Kaolin Clotting Time and Dilute Russell's Viper Venom Time Distinguish Between Prothrombin-Dependent and β2-Glycoprotein I-Dependent Antiphospholipid Antibodies

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Antiphospholipid (aPL) antibodies include anticardiolipin (aCL) and lupus anticoagulant (LA) antibodies. LA antibodies recognize the complex of lipid-bound (human) prothrombin, in this way inhibiting the phospholipid-dependent coagulation reactions, whereas aCL antibodies are directed towards β2-glycoprotein I (β2-GPI) bound to an anionic lipid surface. According to their behavior in coagulation reactions, we have divided aCL antibodies into two groups: aCL-type A, which inhibit the phospholipid-dependent coagulation reactions because they enhance the binding of β2-GPI to the procoagulant phospholipid surface; and aCL-type B antibodies, which are devoid of anticoagulant properties. We report the distinctive laboratory and clinical profiles of 25 patients with well-characterized, phospholipid-dependent inhibitor of coagulation. Fourteen patients had LA antibodies (aCL-type B were concomitantly present in 10 cases, while in the other four, aCL titer was normal), and the other 11 had aCL-type A antibodies. The laboratory evaluation of the two groups showed the dilute Russell viper venom time (dRVVT) to be the most abnormal coagulation test in the aCL-type A–positive group, whereas the kaolin clotting time (KCT) was the most abnormal assay in the LA-positive group. In fact, the ratios of the coagulation times of patient plasma over normal pooled plasma (mean ± standard deviation) for LA versus aCL-type A antibodies were 1.48 ± 0.27 versus 2.20 ± 0.42, $P = .0001$, and 2.22 ± 0.42 versus 1.50 ± 0.42, $P = .0003$, for the dRVVT and KCT, respectively. No differences were observed either in the ratios of the activated partial thromboplastin times and the prothrombin times or the plasma levels of β2-GPI and prothrombin. Conversely, aCL titers were significantly higher in aCL-type A–positive patients (147 ± 44 U) than in the LA-positive group (61 ± 55 U; $P = .0003$). We ruled out the possibility that platelet contamination of plasma could account for the observed coagulation profiles, as the two patterns were reproduced in platelet-free plasma. In addition, we performed clotting tests in plasma in the presence of phospholipids and calcium after addition of factor IXa or factor Xa. The assay performed with factor Xa was more sensitive to the presence of aCL-type A antibodies, while the assay performed with factor IXa was preferentially sensitive to LA-containing plasmas, supporting the earlier findings with the dRVVT and KCT assays. Clinically, the two groups of patients were significantly different only with respect to the history for thrombosis, which was positive in 3 of 14 LA-positive cases (21%) versus 8 of 11 aCL-type A–positive patients (73%; $P = .0308$). These clinical and laboratory associations, which must be validated on larger groups of patients, might be helpful in the identification of those aPL-positive patients at particular risk for thromboembolic events.

ANTIPHOSPHOLIPID (aPL) antibodies represent a heterogeneous family of antibodies that includes lupus anticoagulant (LA) and anticardiolipin (aCL) antibodies. We and others have demonstrated that aPL antibodies are not directed primarily against negatively charged phospholipids, but recognize different phospholipid/protein complexes.[1-7] In particular, LA antibodies recognize the (human) prothrombin/phospholipid complex, in this way inhibiting the phospholipid-dependent coagulation reactions,[5] whereas aCL antibodies are directed towards β2-glycoprotein I (β2-GPI) bound to an anionic lipid surface.[1-4] According to their behavior in coagulation reactions, we have divided aCL antibodies into two groups[7,8]: aCL-type A, which inhibit coagulation reactions because they enhance the binding of β2-GPI to the procoagulant phospholipid surface; and aCL-type B antibodies, which are devoid of anticoagulant properties. On the basis of these observations, it appears that two inhibitors of coagulation—LA and aCL-type A antibodies—exist among the family of aPL antibodies. In spite of their different mechanisms of action, LA and aCL-type A antibodies exert their in vitro anticoagulant effect on the same clotting tests. In fact, they both prolong the clotting time of the phospholipid-dependent coagulation assays (ie, the activated partial thromboplastin time [aPTT], kaolin clotting time [KCT], dilute Russell viper venom time [dRVVT], and tissue thromboplastin inhibition test [TTIT]).[5-7] This prolongation is not corrected after mixing patients' plasma with normal plasma, while the addition of platelets or exogenous phospholipids induces a relative correction of the defect.[9] Clearly, plasmas containing either LA or aCL-type A antibodies meet the criteria of the Scientific and Standardization Committee Subcommittee for Standardization of Lupus Anticoagulants.[9] As a consequence, the term "lupus anticoagulants" is indiscriminately and improperly used to refer to all the phospholipid-dependent inhibitors of coagulation.

Recently, our group observed that in a small, unselected group of patients with aPL antibodies, LA antibodies can be detected in approximately two thirds of the cases, while aCL-type A antibodies account for the remaining phospholipid-dependent inhibitors of coagulation.[5] We have now extended the identification of the type of coagulation inhibitor to a larger group of aPL patients, and we report their clinical and laboratory characteristics. We observed that LA– and aCL-type A–containing plasmas had distinct coagulation profiles. Moreover, when the clinical manifestations of the patients were evaluated with respect to the type of the inhibitor of coagulation, the presence of aCL-type A antibodies, more

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than that of LA antibodies, was associated with thromboembolic events.

MATERIALS AND METHODS

Patients. Twenty-five patients with phospholipid-dependent inhibitors of coagulation were included in the present investigation: eight men and 17 women, aged 14 to 69 years (mean, 39 years). One patient had autoimmune hemolytic anemia, and two had non-Hodgkin's lymphomas. Eight patients were thrombocytopenic (less than 150×10^9 platelets per liter). Clinical history was positive for thromboembolic complications in 11 patients. In particular, one patient had had recurrent deep vein thrombosis (2DVT); one patient, 2DVT and one cerebral ischemic stroke; two patients, 1DVT and one pulmonary embolism; one patient, 1DVT and one thrombosis of the femoral artery; one patient, DVT and transient ischemic attacks (TIA); one patient, thrombosis of the femoral artery; and three patients, cerebral ischemic stroke. One patient experienced recurrent TIA. The thromboembolic events had been documented by phlebography, Doppler ultrasound, perfusion scintigraphy, and/or computed tomography (CT) scan. In cases of TIA, CT scan was negative.

Diagnosis of the phospholipid-dependent inhibitors of coagulation. The criteria proposed by the SSC Subcommittee for Standardization of Lupus Anticoagulants were used for the diagnosis of the phospholipid-dependent inhibitors of coagulation.[9] Venous blood (9 vol) was collected by clean venipuncture into plastic tubes containing 0.13 mol/L trisodium citrate (1 vol) and centrifuged at 3,000*g* for 20 minutes at 4°C. Platelet-poor plasma was divided into small aliquots in plastic tubes and stored at −80°C until processing. Blood from a pool of 20 normal, healthy subjects was prepared in the same way.

The following panel of coagulation tests was performed with each patient's plasma: aPTT (Thrombofax; Ortho Diagnostics, Milan, Italy), aPTT with kaolin as activator of the contact phase of the intrinsic pathway of coagulation (KPTT; PTT Reagenz/t; Boehringer, Mannheim, Germany), and prothrombin time (PT; Thromborel; Istituto Behring, Scoppito, Italy) were performed according to the manufacturers' instructions; KCT was performed according to Exner et al[10]; dRVVT was performed according to Thiagarajan et al[11] using 1:200 diluted Russell viper venom (Wellcome, Greenville, NC) and 1:8 diluted Pathrontin (Istituto Behring). Two replicates were performed for each assay, and the mean value was calculated. The results were expressed as ratios of the mean clotting time of patient's plasma to the mean clotting time of normal pooled plasma. When the ratio exceeded 1.2, the tests were repeated on the 1:1 mixing of patient's with normal pooled plasma. In none of the cases, did mixing with normal pooled plasma correct the prolongation of the patient's coagulation time. The intraassay variability was less than 5% for each coagulation test.

Some coagulation tests were performed also with patients' and normal pooled plasma filtered through 0.2-μm Minisart N filter (Sartorius, Florence, Italy) to remove platelet contamination before storage.

Other coagulation tests. Normal pooled or patient's plasma (50 μL) was mixed with 25 μL of either purified human factor IXa (10 μg/mL) or factor Xa (3.75 mU/mL; both coagulation factors from Stago, Asnieres, France) and 25 μL of Pathrontin as a source of procoagulant phospholipids. After 30 seconds' incubation, 50 μL 0.025-mol/L CaCl2 was added, and the coagulation time was recorded.

All tests were performed in duplicate using a Mechrolab Clot Timer 202 A (Heller Laboratories, Santa Rosa, CA).

Measurement of IgG and IgM aCL antibodies. IgG and IgM aCL titers were measured essentially according to the enzyme-linked immunosorbent assay (ELISA) procedure described by Loizou et al.[12] IgG and IgM aCL antibodies were expressed as G phospholipid (GPL) and M phospholipid (MPL) units, according to Harris et al.[13] Values exceeding 15 GPL or 15 MPL units were considered abnormal.

Purification and characterization of the anticoagulant activity of total IgG and aCL antibodies. Total IgG were affinity-purified from plasma over Protein A-sepharose CL-4B (Pharmacia Fine, Uppsala, Sweden). The protein content of the preparations was assayed according to Sedmak and Grossberg[14] and ranged from 13.5 to 32 mg/mL. The anticoagulant effect of IgG preparations was evaluated by dilute aPTT (with 1:10 diluted Thrombofax), using a mixture of 1 vol antibodies and 2 vol normal pooled plasma or bovine plasma. Total IgG were evaluated in the coagulation tests at comparable concentrations, which ranged from 13.5 to 16.0 mg/mL. Normal IgG (18.6 mg/mL), prepared in a similar way as normal pooled plasma, was used as a control in each experiment.

IgG aCL antibodies were purified from patient's plasma by adsorption to cardiolipin-containing liposomes and subsequent affinity-chromatography over Protein A-sepharose CL-4B, as previously described.[2] Their protein concentrations ranged from 0.336 to 1.5 mg/mL. For reason of comparison in coagulation assays, aCL preparations were adjusted to the same titer using the standard ELISA (see above). Their anticoagulant activity was evaluated at concentrations ranging from 0.065 to 0.490 mg/mL by dilute aPTT. The aCL antibodies were divided into two groups. The aCL-type A antibodies[7] prolonged the dilute aPTT of both human and bovine plasma at concentrations ranging from 0.065 to 0.150 mg/mL. The anticoagulant effect of these antibodies had been previously shown to be critically dependent on the presence of β2-GPI. The aCL-type B antibodies did not prolong the dilute aPTT. They were used in the coagulation tests at concentrations ranging from 0.336 to 0.490 mg/mL.

Characterization of the phospholipid-dependent inhibitor of coagulation. After affinity-purification of aCL antibodies, the anticoagulant activity of the supernatant plasmas was evaluated by dilute aPTT performed in human and bovine plasmas, mixing 1 vol supernatant with 2 vol normal pooled plasma.

Plasmas were considered to contain LA antibodies when the following conditions were fulfilled.[5] (1) The supernatant plasma retained its anticoagulant activity, in spite of the loss of aCL activity. As a control, the anticoagulant activity was compared with that expressed by the supernatant after plasma incubation with liposomes containing phosphatidylcholine and cholesterol (molar ratios, 15:4) but not cardiolipin. (2) Total IgG fractions were able to prolong the dilute aPTT performed in human but not bovine plasma. (3) Affinity-purified aCL antibodies did not inhibit the dilute aPTT performed both in human and in bovine plasma (aCL-type B antibodies).

Plasmas were considered to contain aCL-type A antibodies when the following conditions were fulfilled.[7,8] (1) After adsorption with cardiolipin liposomes, the supernatant plasma lost both the anticoagulant and the aCL activities; and (2) affinity-purified aCL antibodies inhibited the dilute aPTT performed both in human and in bovine plasma.

Measurement of β2-GPI in plasma. The plasma content of β2-GPI was measured by rocket immunoelectrophoresis, using rabbit polyclonal anti-β2-GPI antiserum (Istituto Behring) as previously described.[15] Normal pooled plasma was arbitrarily considered to contain 100% of β2-GPI.

Measurement of prothrombin. The plasma content of prothrombin was assayed using factor II-deficient plasma (Istituto Behring), according to the manufacturer's instructions.

Statistical analysis. The Student's *t*-test for paired and unpaired data and the χ^2 test (with continuity correction) were used. A P value less than .05 was considered significant. The 95% confidence intervals were calculated according to Gardner and Altman.[16]

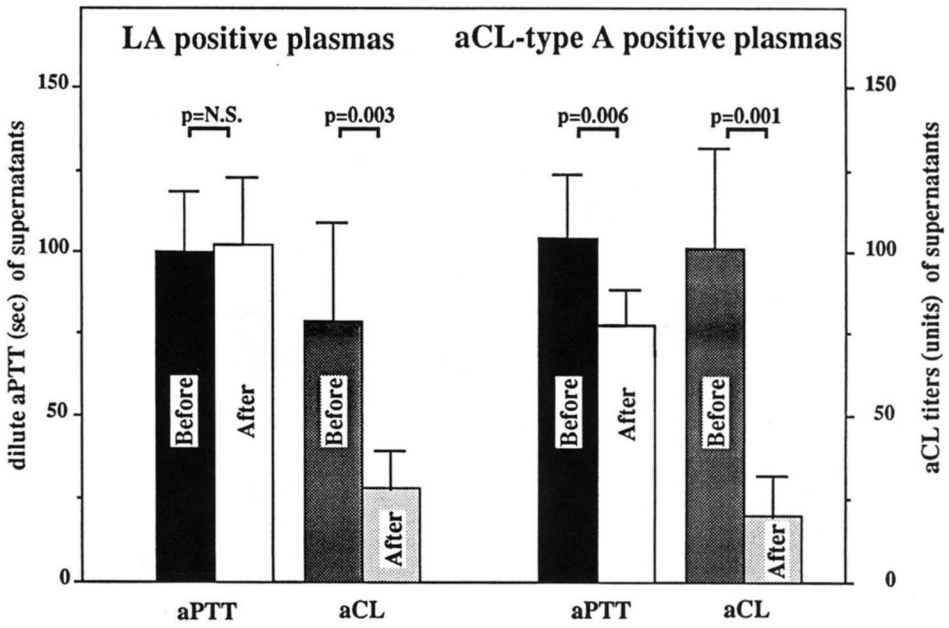

Fig 1. Anticoagulant and aCL activities were measured in plasmas before and after incubation with liposomes containing cardiolipin. As a control, plasmas were similarly incubated with liposomes that did not contain cardiolipin. Anticoagulant activity was measured by dilute aPTT; aCL activity, by ELISA. Bars represent the mean (+SD) of each group. The control aPTT ranged between 60 and 70 seconds. LA-positive plasmas were found in 14 patients, whereas aCL-type A–positive plasmas comprised the remaining 11 cases. The aCL-type B antibodies were present in 10 of the 14 LA-positive plasmas.

RESULTS

Characterization of the phospholipid-dependent inhibitors of coagulation. The plasmas of 25 patients with phospholipid-dependent inhibitors of coagulation were incubated with cardiolipin-containing liposomes. After sedimentation of the lipids, the supernatant plasmas were evaluated for the residual anticoagulant and aCL activities. It was found that in 14 cases the anticoagulant activity remained unchanged in the supernatant. Of these 14 plasmas, 10 contained aCL antibodies that could be partially adsorbed by the liposomes (Fig 1). In the other four cases, aCL plasma titers were normal. In particular, before liposome adsorption, the aPTTs ranged from 67.0 to 123.0 seconds (mean ± SD, 100.4 ± 18.1 seconds), and after adsorption, they varied from 64.5 to 124.0 seconds (102.2 ± 20.3 seconds). Virtually no shortening of the aPTT was caused by cardiolipin adsorption in this group of plasmas. The aPTT of normal pooled plasma ranged from 60.0 to 70.0 seconds, irrespective of liposome adsorption. This strongly points towards the LA nature of the phospholipid-dependent inhibitor of coagulation present in these 14 plasmas, which was further demonstrated by the ability of the total IgG fractions to prolong the dilute aPTT performed in human but not in bovine plasma. In line with these findings, the affinity-purified aCL antibodies did not prolong the dilute aPTT, thus showing the behavior of aCL-type B antibodies (Table 1). It must be emphasized that aCL-type B preparations were used in the coagulation tests at higher protein concentrations than aCL-type A preparations (see Materials and Methods) to reach approximately the same ELISA titers.

In the other 11 patients' plasmas, the anticoagulant and aCL activities cosedimented with the liposomes (Fig 1). In fact, before liposome adsorption, the aPTTs ranged from 83.0 to 133.0 seconds (mean, 104.5 ± 19.6 seconds), and after adsorption, they varied from 63.8 to 90.3 seconds (mean, 78.1 ± 10.2 seconds). The shortening of aPTT ranged from 13.0 to 55.0 seconds. The aPTT of normal pooled plasma ranged from 60.0 to 70.0 seconds also for this group of experiments. The aCL antibodies, affinity-purified from these patients' plasmas, prolonged the dilute aPTT of both human and bovine plasmas (Table 1). This confirms the aCL-type A nature of the coagulation inhibitor of these patients. Unexpectedly, the total IgG fractions of 2 of these 11 patients were able to prolong the aPTT in human but not in bovine plasma, although the aCL antibodies, affinity-purified from the same patient's plasma, displayed the behavior of aCL-type A antibodies.

Coagulation profiles of LA– and aCL-type A–positive patients. The ratios of the coagulation times of patient plasma over normal pooled plasma for various coagulation tests and the titers of aCL antibodies of the 25 patients are shown in Table 2. Patients 1 to 14 belong to the LA-positive group, while patients 15 to 25 represent the aCL-type A–positive group. Analysis of the KCT and dRVVT ratios showed the existence of two distinct profiles that correlated with the type of phospholipid-dependent inhibitor of coagu-

Table 1. Anticoagulant Effect of Total IgG and aCL-Type A and B Antibodies on the Dilute aPTT Performed in Human and Bovine Plasma

	aPTT Range (s)	
Immunoglobulins	Human Plasma	Bovine Plasma
LA		
Total IgG (n = 8)	78.2-160.7	75.0-78.6
aCL (n = 5)	54.3-58.6	74.2-79.8
aCL-type A		
Total IgG (n = 8)	88.5-104.0	75.3-182.0
aCL (n = 8)	74.5-99.5	92.0-160.1
Normal IgG	55.0-64.0	72.0-82.0

Table 2. Coagulation Profile and Levels of aCL Antibodies in 25 Patients With aPL Antibodies

Patient No.	aPTT	KPTT	KCT	dRVVT	aCL Antibodies (U)
LA-positive plasma					
1	1.72	1.42	2.89	1.48	84
2	1.77	1.61	2.84	1.38	9
3	1.33	1.03	2.48	1.35	25
4	1.00	0.94	1.71	1.33	70
5	1.28	1.22	2.13	1.42	36
6	1.29	1.07	1.84	1.35	62
7	1.46	1.24	2.14	1.37	94
8	1.24	1.38	1.86	1.20	5
9	1.42	1.22	1.91	1.20	120
10	1.32	1.22	2.04	1.48	15
11	1.93	1.48	2.55	2.01	96
12	1.28	1.34	1.84	1.28	12
13	1.36	1.21	2.28	1.85	200
14	1.16	1.86	2.82	1.96	30
aCL-type A-positive plasma					
15	1.31	1.32	1.43	1.81	130
16	1.15	1.14	1.08	1.97	120
17	1.18	1.01	1.40	1.75	150
18	1.87	1.46	2.43	2.83	98
19	1.94	1.26	1.10	2.05	150
20	1.84	1.62	1.92	2.43	250
21	1.22	1.23	1.09	1.63	165
22	1.44	1.25	1.20	1.97	125
23	1.50	1.96	1.71	2.45	180
24	2.09	1.79	1.74	2.64	150
25	1.19	1.12	1.40	2.68	96
Normal pooled plasma	≤1.2	≤1.2	≤1.2	≤1.2	≤15

Results are expressed as ratios of mean clotting time of patients plasma to mean clotting time of normal pooled plasma, except for levels of aCL antibodies, which are expressed as units.

lation: the KCT ratio was the most abnormal in each LA-positive patient, while in each aCL-type A–positive patient, the dRVVT ratio was the most abnormal. The findings of Table 2 represent the tests performed on the occasion of the first diagnosis of the phospholipid-dependent inhibitor of coagulation for each patient; the two coagulation patterns were persistently reproduced on several sequential occasions. When the two groups were evaluated for their coagulation tests, the ratios (mean ± SD) for LA versus aCL-type A antibodies were 1.48 ± 0.27 versus 2.20 ± 0.42 (P = .0001) and 2.22 ± 0.42 versus 1.50 ± 0.42 (P = .0003) for the dRVVT and KCT ratios, respectively. Conversely, no differences were observed with respect to the ratios of the aPTT, regardless of the partial thromboplastin reagent used (data not shown). The PT ratios and the plasma levels of prothrombin were similar in the two groups and within the normal range (data not shown). The content of β2-GPI in the LA-positive plasmas (160% ± 65%, mean ± SD) was higher than that of aCL-type A–positive plasmas (132% ± 61%), but this difference was not statistically significant.

Four of the 14 LA-positive patients had normal levels of IgG and IgM aCL antibodies measured by ELISA. By definition, all aCL-type A–positive patients had high levels of aCL antibodies. The aCL titers in plasma were significantly higher in aCL-type A–positive patients (147 ± 44 U) than in the LA-positive group (61 ± 55 U; P = .0003). No correlations were found between aCL titers, platelet count, and the ratios of the phospholipid-dependent coagulation tests (data not shown).

Effect of plasma filtration on phospholipid-dependent coagulation tests. To evaluate whether platelet contamination could be responsible for the different coagulation profiles of LA– and aCL-type A–positive plasmas, phospholipid-dependent coagulation tests were performed in plasmas subjected to filtration through a 0.22-μm filter before storage at −80°C. Table 3 shows the results obtained with the plasmas of four patients (patients 7, 14, 19, and 23 of Table 2). The KCT performed in platelet-free plasma were much longer than those performed in platelet-poor plasma, while no substantial differences were observed with respect to the aPTT and dRVVT. However, when the results were expressed as ratios, the coagulation profiles displayed by platelet-free LA– and aCL-type A–positive plasmas were very similar to those obtained with platelet-poor plasmas (Tables 2 and 3).

Effect of addition of factors IXa and Xa on phospholipid-dependent coagulation tests in plasma. One of the major differences between the KCT and the dRVVT is that the former test proceeds through both the phospholipid-dependent generation of factor Xa by factors IXa and VIIIa and the phospholipid-dependent activation of prothrombin by factors Xa and Va, while the dRVVT evaluates selectively the conversion of prothrombin to thrombin by factors Xa and Va on a phospholipid surface. Thus, to investigate the reason(s) for the different sensitivity of the KCT and dRVVT to the presence of LA and aCL-type A antibodies, respectively, we performed clotting tests in plasma in the presence of phospholipids and calcium after addition of either factor IXa or factor Xa. Table 4 shows the results of the clotting assays performed in the plasmas of five patients (patients 7, 9, 19, 20, and 23 of Table 2): the assay performed with factor Xa was more sensitive to the presence of aCL-type A antibodies, while the assay performed with factor IXa was preferentially sensitive to LA-containing plasmas.

Clinical data of LA– and aCL-type A–positive patients. The two groups of patients did not differ with respect to the associated diseases (Table 2). Similarly, no differences were found with respect to both the number of thrombocytopenic

Table 3. Effect of Plasma Filtration on Phospholipid-Dependent Coagulation Tests

Patient No.*	aPTT	KCT	dRVVT
LA-positive			
7	43.1 (1.23)	299.1 (2.26)	59.2 (1.79)
14	53.7 (1.52)	296.1 (2.24)	47.7 (1.71)
aCL-type A–positive			
19	79.6 (2.26)	118.8 (0.90)	71.2 (2.55)
23	71.7 (2.03)	186.2 (1.41)	74.2 (2.66)
Normal pooled plasma (range)	34.0-38.0	121.4-139.3	27.9-33.1

Data are given as seconds (ratio).

* Patient numbers identify the same patients in Table 2.

Table 4. Coagulation Tests Performed in LA- and aCL-Type A-Positive Plasmas in the Presence of Factors IXa and Xa

Patient No.*	+Factor IXa	+Factor Xa
LA-positive		
7	125.0 (2.40)	67.0 (1.73)
9	107.7 (2.07)	67.5 (1.74)
aCL-type A-positive		
19	86.0 (1.65)	97.0 (2.50)
20	104.1 (2.00)	115.5 (2.97)
23	114.3 (2.20)	103.6 (2.80)
Normal pooled plasma (range)	51.9-52.0	37.0-40.6

Data are given as seconds (ratio).

* Patient numbers identify the same patients as in Table 2.

cases among LA- and aCL-type A-positive patients and the degree of thrombocytopenia (data not shown). Clinical history was positive for thromboembolic complications in 3 of 14 LA-positive patients (21%; 95% confidence intervals, 0 to 42%), versus 8 of 11 aCL-type A-positive cases (73%; 95% confidence intervals, 47% to 99%). This difference was statistically significant (52%; 95% confidence intervals, 18% to 86%, $P = .0308$).

DISCUSSION

The existence of at least three types of aPL antibodies that differ with respect to their antigenic targets, binding properties in solid phase immunoassays, and anticoagulant activity has been reported by several groups of investigators.[1-7] The reactivity towards phospholipid-bound (human) prothrombin and β2-GPI of LA and aCL antibodies, respectively, is responsible for the differences between the antibodies. To help in the identification of the different types of aPL antibodies, we have introduced a new terminology—LA, aCL-type A, and aCL-type B antibodies—that is mostly based on the behavior of the antibodies in coagulation reactions.[5,7] In the present study, 25 plasmas containing a phospholipid-dependent inhibitor of coagulation have been investigated and identified as LA-positive in 14 cases and aCL-type A-positive in the other 11. The aCL-type B antibodies were concomitantly present in 10 of the 14 LA-containing plasmas. In most cases, the identification of the antibodies required the isolation of the total IgG fractions and/or the affinity-purification of the aCL antibodies. An unexpected finding was obtained with two aCL-type A-positive plasmas, whose total IgG fractions were unable to exert anticoagulant effect in bovine plasma. At present, we do not have an explanation for this phenomenon, the more so as the aCL-type A IgG, affinity-purified from the same two plasmas, were able to prolong the dilute aPTT performed in both human and bovine plasma.

The identification of the nature of the phospholipid-dependent inhibitor of coagulation appears to be a rather expensive, time-consuming, and sometimes cumbersome process. In this respect, practical help might come from our observation that the two inhibitors are associated with distinct coagulation profiles, characterized by the peculiar sensitivity of the KCT and dRVVT to the presence of LA and aCL-type A antibodies, respectively. The two groups were significantly different also with respect to the titers, but not the isotypes, of aCL antibodies, with higher levels being found in the aCL-type A-positive patients. Nevertheless, the titers of aCL antibodies cannot be useful to predict to which of the two groups a particular plasma will belong, as in some LA-positive patients very high titers may be found (see Table 2). Moreover, in neither group did the ratios of the coagulation tests correlate with the titers of aCL antibodies.

The possibility that the two coagulation profiles might correlate with the levels of β2-GPI and/or prothrombin was also evaluated, as abnormal plasma concentrations of both proteins have been reported in aPL-positive patients.[15,17-25] In particular, the association between high β2-GPI levels and lupus anticoagulants but not aCL antibodies has been observed by our group.[15] We must emphasize that, at that time, the existence of the two inhibitors was still unknown, and for this reason, the name "lupus anticoagulants" was used to refer to both types of antibodies. Now we can partially confirm that observation, as in the LA-positive group, the levels of β2-GPI were higher than in the aCL-type A-positive group, although the difference did not reach statistical significance.

On the other side, hypoprothrombinemia has been reported in some aPL-positive patients[20-25] due to non-neutralizing antibodies that accelerate the scavenging of prothrombin from circulation.[23] However, in our two groups of patients, the levels of prothrombin as well as their PTs were normal.

Among the clotting tests used to diagnose the phospholipid-dependent inhibitors of coagulation (see Table 2), the aPTTs were not useful to distinguish between LA and aCL-type A antibodies. In general, the sensitivity of the clotting assays to the inhibitors is believed to increase with the decrease of the procoagulant lipid (ie, phosphatidylserine) concentration.[26] In this respect, the dRVVT is performed with a rather dilute partial thromboplastin,[11] while the KCT does not require any added phospholipid.[10] For this reason, the KCT, more than the dRVVT, is affected by the platelet contamination of plasma, which represents an important source of procoagulant phospholipids. As in our laboratory the coagulation tests are routinely performed in platelet-poor and not platelet-free plasma, one might argue that the plasma contamination by residual platelets could account for the two coagulation profiles. However, this was not the case, because the results in terms of specific sensitivity of the KCT to LA antibodies and of the dRVVT to aCL-type A antibodies were even amplified when the assays were repeated after plasma filtration.

Apart from the difference in phospholipid requirement, the dRVVT and KCT differ also in that the former evaluates selectively the conversion of prothrombin to thrombin by factors Xa and Va, while the latter one is an overall assay, which proceeds through both the generation of factor Xa by factors IXa and VIIIa and the activation of prothrombin by factors Xa and Va. As these reactions are phospholipid-dependent, the effects of aCL and LA antibodies on both factor X and prothrombin activation have been evaluated by several groups of researchers. In this respect, it has been demonstrated that LA antibodies inhibit the activation of

both (human) prothrombin[5] and factor X,[27] via recognition of the complex of lipid-bound/(human) prothrombin. On the other side, aCL-type A antibodies have been shown not only to inhibit the generation of thrombin in a β2-GPI–dependent fashion,[6-8] but also to greatly delay the inhibitory effect of β2-GPI on the generation of factor Xa by factors IXa and VIII.[28,29] Therefore, we might hypothesize that in a plasma system, the final effect of aCL-type A antibodies derives from the sum of two opposite actions, which could, at least in part, annihilate each other. This could provide an explanation for why an overall test like the KCT may be normal or hardly prolonged in some aCL-type A–containing plasmas (eg, patients 16, 19, 21, and 22 of Table 2), while the dRVVT, which is independent of factor X activation, is particularly sensitive to the presence of this type of phospholipid-dependent inhibitor of coagulation. In line with this hypothesis, the opposite would stand for LA antibodies, as their inhibitory activity on two consecutive coagulation reactions would be amplified in the KCT but not in the dRVVT. Our experiments performed in patients' plasmas in the presence of either factor IXa or factor Xa and phospholipids, although indirectly, support this hypothesis. In fact, the inhibitory effect of aCL-type A antibodies was more expressed in the clotting system that started after addition of factor Xa (thus, mimicking the conditions of the dRVVT) than in the system with factor IXa, while the opposite was true for the LA-containing plasmas.

Finally, we observed that the prevalence of patients with thrombosis was statistically correlated with the presence of aCL-type A but not of LA antibodies. Even though care must be exercized in the interpretation of these findings, which suffer from the limitations of a retrospective analysis and from the small sample size, the presence of aCL-type A antibodies, more than that of LA antibodies, might represent a useful marker for the identification of those aPL-positive patients prone to thrombosis. This hypothesis must be validated by prospective studies on large groups of patients.

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