance depends on the method of detection applied. Positive results on the aPS/PT test can serve as a marker of thrombotic events in patients with autoimmune diseases.**

The specificity of antiphospholipid antibodies, which are associated with thrombotic events, is not directed to phospholipids, but rather to phospholipid-binding proteins or phospholipid–protein complexes (1,2). The presence of antiphospholipid antibodies is associated with the occurrence of arterial/venous thrombosis, recurrent fetal loss, neurologic disorders, and thrombocytopenia. The term "antiphospholipid syndrome" (APS) has been used to describe a condition in which these clinical manifestations are linked with the persistence of antiphospholipid antibodies, and this is now recognized as one of the most common causes of acquired thrombophilia (3,4). In clinical practice, the detection of both anticardiolipin (aCL) antibodies by enzyme-linked immunosorbent assay (ELISA) and lupus anticoagulant (LAC) by clotting assays has been standardized for the diagnosis of APS. However, the family of antiphospholipid antibodies has recently been expanded to include a heterogeneous group of autoantibodies whose specificity is directed to proteins involved in coagulation or to a complex of these proteins with phospholipids.

Among the phospholipid-binding proteins, β_2-glycoprotein I (β_2GPI), which bears the epitopes for aCL binding, has been extensively studied. These epitopes are exposed when β_2GPI binds to negatively charged phospholipids such as cardiolipin, or binds to irradiated plastic plates (5), thus behaving as a cofactor for aCL binding. Studies have highlighted the significance of using anti-β_2GPI antibodies as an alternative ELISA method that has higher specificity than that of

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the conventional aCL ELISA (6,7). β_2GPI has also been shown as a cofactor in LAC activity (8).

Prothrombin, another phospholipid-binding protein, was first proposed as a possible cofactor in LAC activity by Löliger in 1959 (9). In subsequent years, the interest regarding this protein has increased and several groups have investigated the characteristics of antiprothrombin antibodies. These antibodies are commonly detected by ELISA methods in which irradiated plates are utilized (10) or in which prothrombin is detected in complex with phosphatidylserine (PS/PT) (11). In fact, antiprothrombin antibodies cannot bind when prothrombin is immobilized onto nonirradiated plates (10,11), but binding is observed if prothrombin is immobilized on a suitable anionic surface, adsorbed on gamma-irradiated plates, or exposed to immobilized anionic phospholipids. An analogy between the behavior of these antibodies and that of anti-β_2GPI has been suggested. Antiprothrombin antibodies may be directed against cryptic epitopes or neoepitopes (as antigens) that are exposed when prothrombin binds to anionic phospholipids, and/or may act as low-affinity antibodies that bind bivalently to immobilized prothrombin.

Prothrombin appears to be a common antigenic target of antiphospholipid antibodies, since antiprothrombin antibodies are detected in ~50–90% of patients with other antiphospholipid antibodies (12). Because antiprothrombin antibodies have heterogeneous immunologic and functional properties, their clinical significance has not been established. Some studies, including our previous study on 207 patients with systemic lupus erythematosus (SLE) (13), have shown positive, albeit weak, correlations with the clinical manifestations of APS. However, other investigators have found no correlations (14), and therefore the value of antiprothrombin antibodies as markers of APS remains to be determined. Moreover, the difference in the clinical significance between antiprothrombin antibodies that are detected using the irradiated ELISA plate system (aPT) and those detected using the phospholipid-bound prothrombin system (aPS/PT) has not yet been determined.

To clarify the clinical value of antiprothrombin detection, we investigated the significance of antiprothrombin antibodies detected using the 2 different methods in a cohort of patients with systemic autoimmune diseases. We also examined the clinical significance of antiprothrombin antibodies in relation to manifestations of APS, in comparison with that of antiphospholipid antibodies detected by established methods.

Table 1. Profile of 265 patients with systemic autoimmune diseases*

Diagnosis, no. of patients	
Primary APS	21
SLE with APS	24
SLE without APS	79
RA	46
Primary Sjögren's syndrome	36
Polymyositis/dermatomyositis	14
Scleroderma	21
Behçet's disease	7
Vasculitis syndrome	3
Other	14
Mean age (range), years	47 (17–85)
No. female:no. male	234:31
Clinical features of APS, no. (%)†	62 (23)
Arterial thrombosis	36 (14)
Venous thrombosis	17 (6)
Pregnancy loss	15/177 (8)
Thrombocytopenia (<100,000/μl), no. (%)	38 (14)
Clinical features and/or thrombocytopenia, no. (%)	90 (34)

* APS = antiphospholipid syndrome; SLE = systemic lupus erythematosus; RA = rheumatoid arthritis.
† Thrombocytopenia is excluded on the basis of the 1999 preliminary criteria for APS (16). Some patients had more than 1 clinical feature.

PATIENTS AND METHODS

Patients. The study population comprised 265 consecutive Japanese patients with systemic autoimmune diseases who were examined in the rheumatology clinic of Hokkaido University Hospital. Clinical records were carefully reviewed retrospectively and/or patients were interviewed at the same time as sample collection. This was a retrospective cross-sectional study in which the time interval between events and time of sampling varied from 8 years to 3 months. Arterial events comprised stroke, myocardial infarction, and iliac artery occlusion, confirmed by computed tomography scan, magnetic resonance imaging, or angiography. Deep vein thrombosis and pulmonary thrombosis were defined as venous thrombosis, and were confirmed by angiography or scintigram. The clinical profiles of these patients are described in Table 1.

In Table 1, patients with diagnoses of primary APS or SLE with APS represent a group of subjects who were already diagnosed as having APS based on the clinical features of APS and positive findings of aCL or LAC (15) when their blood samples were collected, whereas the numbers of patients listed as having the clinical features of APS and thrombocytopenia represent those patients who had such clinical features regardless of the presence of aCL or LAC.

In the previous proposed criteria for APS (15), thrombocytopenia was considered to be one definite clinical feature of APS. However, in the current preliminary criteria for APS, thrombocytopenia was excluded from the major clinical features of APS (16). Therefore, in this study, data from the following subgroups were analyzed: 1) Patients with the clinical features of APS (i.e., patients with arterial thrombosis, venous thrombosis, and/or pregnancy loss), and 2) those with the clinical features of APS and/or thrombocytopenia. Pregnancy loss was defined according to the current preliminary criteria for APS (16).

Table 2. Representative results of the assay for aPS/PT in comparison with other assay results

Patient	PS/PT*	PS*	aPS/PT†	aCL‡	LAC
IgG					
1	0.812	0.004	54	1.0	+
2	0.800	0.013	36	3.6	+
3	1.294	0.014	90	6.7	+
4	1.244	0.055	100	30	+
5	0.488	0.023	22	>63	+
6	0.560	0.018	18	>63	+
IgM					
7	1.242	0.016	100	>89	+
8	0.416	0.037	37	>89	+
9	0.672	0.148	82	22	+
4§	0.306	0.013	23	4	+

* Values are the optical density at 405 nm in wells containing phosphatidylserine–prothrombin complex (PS/PT) or phosphatidylserine without prothrombin (PS).
† Values are the units of antiphosphatidylserine–prothrombin antibodies (aPS/PT).
‡ Values are IgG or IgM phospholipid units of anticardiolipin antibodies (aCL).
§ Patient 4 was also tested for IgG.

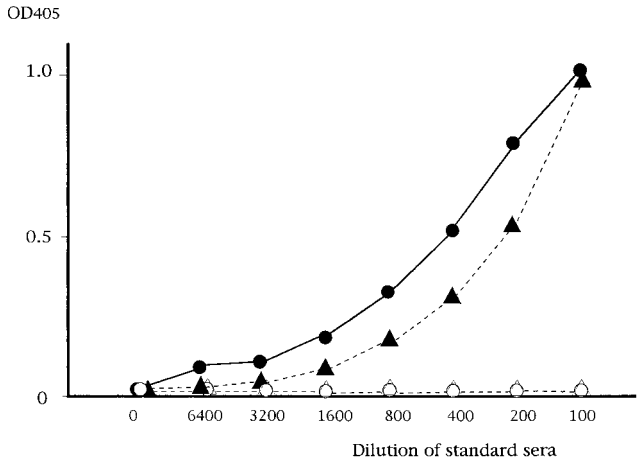

Figure 1. Standard curves for IgG (●) and IgM (▲) antiphosphatidylserine–prothrombin antibodies (aPS/PT). Each dilution of the standard sera was tested for aPS/PT. Values from sample blanks (wells with phosphatidylserine alone) in each dilution for IgG (○) and IgM (△) are also shown. OD405 = optical density at 405 nm.

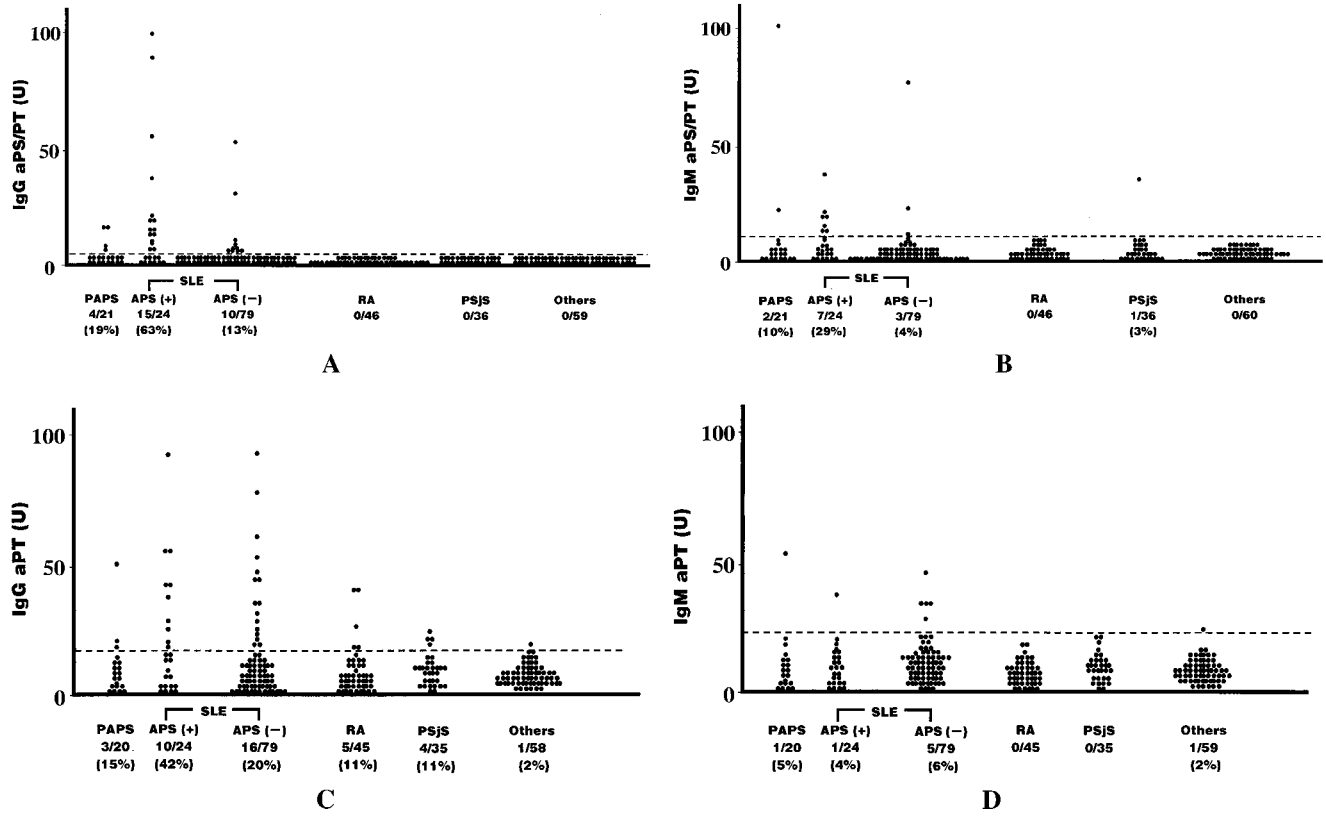

Figure 2. Distribution of antiprothrombin antibodies in systemic autoimmune diseases. IgG and IgM antibodies against the phosphatidylserine–prothrombin complex (aPS/PT) and IgG and IgM antibodies against prothrombin fixed on irradiated plates (aPT) were determined by enzyme-linked immunosorbent assay. The titers of **A,** IgG aPS/PT, **B,** IgM aPS/PT, **C,** IgG aPT, and **D,** IgM aPT are shown. The dashed line indicates the cutoff for positivity. U = units; PAPS = primary antiphospholipid syndrome; SLE = systemic lupus erythematosus; RA = rheumatoid arthritis; PSjS = primary Sjögren's syndrome.

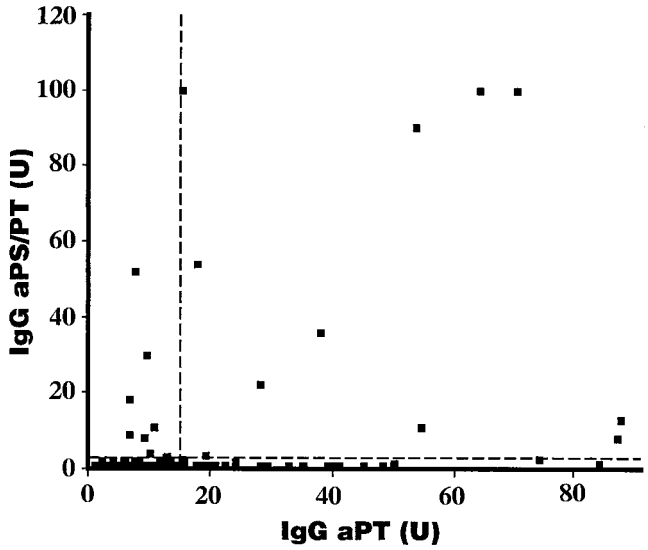

Figure 3. Relationship between the titers of IgG aPS/PT and those of IgG aPT. Many patients with discrepant results (i.e., only 1 of the determinations yielded positive results) were found. Dashed lines indicate the cutoffs for positivity. See Figure 2 for definitions.

IgG and IgM aPS/PT ELISA. Nonirradiated microtiter plates (Sumilon type S; Sumitomo Bakelite, Tokyo, Japan) were coated with 30 μl of 50 μg/ml phosphatidylserine and dried overnight at 4°C. To avoid nonspecific binding of proteins, wells were blocked with 150 μl of Tris buffered saline (TBS) containing 1% fatty acid–free bovine serum albumin (BSA) (A-6003; Sigma, St. Louis, MO) and 5 m*M* $CaCl_2$ (BSA-Ca). After 3 washes in TBS containing 0.05% Tween 20 (Sigma) and 5 m*M* $CaCl_2$, 50 μl of 10 μg/ml human prothrombin (Diagnostica Stago, Asnieres, France) in BSA-Ca was added to half the number of plates and the same volume of BSA-Ca alone (as sample blank) was added to the other half. After 1 hour of incubation at 37°C, plates were washed and 50 μl of patient serum diluted 1:100 in BSA-Ca was added in duplicate. Plates were incubated for 1 hour at room temperature, followed by the addition of alkaline phosphatase–conjugated goat anti-human IgG and substrate.

The optical density in wells containing phosphatidylserine alone was subtracted from that in the wells containing PS/PT complex. The titer of aPS/PT antibodies in each sample was derived from the standard curve according to dilutions of the positive control. A normal range was established using serum from 36 healthy controls, with a cutoff of 2.0 units for IgG and 13 units for IgM (being 5 SD above the mean of controls). None of the patients showed IgA binding to the PS/PT complex, and therefore an IgA aPS/PT assay was not established.

IgG and IgM aPT ELISA. An ELISA utilizing irradiated ELISA plates was performed as previously described (13) to detect aPT antibodies. Briefly, irradiated microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight with 10 μg/ml of purified human prothrombin (80 μl/well) in phosphate buffered saline (PBS) at 4°C. Wells were blocked with 0.5% gelatin for 1 hour at 37°C. After 3 washes with PBS containing 0.1% Tween 20, 50 μl of serum diluted 1:100 in PBS containing 1% BSA was added in duplicate. Plates were incubated for 1 hour at room temperature, followed by the addition of alkaline phosphatase–conjugated goat anti-human IgG or IgM and substrate. The optical density at 405 nm was measured and converted to units, and a sample showing high binding served as a standard.

IgG, IgM, and IgA aCL ELISA and determination of aCL–β_2GPI dependency. IgG, IgM, and IgA aCL were measured according to the standard aCL ELISA (17). β_2GPI dependency of aCL activity was tested as previously described (18).

Determination of LAC. Blood samples from patients not taking warfarin were collected in precooled tubes containing 1:10 volume of 0.105*M* sodium citrate, and immediately centrifuged at 3,000 revolutions per minute for 15 minutes. After filtration, aliquots of platelet-free plasma were stored at −30°C until use for the LAC clotting tests.

Three clotting tests were performed for LAC determination, using an opto-mechanical coagulation analyzer (Behring Fibrintimer II; Behring Diagnostics, Marburg, Germany) according to the guidelines recommended by the Subcommittee on Lupus Anticoagulant/Phospholipid-Dependent Antibodies (19). For the activated partial thromboplastin time (aPTT) test, a sensitive reagent with low phospholipid concentration (Diagnostica Stago) was used for screening. A mixing test (plasma sample:normal pooled plasma 1:1, 1:4, and 1:9) and phospholipid addition test were used to confirm the presence of LAC. A kaolin clotting time (KCT) test and confirmation via a mixing study were performed in the same manner. The dilute Russell viper venom time (dRVVT) test was used to screen and confirm the presence of LAC, using RVV-screen and RVV-confirm reagents (American Diagnostica, Greenwich, CT).

Statistical analysis and clinical significance. The relative risks were approximated by odds ratios (ORs) with 95% confidence intervals (95% CI). The association was considered

Table 3. Distribution of positivity for anticardiolipin and lupus anticoagulant*

	Conventional	β_2GPI dependent
Anticardiolipin antibodies (n = 25)		
IgG/IgM/IgA†	73 (28)	33 (13)
IgG	45 (18)	23 (9)
IgM	25 (10)	12 (5)
IgA	28 (11)	8 (3)
Lupus anticoagulant (n = 253)		
aPTT/KCT	69 (27)	
dRVVT	49 (19)	
aPTT/KCT and/or dRVVT (all)	77 (30)	
aPTT/KCT alone	20 (8)	
dRVVT alone	8 (3)	
aPTT/KCT and dRVVT	49 (19)	

* Values are the number (%) of patients. β_2GPI = β_2-glycoprotein I; aPTT = activated partial thromboplastin time; KCT = kaolin clotting time; dRVVT = dilute Russell viper venom time.
† Some patients had more than 1 isotype.

Table 4. Clinical significance of the presence of antiprothrombin antibodies or other antiphospholipid antibodies in relation to manifestations of the antiphospholipid syndrome (APS) or thrombocytopenia*

Category, assay, result	Manifestations†						
	Yes	No	*P*	Odds ratio	95% CI	Sensitivity, %	Specificity, %
Manifestations of APS or thrombocytopenia							
aPS/PT							
IgG							
+	19	10	0.0003	4.42	1.96–9.97	21.1	93.9
−	71	165					
IgG/IgM							
+	22	12	0.0001	4.39	2.06–9.38	24.4	93.1
−	68	163					
aPT							
IgG							
+	16	23	NS	1.44	0.72–2.90	18.8	86.1
−	72	150					
IgG/IgM							
+	20	24	NS	1.83	0.94–3.53	22.7	86.1
−	68	149					
aCL							
IgG							
+	26	19	0.0004	3.36	1.73–6.45	28.9	89.1
−	64	156					
IgG/IgM/IgA							
+	38	35	0.0002	2.92	1.67–5.11	42.2	80.0
−	52	140					
β_2GPI IgG							
+	16	6	0.0002	6.09	2.29–16.18	17.8	96.6
−	74	169					
β_2GPI IgG/IgM/IgA							
+	21	12	0.0003	4.13	1.93–8.87	23.3	93.1
−	69	163					
LAC							
dRVVT							
+	29	20	<0.0001	4.13	2.16–7.91	35.4	88.3
−	53	151					
All							
+	41	36	<0.0001	3.75	2.13–6.62	50.0	78.9
−	41	135					
Manifestations of APS (thrombocytopenia excluded)							
aPS/PT							
IgG							
+	13	16	0.0079	3.10	1.40–6.88	21.0	92.1
−	49	187					
IgG/IgM							
+	16	18	0.0011	3.57	1.69–7.54	25.8	91.1
−	46	185					
aPT							
IgG							
+	9	30	NS	1.01	0.45–2.57	15.0	85.1
−	51	171					
IgG/IgM							
+	11	33	NS	1.14	0.54–2.43	18.3	83.6
−	49	168					
aCL							
IgG							
+	19	26	0.0021	3.01	1.53–5.93	30.6	87.2
−	43	177					
IgG/IgM/IgA							
+	27	46	0.0022	2.63	1.44–4.80	43.5	77.3
−	35	157					

Table 4. (*Cont'd*)

Category, assay, result	Manifestations†		*P*	Odds ratio	95% CI	Sensitivity, %	Specificity, %
	Yes	No					
β_2GPI IgG							
+	13	9	0.0001	5.72	2.31–14.15	21.0	95.6
−	49	194					
β_2GPI IgG/IgM/IgA							
+	14	19	0.0111	2.82	1.32–6.04	22.6	90.6
−	48	184					
LAC							
dRVVT							
+	21	28	0.0001	3.77	1.92–7.41	38.9	85.9
−	34	171					
All							
+	29	48	<0.0001	3.65	1.95–6.82	53.7	75.9
−	25	151					

* 95% CI = 95% confidence interval; NS = not significant (see Tables 2 and 3 for other definitions).
† Values are the no. of patients.

significant if the *P* value as determined by Fisher's exact test was less than 0.05. In each test, the sensitivity (or prevalence; i.e., patients with clinical manifestations of APS and a positive antibody test result divided by all patients with clinical manifestations of APS) and specificity (patients with a negative antibody test result without clinical manifestations of APS divided by all patients without clinical manifestations of APS) were calculated as markers of clinical significance of the test.

A multiple logistic regression analysis was used to compare the correlation values between aPS/PT and aCL–β_2GPI in relation to APS, since both types of antibodies were correlated with APS but were not linked to each other.

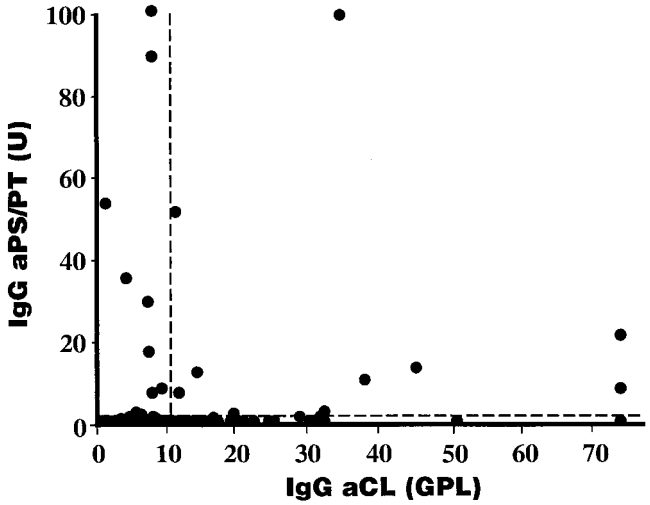

Figure 4. Relationship between IgG aPS/PT and anticardiolipin antibodies (aCL). No correlation was found between the titers of aPS/PT and those of aCL in patients with aPS/PT or aCL. Dashed lines indicate the cutoffs for positivity. GPL = IgG phospholipid units (see Figure 2 for other definitions).

RESULTS

Distribution of IgG and IgM antiprothrombin antibodies. Representative raw data on the antiprothrombin antibody distribution in some samples as determined against a standard curve are shown in Table 2 and Figure 1. The interassay coefficient was 7.5%, and the intraassay coefficient was 5.9%.

A positive titer of IgG aPS/PT was found in 29 of 265 patients (10.9%), and a positive titer of IgM aPS/PT in 13 (4.9%). Twenty-one had IgG alone, 5 IgM alone, and 8 had both IgG and IgM. The distribution of aPS/PT for each disease is shown in Figures 2A and B. IgG aPS/PT was found in patients with primary APS (19%) and APS secondary to SLE (63%), but was rare in the other diseases. IgM aPS/PT was also detected mainly in primary APS (10%) and in APS secondary to SLE (29%).

IgG and IgM aPT were detected in 39 (14.9%) and 8 (3.1%) of 261 patients, respectively. Thirty-six had IgG alone, 5 had IgM alone, and 3 were positive for both. Their distributions are shown in Figures 2C and D. IgG aPT was found in patients with various diseases, including rheumatoid arthritis (RA) and primary Sjögren's syndrome (SS), but the prevalence of IgM aPT was low.

Relationship between aPS/PT and aPT. Eleven of 240 patients (4.3%) had both IgG aPS/PT and aPT, 18 (7.0%) had only IgG aPS/PT, and 28 (10.9%) had only IgG aPT (57 [22.3%] were positive for IgG aPS/PT and/or aPT). The relationship between the titers of IgG aPS/PT and those of IgG aPT is shown in Figure 3. Although 11 patients were positive for both aPS/PT and aPT, there were many patients with discrepant results

Table 5. Relationship of lupus anticoagulant by the dilute Russell viper venom time test to antiprothrombin or to anticardiolipin antibodies*

Assay, result	Manifestations†		*P*	Odds ratio	95% CI	Sensitivity, %	Specificity, %
	Yes	No					
aPS/PT							
IgG							
+	24	5	<0.0001	38.2	13.4–109.1	49.0	97.5
−	25	199					
IgG/IgM							
+	24	9	<0.0001	20.8	8.70–49.7	49.0	95.6
−	25	195					
aPT							
IgG							
+	15	22	0.0015	3.51	1.65–7.47	31.3	88.8
−	33	170					
IgG/IgM							
+	20	24	0.0054	3.00	1.43–6.19	30.6	87.1
−	68	149					
aCL							
IgG							
+	19	24	<0.0001	4.75	2.32–9.71	38.8	88.2
−	30	180					
IgG/IgM/IgA							
+	22	49	0.0061	2.58	1.35–4.92	44.9	76.0
−	27	155					
β_2GPI IgG							
+	14	6	<0.0001	13.2	4.75–36.7	28.6	97.1
−	35	198					
β_2GPI IgG/IgM/IgA							
+	18	13	<0.0001	8.53	3.80–19.13	36.7	93.6
−	31	191					

* 95% CI = 95% confidence interval (see Tables 2 and 3 for other definitions).
† Values are the no. of patients.

(i.e., only 1 of the antibody determinations yielded positive results).

Prevalence of aCL, β_2GPI-dependent aCL, and LAC. In the same serum samples, IgG, IgM, and IgA aCL and their β_2GPI dependency were determined, and those profiles are described in Table 3. Antibodies to aCL, using the conventional determination, were found in 28% of all patients, and β_2GPI-dependent aCL in 13% of the patients. LAC was subcategorized into 2 groups. The first group comprised LAC detected by aPTT/KCT. Both aPTT and KCT represent an evaluation of the intrinsic pathway of the coagulation system. For the second LAC group, dRVVT, which represents an evaluation of only the final common pathway between the intrinsic and extrinsic systems, was used. LAC was tested in 253 patients who were not receiving warfarin, and the results are summarized in Table 3. Patients with LAC detected by either aPTT/KCT or dRVVT or both were defined as LAC (all).

Clinical significance of antiprothrombin antibodies and other antiphospholipid detection. Findings with regard to the clinical significance of aPS/PT, aPT, aCL, and LAC in relation to the manifestations of APS are described in Table 4. With respect to aPS/PT and aPT, the calculation was done both for IgG alone and for IgG or IgM. The markers of significance of aCL or β_2GPI-dependent aCL were also shown for IgG alone and for IgG/IgM/IgA, and those of LAC for dRVVT alone and for LAC (all) (aPTT/KCT and/or dRVVT). In Table 4, the clinical manifestations of APS included arterial thrombosis, venous thrombosis, pregnancy loss, and thrombocytopenia. Calculations were also performed with thrombocytopenia excluded (Table 4).

Both IgG aPS/PT and IgG/IgM aPS/PT showed a highly significant correlation with the clinical manifestations of APS (either including or excluding thrombocytopenia). In contrast, neither IgG aPT nor IgG/IgM aPT showed a significant correlation. In Table 4, the OR for the presence of IgG aPS/PT and that for IgG/IgM aPS/PT in relation to APS and/or thrombocytopenia were consistent with the ORs in well-established and standardized antiphospholipid antibody determinations (i.e., aCL and LAC), and the specificity was as high as that for β_2GPI-dependent aCL, a specific marker for APS.

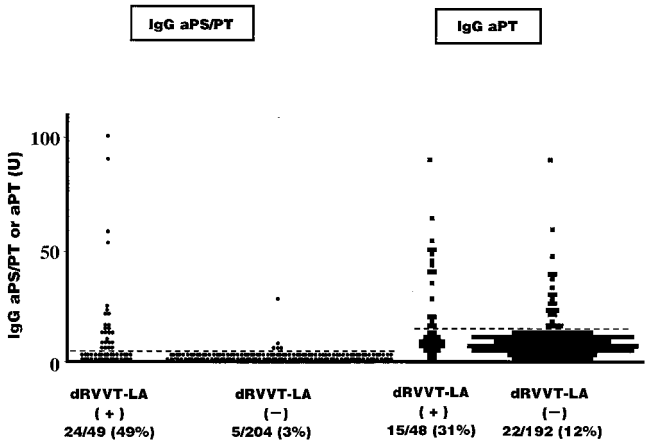

Figure 5. Relationship between lupus anticoagulant (LA) detected by the dilute Russell viper venom time (dRVVT) test and IgG antiprothrombin antibodies detected by enzyme-linked immunosorbent assay for aPS/PT or aPT. Patients positive for LA on the dRVVT test had significantly high titers of IgG aPT, but even higher titers of IgG aPS/PT. Dotted line indicates the cutoff for positivity. See Figure 2 for other definitions.

Relationship between aPS/PT and aCL. The relationship between the titers of IgG aPS/PT and those of IgG aCL is shown in Figure 4. Ten patients had both IgG aPS/PT and IgG aCL, but no correlations were found between the titers of aPS/PT and those of aCL in patients with aPS/PT or aCL.

Relationship of LAC to antiprothrombin antibodies and to aCL. Correlations between LAC as detected by the dRVVT test and each ELISA determination for antiprothrombin antibodies are summarized in Table 5. Both IgG aPS/PT and IgG/IgM aPS/PT strongly correlated with the presence of LAC by dRVVT. The ORs for the presence of aPS/PT (both IgG and IgG/IgM) were much higher than those for other antiphospholipid antibody determinations. IgG and IgG/IgM aPT also showed a significant correlation with the presence of LAC by dRVVT. The distributions of IgG aPS/PT and IgG aPT according to the presence or absence of LAC are shown in Figure 5. The correlation between LAC as detected by all tests and the presence of aPS/PT was weaker than that between LAC as detected by the dRVVT test and the latter (results not shown).

Multivariate analysis. The results of the multiple logistic regression analysis are shown in Table 6. Slightly higher correlations were shown between aPS/PT and APS or LAC than between aCL–β_2GPI and APS or LAC.

Table 6. Multivariate analysis*

Category, assay	Odds ratio	95% CI	*P*
Manifestations of APS or thrombocytopenia			
IgG/IgM aPS/PT	3.36	1.52–7.43	0.0025
IgG/IgM/IgA aCL–β_2GPI	3.07	1.38–6.86	0.0059
Manifestations of APS (thrombocytopenia excluded)			
IgG/IgM aPS/PT	2.92	1.33–6.40	0.0091
IgG/IgM/IgA aCL–β_2GPI	2.06	0.91–4.66	0.0887
Relationship with LAC by dRVVT			
IgG/IgM aPS/PT	9.39	3.74–23.55	<0.0001
IgG/IgM/IgA aCL–β_2GPI	4.01	1.64–9.78	0.0023

* 95% CI = 95% confidence interval (see Tables 2 and 3 for other definitions).

DISCUSSION

In this study, the clinical significance of antiprothrombin antibodies was evaluated in a large number of patients with systemic autoimmune diseases. The results obtained closely depended on the assay performed. There was a correlation between aPS/PT antibodies and the clinical manifestations of APS, and the importance of aPS/PT as a marker for APS was demonstrated for the first time.

In 1991, Bevers et al (20) highlighted the importance of antibodies against prothrombin in the activity of LAC, as based on data on 16 patients with both aCL and LAC. After incubation with cardiolipin-containing liposomes, LAC activity remained in the supernatant in samples from 11 of 16 patients. These 11 samples demonstrated LAC activity in a phospholipid-bound, prothrombin-dependent manner. Subsequently, Oosting et al (21) showed that 4 of 22 samples containing LAC inhibited endothelial cell–mediated prothrombinase activity, and the IgG fraction containing LAC activity bound to the phospholipid–prothrombin complex. Based on these reports, the consensus was that antibodies against phospholipid-bound prothrombin, as well as against phospholipid-bound β_2GPI, are one of the components responsible for LAC activity.

In 1995, Arvieux et al (10) showed that antiprothrombin antibodies could be detected using a standard ELISA and prothrombin coated onto irradiated plates. Studies were done to investigate the clinical significance of antiprothrombin antibodies detected by ELISA. Petri et al (22) reported that antiprothrombin antibodies have potential value for predicting thrombosis in a cohort of patients with SLE. Puurunen et al (23) also found a positive correlation between the presence of antiprothrombin antibodies, as well as anti-β_2GPI antibodies,

and deep vein thrombosis in subjects with SLE. We also found a correlation between the presence of antiprothrombin antibodies and the occurrence of vascular events (arterial thrombosis, venous thrombosis, and pregnancy problems in general) in 207 patients with SLE (13). Outside the autoimmune setting, Vaarala et al (24) showed that high levels of antiprothrombin antibodies conferred a 2.5-fold increase in the risk of myocardial infarction or cardiac death in middle-aged men. Palosuo et al (25) studied 265 middle-aged men with deep vein thrombosis or pulmonary embolism and found that the risk of thrombotic events was significantly increased in carriers of antiprothrombin antibodies.

Conversely, Pengo et al (14) found no correlation between the presence of antiprothrombin antibodies and thrombosis in 22 patients with APS. Horbach et al (26) investigated the clinical significance of antiprothrombin antibodies in 175 patients with SLE and found that both IgG and IgM aPT were more frequent in patients with a history of venous thrombosis, but the correlation was not significant when examined by multivariate analysis. Forastiero et al (27) reported a weak association between the presence of antiprothrombin antibodies and venous thrombosis in 233 patients with LAC and/or aCL, but the correlation was not statistically significant. Galli et al (11) found antiprothrombin antibodies in 58% of APS patients, but they found no correlation between thrombotic events and the presence of these antibodies. There are other reports in which no correlation was found between antiprothrombin antibodies and thrombotic events (28,29).

In those previous studies, the ELISA was performed by using, as antigen, prothrombin coated on irradiated plates (the aPT system). Although some of the available data supported a potential correlation between antiprothrombin antibodies and some of the clinical features of APS, the value of antiprothrombin antibodies as a marker of APS has remained questionable.

The binding of autoimmune aCL (anti-β_2GPI) to the cardiolipin–β_2GPI complex showed a high correlation with that to β_2GPI coated on irradiated ELISA plates (5), and 5 human monoclonal aCL (anti-β_2GPI) derived from APS patients bound both to the cardiolipin–β_2GPI complex and to β_2GPI on irradiated plates (30). Therefore, autoantibodies detected by aCL–β_2GPI and anti-β_2GPI using irradiated plates are likely to be comparable. In contrast, a major discrepancy was found between the titers of aPS/PT and those of aPT in our study, although some patients were positive for both. The interaction between negatively charged phospholipids and β_2GPI is not calcium dependent, and the phospholipid-binding sites on β_2GPI are highly cationic (31). In contrast, the phospholipid-binding site on prothrombin, the Gla domain, requires calcium-ion for phosphatidylserine binding. Therefore, the interaction between prothrombin and the oxidized plastic surface may differ from that between β_2GPI and the latter, and may lead to the difference in epitope expression between prothrombin bound to phosphatidylserine and that to the irradiated surface, unlike that of β_2GPI.

It was not aPT, but aPS/PT, that correlated with the clinical manifestations of APS, which is the most important finding of this study. In our previous study on 72 patients with APS (including thrombocytopenia) among 207 patients with SLE, aPT significantly correlated with the clinical features of APS (13). However, we could not confirm this correlation in the present series of patients with systemic autoimmune disorders, mainly because of the low specificity of aPT compared with that of aPS/PT. In this study, IgG aPT were found not only in cases of primary APS or SLE, but also in RA or in primary SS, in which the prevalence was 11%. Furthermore, the correlation of LAC to aPS/PT was much stronger than that to aPT, although both correlations were statistically significant. When we focused on patients with SLE and/or APS, the results were not different. The correlation between aPS/PT and APS was significant (IgG/IgM aPS/PT and APS including thrombocytopenia OR 8.29 [95% CI 3.03–22.71], $P < 0.0001$), but that between aPT and the latter was not (IgG/IgM aPT and APS including thrombocytopenia OR 1.89 [95% CI 0.71–5.06], P not significant).

In this study, the clinical manifestations of APS were defined in 2 different categories: including and excluding patients with thrombocytopenia, and with or without any of the clinical manifestations of arterial thrombosis, venous thrombosis, and pregnancy loss. The ORs for both IgG and IgG/IgM aPS/PT were comparable. The same tendency was found for aCL or LAC. Therefore, aPS/PT, as well as aCL and LAC, can serve as a marker of APS regardless of the definition of APS.

In the aPS/PT assay, phosphatidylserine was coated on the plates. Endogenous β_2GPI present in diluted serum samples might bind to phosphatidylserine and expose epitopes for aCL (anti-β_2GPI), leading to interference with the determination of antibodies that recognize phosphatidylserine-bound prothrombin. However, in the presence of a high concentration of Ca^{++} (5 m*M*) in all diluents and the washing buffer, the binding of such a low concentration of β_2GPI to phosphatidylserine was negligible (results not shown). No correlation was found between the titers of aPS/PT and those of

aCL (Figure 4), and therefore the correlation between aPS/PT and APS that we found was not due to the cross-reaction of aCL, nor was it due to detection of aCL (anti-β_2GPI) bound to the phosphatidylserine–β_2GPI complex.

The aPS/PT antibodies showed a strong correlation with the presence of LAC by dRVVT. Galli et al (33) found that aCL predominantly correlated with the presence of LAC by dRVVT, but our data showed that both aPS/PT and aCL–β_2GPI strongly correlated with LAC by dRVVT. Our results seem to be reasonable since aPS/PT may hamper thrombin generation from phospholipid-bound prothrombin and the dRVVT test detects exactly such a pathway by directly converting factor X to Xa. LAC as detected by the KCT/aPTT tests showed a weaker correlation both with aPS/PT and with aCL–β_2GPI (results not shown). The dRVVT reagent is, in general, more sensitive for detecting inhibitors compared with kaolin or standard aPTT reagents (32), but the KCT/aPTT system can detect a wider spectrum of inhibitors because this system is affected by inhibitors through all intrinsic pathways of coagulation. Thus, we used 3 reagents for LAC detection, and Table 4 shows that the detection of LAC by all 3 tests was more prevalent in relation to APS than was LAC by dRVVT alone, but the latter method of detection was more specific for APS than the 3 tests for LAC combined. Unlike the findings of a previous study (33), we could not confirm the predominance of LAC by KCT in patients with antiprothrombin antibodies as detected by either the aPS/PT or the aPT system, compared with patients with aCL. β_2GPI may affect not only prothrombinase activity, but also the tenase or FXII activator; we may therefore consider that aCL (anti-β_2GPI) had an effect on the LAC activity as detected by the KCT/aPTT system as well as the dRVVT test. Therefore, aPS/PT, as well as aCL, strongly correlated with the presence of LAC regardless of the method used.

In a recent review (34), it was described that LAC represents a phenomenon that can be extremely frustrating to laboratory investigators as well as clinicians, because of its high heterogeneity and the difficulty in standardizing the technique for its detection. On the basis of our finding of a strong correlation between aPS/PT and LAC (Table 5 and Figure 5), we suggest that testing aPS/PT may help to confirm the presence of LAC.

Despite the uncertainties regarding the pathogenesis of autoimmune diseases that involve antiprothrombin antibodies, there is increasing evidence that antiprothrombin antibodies have a role to play in the hypercoagulable state of APS. The antigens are present in plasma or on cell surfaces that are exposed to plasma, and are therefore accessible to circulating antibodies. Some effects on endothelial cells have been proposed: 1) antiprothrombin antibodies inhibit thrombin-mediated endothelial cell prostacyclin release and hamper protein C activation (1); 2) antiprothrombin antibodies could recognize the prothrombin–anionic phospholipid complex on the endothelial cell surface, thus activating endothelial cells and inducing procoagulant substances via prothrombin (35), similar to activating endothelium caused by anti-β_2GPI antibodies via β_2GPI (36,37); or 3) antiprothrombin antibodies could increase the affinity of prothrombin for negatively charged phospholipids. Thus, the prothrombin–antibody complexes would compete with the binding of other coagulation factors for the available surface, resulting in a prolongation of clotting assays that can be neutralized by the addition of extra phospholipids (38). This in vitro phenomenon could be extrapolated to an in vivo scenario: membrane binding of prothrombin–antibody complexes could increase the concentration of prothrombin on a negatively charged cell surface. It may lead to a hypercoagulable state, since antibody-bound prothrombin can also be converted to thrombin (38), resulting in a thrombotic tendency (2).

For daily clinical practice, the sensitive methods should be used first, followed by the specific tests. According to the findings shown in Table 4, conventional aCL and LAC tests (using the standard 3 reagents) should be performed first. The β_2GPI-based assay to detect aCL–β_2GPI (β_2GPI-dependent aCL or anti-β_2GPI using irradiated plates) and the aPS/PT assay should then be conducted for better recognition of APS. Moreover, when we cannot confirm the presence of LAC (because, for example, patients have already received warfarin or plasma samples have been damaged in an unstable clotting assay), the results of the aCL–β_2GPI and aPS/PT assays would be helpful for confirming the presence of LAC. It is likely that aPS/PT are among the antibodies responsible for LAC, and therefore an LAC-positive, aPS/PT-negative finding is a rare pattern. Only 6 patients (2.3%) without LAC and without aCL–β_2GPI had aPS/PT, and 2 of the 6 (33%) had thrombotic events. Since the aPS/PT method is very specific assay for APS, we believe that it provides better recognition of APS than the conventional aCL and LAC tests that may detect many nonspecific antibodies or phenomena. Eight of 26 LAC-positive, aCL–β_2GPI–negative APS patients had aPS/PT. At the moment, however, we cannot substitute the aPS/PT assay system

for LAC testing, since the presence of aCL–β_2GPI and aPS/PT cannot explain all of the LAC phemomena.

In conclusion, detection of aPS/PT can be used not only to confirm the presence of LAC, but also to function, in addition to aCL–β_2GPI, as one of the strong markers of APS, particularly considering the correlation observed between aPS/PT and APS with very high specificity. We propose that the aPS/PT assay should be performed in conjunction with other antiphospholipid antibody detection methods to improve the likelihood of recognizing APS, which would ultimately facilitate the management of the disease.

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