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Interference of lupus anticoagulants in prothrombin time assays: implications for selection of adequate methods to optimize the management of thrombosis in antiphospholipid-antibody syndrome

PATRIZIA DELLA VALLE, LUCIANO CRIPPA, ANNA MARIA GARLANDO, ELISABETTA PATTARINI, OMID SAFA,* SILVANA VIGANÒ D'ANGELO, ARMANDO D'ANGELO

Coagulation Service and Thrombosis Research Unit, Scientific Institute H S. Raffaele, Milan, Italy

**Current address: Oklahoma Medical Research Foundation, 13th NE Street, OKC, OK, USA*

ABSTRACT

Background and Objectives. **Prolonged anticoagulation aiming at International Normalized Ratio (INR) values > 3.0 has been recommended for patients with thrombosis and the antiphospholipid-antibody syndrome. We evaluated the influence of anticoagulant antibodies in two different prothrombin time (PT) assays carried out on plasma from lupus anticoagulant patients on oral anticoagulation.**

Design and Methods. **INR values obtained with a combined (final test plasma dilution 1:20) and a recombinant (final test plasma dilution 1:3) thromboplastin were compared in 17 patients with persistent lupus anticoagulants (LA) receiving oral anticoagulant treatment and monitored for 69.8 patient-years. Doses of anticoagulant drugs were always assigned based on the results obtained with the combined thromboplastin, aiming at a target INR of 2.5 or 3.0 for patients with venous or arterial thromboembolic disease. Paired determinations with both reagents were also obtained throughout the study period in 150 patients on stable oral anticoagulation but free of antiphospholipid antibodies. Total IgG fractions were purified from selected patients to evaluate effect in the two PT assay systems.**

Results. **No patient experienced recurrence of thrombosis or major bleeding complications (95% confidence interval: 0.1-6.5 per 100 patient-years). INR values with the recombinant reagent were significantly higher than with the combined reagent in 8 LA patients (mean ΔINR ranging from 0.17 to 0.54) of the degree of anticoagulation was overestimated in all but one LA patients with the recombinant reagent when compared to the ΔINR observed in non-LA patients (-0.64 ± 0.42). The anti-cardiolipin IgG titer (r_2 = 0.43, p = 0.004) and the anti-β_2GPI IgG titer (r_2 = 0.30, p = 0.023) were positively associated with the mean Δ INR observed in LA patients. When added to plasmas with different levels of vitamin K-dependent factors, total IgG fractions from 6 LA patients with significant overestimation of the INR with the recombinant reagent (mean DINR ranging from 0.17 to 0.54, group 1) and from 7 LA patients with mean ΔINR ≤ 0.0 (ranging from -0.25 to 0.04, group 2) reproduced the effects observed *ex vivo* in the two assay systems. However, when total IgG fractions were tested at the same final concentration in the two PT assay systems, there was no difference in the clotting times determined with total IgG fractions from group 1 and group 2 LA patients. Addition of negatively charged liposomes (0.4 and 0.8 mg/mL final concentrations) to platelet free plasma from LA-free patients on stable oral anticoagulation caused a 20% to 48% prolongation of the prothrombin time determined with the recombinant reagent. In contrast, no significant prolongation of the prothrombin time determined with the recombinant reagent was observed upon addition of negatively charged liposomes to plasma from group 1 LA patients.**

Interpretation and Conclusions. **These results confirm previous suggestions of assay-dependency of INR values in LA patients on oral anticoagulation. For these patients, accurate INR values may be obtained using combined thromboplastin reagents that permit testing at high plasma dilution.**

Key words: antiphospholipid-antibody syndrome, lupus anticoagulants, oral anticoagulation, International Normalized Ratio system, β_2-glycoprotein I, recombinant thromboplastins, combined thromboplastins

Correspondence: Armando D'Angelo, M.D., Servizio di Coagulazione, IRCCS H S. Raffaele, via Olgettina 60, 20132 Milan, Italy. Phone: international+39-2-26432228 – Fax: international+39-2-26432640 – E-mail: armando.dangelo@hsr.it

The antiphospholipid-antibody (APA) syndrome is a disorder characterized by recurrence of clinical events – such as thromboembolic disease or fetal loss – in subjects carrying an antiphospholipid antibody (anticardiolipin antibodies, lupus anticoagulants or both).[1] The syndrome may be primary or secondary to autoimmune diseases,[2] with both forms sharing similar clinical and laboratory features.[3] Venous or arterial thrombosis or both have been described as initial events in affected patients, with venous thromboembolism representing the more common manifestation.[4-6]

In spite of anticoagulant treatment, recurrence of thrombosis is frequent in patients with the APA syndrome,[4,7-11] especially in those carrying lupus anticoagulants (LA)[12,13] and/or high titers of anticardiolipin antibodies.[11,14] In retrospective studies, a lower incidence of recurrences (arterial and venous) was

observed in patients kept at a high intensity of oral anticoagulation,[9,10,15-18] leading to the recommendation that patients with antiphospholipid antibodies who have had a documented major thrombotic event should receive life-long oral anticoagulant treatment to achieve an International Normalized Ratio (INR) of 3.0 or higher.[10,19,20]

Why patients with the APA syndrome should require an intensity of anticoagulation higher than that recommended for patients with the same thrombotic manifestations, but free of APAs (INR values between 2.0 and 3.0[21]), is unknown. Heparin treatment of patients with LA is often inadequate because of the interference of the antibodies in activated partial thromboplastin time assays. Because of the lesser prolongation of the clotting time observed in baseline prothrombin time assays, it is generally believed that interference of LA in the laboratory monitoring of oral anticoagulant treatment does not occur. That this may not be the case was strongly suggested by a preliminary evaluation carried out in our anticoagulation clinic[22] and later confirmed by Moll and Ortel[23] and by another collaborative study.[24] However, a British study did not support the suggestion that the INR may not reflect the true level of anticoagulation in the long-term warfarin treated patient in whom lupus anticoagulant was detected.[25] As a result, there is controversy about management of oral anticoagulation in patients with lupus anticoagulants.[26,27] In the present study, we extended our previous observations and tested the interference of antiphospholipid antibodies on INR results of patients with LA on oral anticoagulant therapy.

Design and Methods

Patients

From 1991 to 1995, we enrolled in our anticoagulation clinic 22 patients with LA and thromboembolic diseases. Five patients were lost early during the follow-up. The characteristics of the remaining 17 patients are reported in Table 1. The APA syndrome was secondary to systemic lupus erythematosus in 5 patients, to polyarteritis nodosa in one patient[28] and was associated to myasthenia in another patient. Two patients with deep vein thrombosis and pulmonary embolism had previously suffered cerebrovascular thrombosis but they had not received anticoagulant treatment. All patients had their diagnosis of thrombosis documented by objective tests (venography or compression ultrasonography and lung scanning for venous thromboembolism; computed tomographic scanning for cerebrovascular occlusive disease; thrombectomy at surgery for peripheral artery occlusive disease). Six patients were referred to our Institution on heparin treatment; anticoagulation in all the remaining patients was started after obtaining a baseline coagulation profile. Within 6 days of intravenous or subcutaneous unfractionated heparin, oral anticoagulation was started and overlapped with heparin until the INR was greater than 2.0 on 3 consecutive days.

Table 1. Demographic characteristics and antiphospholipid-antibody status of patients with lupus anticoagulants.

Pt	Sex	Age (yrs)	APA-syndrome	Thrombotic event*	PT ratio†	aPTT ratio†	PTT-LA ratio††	Staclot-LA ratio		Anti-cardiolipin		Anti-β2-GPI IgG**	Anti-factor II IgG**
								–Hex PL§	+ Hex PL§	IgG^	IgM#		
1	M	46	primary	DVT	nd	nd	2.39	2.55	1.46	73.0	1.0	0.240	0.279
2	F	33	secondary	DVT	1.20	1.25	4.31	3.14	1.50	11.4	0.3	0.059	0.070
3	M	48	secondary	DVT/CVOD	1.17	1.63	2.02	2.66	1.54	41.0	215.0	0.204	0.080
4	M	62	secondary	DVT	1.10	1.81	1.97	2.08	1.55	187.0	3.0	0.625	0.266
5	F	68	secondary	PE/CVOD	nd	nd	2.54	2.45	1.76	9.0	0.0	0.463	0.296
6	F	42	secondary	DVT/PE	nd	nd	1.44	1.52	1.11	2.0	0.0	0.097	0.063
7	M	51	primary	DVT	nd	nd	1.74	1.90	1.10	40.0	3.0	0.298	0.100
8	F	35	primary	DVT	1.22	2.01	2.73	3.02	1.66	210.0	11.0	>2.000	0.815
9	F	15	primary	DVT	1.3	2.37	2.74	3.15	1.11	25.0	13.0	0.314	0.069
10	F	44	secondary	DVT	1.08	1.21	2.79	1.97	0.90	177.0	0.0	0.986	0.200
11	F	42	primary	DVT/PE	1.10	1.20	1.75	3.35	1.02	156.0	48.0	1.136	0.248
12	M	38	secondary	DVT	nd	nd	1.31	1.47	0.82	1.0	2.0	0.030	0.067
13	F	60	primary	PAOD	0.96	1.45	1.89	2.02	0.99	4.0	1.0	0.346	0.219
14	M	37	primary	CVOD	1.03	1.29	1.35	1.32	1.04	1.0	1.0	0.008	0.040
15	F	22	primary	DVT	1.24	1.19	3.32	3.81	1.91	27.0	14.0	0.784	0.142
16	M	64	primary	DVT	nd	nd	3.12	3.63	1.52	287.0	1.0	1.614	0.189
17	M	57	primary	PAOD	1.01	2.29	3.23	3.90	0.95	5.0	5.0	0.044	0.146
Normal values					<1.18	<1.28	<1.25	<1.16	<1.22	<15.0	<12.5	<0.130	<0.120

**DVT, deep vein thrombosis; CVOD, cerebrovascular occlusive disease; PE, pulmonary embolism; PAOD, peripheral artery occlusive disease. †Prothrombin time and activated partial thromboplastin times ratios not determined in patients observed when already on anticoagulant treatment. ‡ PTT-LA ratios in a 50:50 mixture of the patient's plasma with normal pooled plasma; §hexagonal (II) phase phospholipid; ^GPL units; #MPL units. **absorbance at 405 nm.*

Design of the study

In our anticoagulation clinic, patients with persistent LA and a documented major episode of thromboembolism are maintained on long-term oral anticoagulation except in the presence of contraindications or when there is confirmed disappearance of the laboratory features of the APA syndrome (see below). Based on preliminary data obtained by comparison of INR results with different thromboplastin reagents in a patient with a potent LA, in 1991 we started monitoring oral anticoagulant treatment of these patients with a combined thromboplastin reagent aiming at an INR of 2.5 for venous thromboembolic disease and 3.0 for arterial occlusive disease.[29] LA patients were instructed to visit our anticoagulation clinic on the same day of the week at 4 to 5 week intervals after the attainment of stable anticoagulation. At each appointment, patients were questioned about the occurrence of bleeding complications and/or recurrence of thrombosis. Patients with deep vein thrombosis were monitored by compression ultrasonography at least once every year.

In 1992 a recombinant thromboplastin was introduced in our laboratory for the monitoring of oral anticoagulation in non-LA patients. This reagent (Recombiplastin, Ortho Diagnostic Systems, Raritan, NJ, USA) is used by over 30% of Centers affiliated to the Italian Federation of Anticoagulation Clinics (FCSA). At that time we began a pairwise comparison of INR values measured with the combined and with the recombinant reagent in patients with LA. The weekly dose of anticoagulant drugs was always assigned based on the results obtained with the combined reagent. Data obtained during the early phase of anticoagulant treatment (usually the first 1-2 months of treatment) were not considered for analysis, which included a total of 709, paired INR results in the 17 patients.

Laboratory methods

Prothrombin time assay systems. All determinations were carried out by the same automated coagulometer (ACL 300R, Instrumentation Laboratory) using dedicated software programs.

Pro-IL-Complex (Instrumentation Laboratory, Milan, Italy) is a combined reagent – based on the prothrombin-proconvertin time principle[30] – containing bovine brain and polybrene. Citrated plasma (10 µL) is diluted in 50 µL of bovine plasma depleted of vitamin K-dependent factors (source of factor V and fibrinogen) and clotting initiated by addition of 140 µL of thromboplastin. The final test plasma dilution in the assay system is 1:20.

Recombiplastin contains human recombinant tissue factor and synthetic phospholipid. Clotting is initiated by addition of 100 µL of thromboplastin to citrated plasma (50 µL), with a 1:3 final test plasma dilution in the assay system. Six lots of Pro-IL-Complex and 11 lots of Recombiplastin were used throughout the study. INR values were calculated using the instrument-specific International Sensitivity Index values supplied by the manufacturers with each lot of reagent (Recombiplastin: 0.81-0.97; Pro-IL-Complex: 1.12-1.15).

Throughout the study period, plasma samples from 150 patients on stable oral anticoagulation at different target INRs, but free of LA and/or anticardiolipin antibodies were also tested once with both thromboplastin reagents.

Lupus anticoagulant detection. Before 1993, the screening assays used in our laboratory for LA detection included the activated partial thromboplastin time, the diluted Russell's viper venom time and the kaolin clotting time, carried out on citrated plasma after double centrifugation.[31,32] In 1993 a standardized procedure was introduced for the detection of LA in a 50:50 mixture of the test plasma with normal plasma (PTT-LA, Stago, Gennevilliers, France). Confirmation of diagnosis is obtained by a significant shortening (≥ 8 sec) of the abnormal clotting time in presence of hexagonal (II) phase phospholipid (Staclot-LA, Stago, Gennevilliers, France). The procedure is highly sensitive and permits diagnosis of LA in plasma from patients on oral anticoagulant treatment.[33] Values were expressed using ratios of the patient's to normal pooled plasma clotting times as previously described.[34] Persistence of lupus anticoagulants was monitored in all patients at 6 month intervals.

Detection of antibodies to cardiolipin, β_2-GPI and factor II. Anticardiolipin IgG and IgM were tested by commercial ELISA methods (Quanta Lite Aca IgG, IgM, Inova Diagnostics, San Diego, CA, USA). β_2-glycoprotein I (β_2-GPI) and prothrombin were purified from citrated human plasma (3 liters) according to published procedures.[35,36] Plasma IgG reacting to β_2-GPI and prothrombin coated onto 96-well microtiter plates (Nunc-Immuno Plate Maxi Sorp, Roskilde, Denmark) were tested as previously described.[34] Results were considered abnormal when optical densities exceeded by more than 3 standard deviations the mean values observed in 30 normal subjects. All patients with LA had these determinations carried out at least twice at 6 month intervals.

Effect of total IgG fractions in the prothrombin time assay systems. To avoid contamination of IgG fractions with vitamin K-dependent clotting factors, the latter were removed by barium chloride (80 mmol/L final concentration) adsorption of citrated plasma. Total IgG fractions from patients with LA and from a normal pooled plasma obtained from 30 healthy donors were purified by protein G affinity chromatography (Mab Trap G II, Pharmacia Biotech, Uppsala, Sweden) as previously described,[37] recovering a total of 95 to 110 mg IgG in 30 mL of eluting buffer. After extensive dialysis against 0.006 M Tris-HCl buffer, pH 7.5, IgG were transferred to 15 mL conic tubes and freeze-dried under vacuum.

The influence of IgG fractions on prothrombin time

results was determined in plasma previously depleted of IgG by protein G affinity adsorption. The prothrombin times of normal pooled plasma and of plasma pools obtained from 4 patients on oral anticoagulant treatment were not significantly affected by the IgG depletion procedure (see below).

Dose-dependency of the IgG anticoagulant effect was evaluated by redissolving the lyophilized IgG fractions (1-5 mg) of two LA patients (3 and 7, see Tables 1 and 2) in 0.5 mL aliquots of a pooled plasma obtained from anticoagulated patients (INR = 2.50). Dependency of the anticoagulant effect of IgG fractions on the reduction of vitamin K-dependent clotting factors was evaluated by redissolving 5 mg of the patients' IgG in 0.5 mL aliquots of normal plasma and of pooled plasmas with INR values of 1.89, 2.71 and 3.55. The mixtures were then immediately tested in both assay systems.

Because of the large difference in the final total IgG concentrations in the two PT assay systems, different amounts of total IgG fractions purified from 13 LA patients (0.38-5 mg) were redissolved in 0.5 mL aliquots of normal pooled plasma and of a plasma pool obtained from patients on oral anticoagulation (INR = 3.47). The influence of IgG in the PT assay using the recombinant reagent was tested at two average final IgG concentrations corresponding to those expected when using whole plasma in the recombinant (2.65±0.59 mg/mL) and in the combined (0.40±0.09 mg/mL) PT assay systems. For comparison, the two above mentioned final IgG concentrations added to normal pooled plasma were also tested in the PTT-LA assay system.

Effect of phospatidylserine-phosphatidylcholine liposome addition in the prothrombin time assay system with the recombinant reagent. Phosphatidylserine (PS) -phosphatidylcholine (PC) (30:70) mixtures in chloroform were evaporated to dryness under nitrogen, rehydrated in TBS (10 mg/mL) for 30 min with intermittent vortexing and sonicated for 20 min at 4°C. After centrifugation at 2000 × g for 10 min at 20°C, liposome preparations were then diluted in TBS to the concentrations required and used within 24 hours.[38]

PT determinations with Recombiplastin were carried out in platelet free plasma from 40 patients free of anticardiolipin antibodies and in 12 LA patients after addition of buffer or PS-PC liposomes at 0.4 and 0.8 mg/mL final concentration. Liposomes (10 or 20 mL) or a corresponding volume of TBS were added to 0.1 mL of platelet free plasma and incubated for 2 min at 37°C prior to PT determination. Longer incubation times did not affect the results.

Statistical methods

Ninety-five percent confidence intervals were calculated from the binomial distribution. Regression analysis and comparison of slopes was performed by standard statistical methods. The Wilcoxon's rank test was used to compare INR values obtained with

Table 2. Monitoring of oral anticoagulation in patients with and without lupus anticoagulants.

Pt.	Months of follow-up	Anticoagulant drug	Weekly dose (mg)	INR Pro-IL-Complex	INR Recombiplastin	ΔINR*	p†	a‡	b§
1	70	Acenocoumarol	20.7±2.1	2.60±0.71	2.49±0.78	-0.09±0.38	0.02	0.000	-0.046#
2	67	Acenocoumarol	14.5±2.4	2.45±1.12	2.77±1.44	0.30±0.62	0.00001	-0.130	0.333#
3	66	Acenocoumarol	11.0±2.0	2.18±0.37	1.92±0.41	-0.25±0.26	0.00001	-0.087	-0.137°
4	66	Acenocoumarol	14.9±1.3	2.45±0.71	2.83±0.93	0.31±0.57	0.00001	0.244	0.033#
5	65	Acenocoumarol	18.9±1.2	2.62±0.63	2.72±0.65	0.10±0.37	0.047	0.334	-0.137#
6	62	Acenocoumarol	10.0±1.4	2.22±0.89	2.25±0.78	0.04±0.48	ns	0.047	0.004#
7	60	Acenocoumarol	15.6±1.6	2.43±0.78	2.64±0.85	0.22±0.31	0.00001	0.212	0.011#
8	59	Acenocoumarol	14.4±0.6	2.55±0.49	2.76±0.54	0.18±0.26	0.017	0.242	-0.040°
9	56	Acenocoumarol	19.1±1.2	2.42±0.45	2.59±0.60	0.17±0.46	0.007	0.319	-0.104°
10	54	Acenocoumarol	26.7±2.6	2.58±0.99	2.98 ± 0.96	0.35 ± 0.49	0.00001	0.628	-0.151#
11	52	Acenocoumarol	21.5±4.2	2.35±0.83	2.35±0.76	-0.01±0.31	ns	0.189	-0.146#
12	40	Acenocoumarol	25.9±2.4	2.30±0.73	2.12±0.61	-0.17±0.25	0.007	0.098	-0.204°
13	31	Warfarin	30.0±2.6	2.98±0.80	2.94±0.65	-0.02±0.34	ns	0.401	-0.207°
14	26	Warfarin	59.5±9.9	2.14±0.70	1.97±0.64	-0.17±0.11	0.00001	-0.064	-0.092#
15	26	Warfarin	49.2±8.6	1.84±0.48	1.87±0.46	0.02±0.13	ns	0.090	-0.078
16	19	Acenocoumarol	36.1±9.2	2.06±0.56	2.59±0.68	0.54±0.25	0.00001	0.372	0.140#
17	18	Warfarin	52.8±2.5	2.54±0.52	2.31±0.60	-0.14±0.35	0.043	-0.180	0.053#
Patients free of APA-syndrome on oral anticoagulation (n=150)			3.31 ± 0.83	2.66 ± 0.54	-0.64 ± 0.42	0.00001	0.308	-0.412	

*Mean±SD are reported. *INR with Recombiplastin - INR with Pro-IL-Complex; †significance of the difference of ΔINR versus zero. ‡§origin (a) and slope (b) of the regression of ΔINR/(Pro-IL-Complex INR - 1); the significance of the difference in the slope of individual patients versus the slope of the regression in patients free of APA-syndrome on oral anticoagulant therapy is also shown: #p<0.001; °p<0.01.*

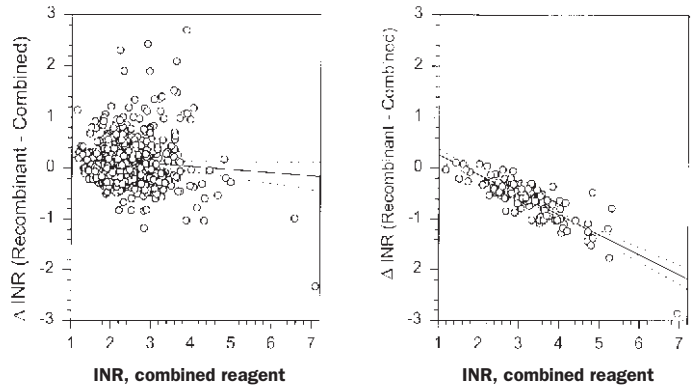

Figure 1. Plot of the differences in INR (ΔINR) with the recombinant and the combined thromboplastin reagent versus the INR obtained with the combined reagent in patients with lupus anticoagulants (left panel) and in patients free of lupus anticoagulants on oral anticoagulation (right panel). Regression lines and 95% confidence limits are shown.

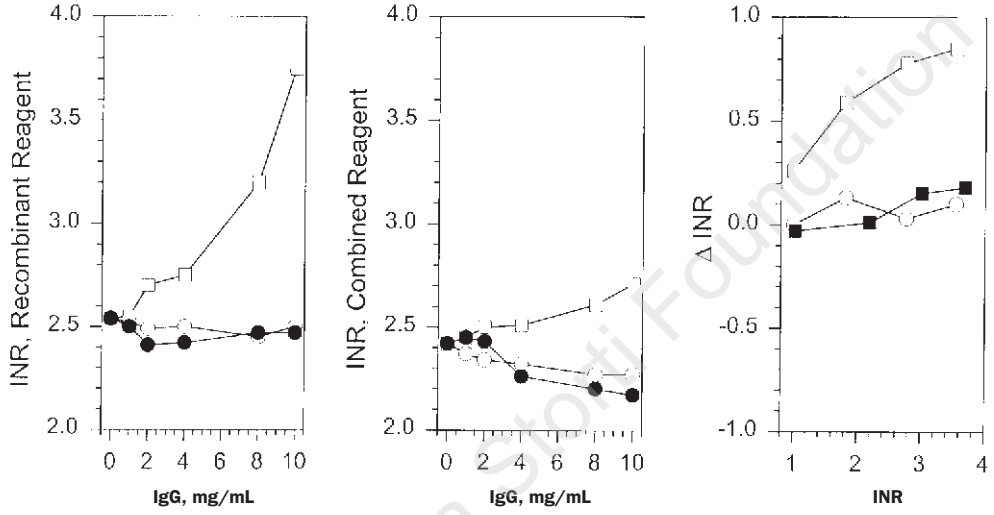

Figure 2. Anticoagulant effect of lupus anticoagulant IgG in the prothrombin time assay systems. Left and middle panels: Dose-dependency of the anticoagulant effect of total IgG fractions purified from patient #7 (open squares), patient #3 (open circles) and from normal pooled plasma (closed circles) in the assay system employing the recombinant (left panel) or the combined reagent (middle panel). All IgG fractions were added to the same plasma pool obtained from patients free of antiphospholipid antibodies on oral anticoagulation. INR denotes International Normalized Ratio. Right panel: Changes in the INR (ΔINR) of normal pooled plasma and of plasma pools obtained from patients receiving different intensities of oral anticoagulant treatment determined with the recombinant (open symbols) and the combined reagent (closed symbols) after the addition of total IgG fractions (10 mg/mL) purified from patients #7 (squares) and #3 (circles).

the two reagents. Results obtained with the combined thromboplastin were subtracted from the corresponding values with the recombinant reagent and the difference (ΔINR) plotted against the INR values of the combined reagent.

Results

The APA status of the 17 patients is shown in Table 1 (median of at least two determinations). The addition of hexagonal (II) phase phospholipid resulted in a significant shortening of the clotting time in all patients, with complete normalization achieved in 9. Seven patients had no anti-cardiolipin antibodies. IgG antibodies reacting to solid phase β_2-GPI or factor II were repeatedly found in 12 and 10 patients.

The total follow-up time amounted to 69.8 patient-years. The average weekly doses of anticoagulant drugs, the average INR values determined with the two reagents and the Δ INR between the two PT systems are shown in Table 2. Five hundred and three out of 709 INR determinations (70.9%) with the combined thromboplastin were within the desired therapeutic ranges. Four patients (#3, #14, #15 and #16) were suboptimally anticoagulated. Neither recurrence of thrombosis nor major bleeding was observed in any patient (95% confidence interval: 0.1-6.5 per 100 patient-years).

INR values with the recombinant reagent were sig-

nificantly higher than with the combined reagent in 8 of the 17 patients (average ΔINR ranging from 0.10 to 0.54) and lower in 5 (average ΔINR from –0.09 to –0.25). Among the parameters of APA status reported in Table 1, only the anti-cardiolipin IgG titer (r_2 = 0.43, p = 0.004) and the anti-β_2GPI IgG titer (r_2 = 0.30, p = 0.023) were positively associated with the average ΔINR observed in LA patients.

INR values determined with the 2 reagents in control patients free of LA were significantly different (Table 2 and Figure 1). For these patients, the slope of the regression of ΔINR versus the INR –1 of the combined reagent was –0.412 (r_2 = 0.71). Thus, for all but one LA patient, the slope of the regression of ΔINR/INR-1 was significantly greater than that of control patients (Table 2 and Figure 1), suggesting a different interference of LA in the two PT assay systems.

That the important differences observed with the 2 prothrombin time assay systems were due to the presence of interfering antibodies was shown by testing total IgG fractions purified from two patients (#3 and #7) who had average ΔINR going in opposite directions (–0.25 and 0.22, n= 58 and 55 respectively). With the recombinant thromboplastin, the INR of a plasma pool from patients on oral anticoagulant treatment increased from 2.54 to 3.75 at a 10 mg/mL concentration of the IgG fraction of patient #7 but did not change with the addition of total IgG purified from patient #3 or from normal pooled plasma (Figure 2, left panel). With the combined thromboplastin, total IgG fraction of patient #7 affected the INR of the same plasma pool to a much lesser extent (from 2.45 to 2.71, Figure 2, middle panel). Dependency of the IgG anticoagulant activity on the reduction of vitamin K-dependent clotting factors in the test plasma was proven by addition of total IgG fraction (10 mg/mL) to normal plasma and to plasma pools from patients on oral anticoagulation with different INR values. The changes in the INR observed with total IgG fraction of patient #7 were positively related to the baseline INR (Figure 2, right panel). The total IgG fraction from patient #3 was ineffective in increasing INR values of any plasma pool with either thromboplastin reagent.

Because of the widely different final IgG concentration in the two PT assay systems, we also tested total IgG fractions from additional LA patients at a similar final concentration. LA patients are grouped in Table 3 according to the presence (#2, #7, #8, #9, #10, #16, group 1) or absence (#1, #3, #6, #11, #13, #15, #17, group 2) of a significant overestimation ($p \leq 0.01$) of the INR with the recombinant versus the combined reagent. At average final concentrations of 2.76 and 2.55 mg/mL – corresponding to the 10 mg/mL concentration of Figure 2 – total IgG fractions from group 1 LA patients caused a significantly greater prolongation of the PTT-LA than did total IgG fractions from group 2 LA patients. At the same average final concentrations, total IgG fractions from group 1 LA patients also prolonged to a greater extent the PT of normal pooled plasma and of warfarin plasma when tested with the recombinant reagent (Table

Table 3. Effect of the addition of total IgG fractions to normal and warfarin plasma in different assay systems.

	IgG	*PTT-LA (sec)*	*Recombiplastin (sec)*		*IgG*	*PTT-LA (sec)*	*Recombiplastin (sec)*		*Pro-IL complex (sec)*	
	*(mg/mL)**	*NPP*	*NPP*	*WP*	*(mg/mL)**	*NPP*	*NPP*	*WP*	*NPP*	*WP*
NPP IgG	3.00	38.3	12.7	40.2	0.45	36.1	12.2	39.4	38.7	119.0
P-IgG	0.00	38.7	12.0	40.0	0.00	36.1	12.2	40.1	39.2	121.2
Pat 2	2.38	136.6	14.5	44.2	0.36	46.0	12.4	39.7	39.4	122
Pat 7	3.03	107.2	13.7	46.8	0.45	42.4	12.4	40.0	39.4	124
Pat 8	2.07	107.2	13.9	43.9	0.31	47.5	12.3	40.1	38.7	120
Pat 9	3.19	116.0	14.3	42.9	0.48	45.7	12.4	39.4	37.7	119
Pat 10	3.15	160.6	15.8	47.6	0.47	53.8	12.5	39.5	39.2	120
Pat 16	2.76	171.2	16.1	48.3	0.41	58.2	12.7	40.2	39.7	124
mean±SD	2.76±0.45	133.1±27.8	14.7±1.0	45.6±2.2	0.41±0.07	48.9±5.9	12.5±0.1	39.8±0.3	39.0±0.7	121.5±2.2
Pat 1	1.64	64.4	13.0	42.0	0.25	40.2	12.3	38.8	39.2	122
Pat 3	3.15	63.7	12.9	41.1	0.47	39.4	12.7	39.2	39.2	123
Pat 6	1.65	46.6	13.0	41.6	0.25	36.8	12.6	39.0	37.4	120
Pat 11	3.10	51.9	12.8	42.2	0.47	40.2	12.2	39.5	39.2	119
Pat 13	2.67	46.1	12.8	40.0	0.40	35.4	12.0	39.3	38.7	119
Pat 15	2.32	106.1	14.1	42.1	0.35	44.6	12.5	39.9	39.4	125
Pat 17	3.33	99.1	13.4	41.6	0.50	42.4	12.5	39.4	39.4	123
mean±SD	2.55±0.69	68.3±24.7	13.1±0.5	41.5±0.8	0.38±0.11	39.9±3.1	12.4±0.2	39.3±0.4	38.9±0.7	121.6±2.3
p†	ns	0.0013	0.012	0.0051	ns	0.012	ns	ns	ns	ns

*Clotting times were determined after the addition of patients' total IgG fractions to normal pooled plasma (NPP) or to a pool of plasmas from patients on stable oral anticoagulation (WP, INR = 3.47) using the different assay systems. Total IgG concentrations reported refer to the final concentration in the PT assay systems. Patients are grouped according to the presence of absence of a significant overestimation (p <0.01 or less) in INR values with the recombinant vs the combined thromboplastin reagent. NPP-IgG: total IgG fraction from normal pooled plasma; P-IgG normal or warfarin plasma depleted of IgG. * final total IgG concentration in the assay. †significance of the difference between the two groups of patients with lupus anticoagulants.*

3). When IgG fractions were tested in the PTT-LA system at 3/20 lower concentration - corresponding to the actual final IgG concentration in the PT system using the combined reagent - there was still a significantly greater prolongation of the clotting time with total IgG fractions from group 1 LA patients. However, there was no difference in the clotting times determined with total IgG fractions from group 1 and group 2 LA patients irrespective of the PT assay system (Table 3). These results strongly suggest that the final concentration of anticoagulant antibodies – rather than differences in the phospholipid composition of the two thromboplastin reagents – is the major determinant of the differences in INR values observed with the two PT assay systems.

A standpoint of the diagnosis of lupus anticoagulant antibodies is represented by partial or complete correction of clotting time prolongations by addition of negatively charged phospholipid. When added to platelet-free plasma from patients on stable oral anticoagulation, but free of anticardiolipin and LA antibodies, PC-PS liposomes caused the expected dose-dependent prolongation of the PT determined with the recombinant reagent (Figure 3, left panel). At either liposome concentration, the prolongation of the clotting time was linearly related to the clotting time determined in platelet-free plasma. When PC-PS liposomes were added to platelet-free plasma from LA patients, the clotting time prolongation observed with plasmas from group 2 LA patients (open circles) was not significantly different from that observed in LA-free patients. In contrast, platelet-free plasma from group 1 LA patients did not show prolongation of the recombinant PT at either liposome concentration (Figure 3, middle and right panels). For LA-free patients, the slopes of the regression of prothrombin times with the addition of PC-PS liposomes versus prothrombin times in platelet-free plasma were 1.20 (at 0.4 mg/mL PC-PS liposome concentration) and 1.48 (at 0.8 mg/mL PC-PS liposome concentration). For group 2 LA patients, corresponding regression slopes were 1.23 and 1.38 (p = 0.096 vs LA-free patients). For group 1 LA patients, regression slopes were 1.00 and 1.01 (p = 0.0001 vs LA-free patients). These results indicate that by increasing the negatively-charged phospholipid content of the recombinant reagent, substantially lower INR values are observed in LA patients.

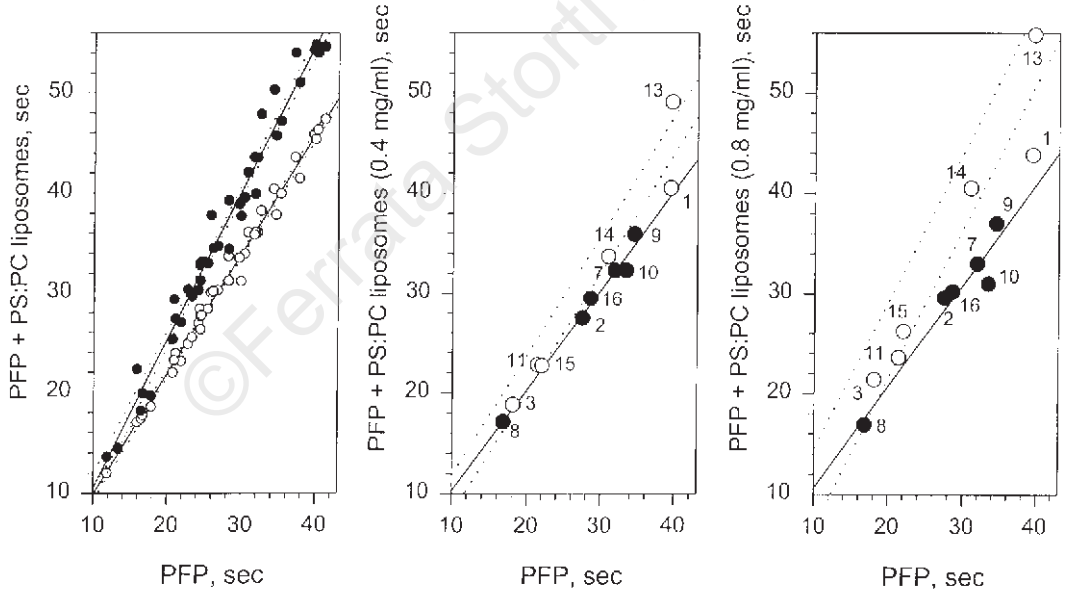

Figure 3. Effect of the addition of PC-PS liposomes on the prothrombin time assay system employing the recombinant reagent. Left panel: regression analysis of clotting times measured in 40 LA free patients on stable oral anticoagulation after addition of 0.4 mg/mL (open circles) or 0.8 mg/mL (closed circles) PC-PS liposomes versus the clotting times measured in platelet-free plasma (PFP). Regression lines and 95% confidence limits are shown. Middle panel: effect of the addition of 0.4 mg/mL PC-PS liposomes to platelet-free plasma from group 1 (closed circles) and group 2 (open circles) LA patients. The two groups of LA patients are defined as in Table 3 and LA patients are numbered according to Tables 1-3. Regression analysis of clotting times measured after the addition of PC-PS liposomes versus the clotting times measured in platelet-free plasma is shown for group 1 LA patients only. Dotted lines show the 95% prediction interval of the regression observed in LA-free patients at 0.4 mg/mL PC-PS liposome concentration. Right panel: effect of the addition of 0.8 mg/mL PC-PS liposomes to platelet-free plasma from group 1 (closed circles) and group 2 (open circles) LA patients. Regression analysis of clotting times measured after the addition of PC-PS liposomes versus the clotting times measured in platelet-free plasma is shown for group 1 LA patients only. Dotted lines show the 95% prediction interval of the regression observed in LA-free patients at 0.8 mg/mL PC-PS liposome concentration.

Discussion

Adoption of the INR system has contributed to a reduction in the number of patients unnecessarily overanticoagulated.[29] On the other hand, the high precision of currently available thromboplastin reagents has permitted the emergence of differences among reagent-instrument combinations[39,40] which may result in serious discrepancies in the treatment of patients, given the narrow range of therapeutic intervals indicated for the different clinical settings.[29,41] Concern about consistency of results obtained with the same thromboplastin by different coagulometers has led to the certification of instrument-specific International Sensitivity Index (ISI) values. That such an approach may not be successful in routine laboratory practice is suggested by the consistent finding of substantially different INR values observed with commercial thromboplastin reagents in patients on oral anticoagulation, irrespective of the presence of antiphospholipid antibodies in their plasma (refs. #23-26 and this manuscript). The introduction of lyophilized plasma samples with certified INR values for local calibration of reagent-instrument combinations[24,25] may help to overcome this discrepancy, but would fail to ensure commutability of prothrombin time results for patients with LA on oral anticoagulant treatment.

In this paper we show that INR determinations obtained with a recombinant PT reagent substantially overestimate the actual degree of anticoagulation of most LA patients and that this is due – irrespective of correct ISI assignment – to interference of lupus anticoagulant IgG in PT assays carried out at low test plasma dilution, as occurs with plain and recombinant thromboplastin reagents.

Since the reports by us[26] and by Moll and Ortel[23] which questioned the validity of the INR system in the monitoring of oral anticoagulation in patients with lupus anticoagulants, the issue of INR determinations in patients with LA has been approached by other authors. Lawrie *et al*.[25] observed substantial equivalence of INR determinations obtained with 8 plain or recombinant thromboplastin reagents in patients with and without LA after local ISI assignment with the use of INR calibrated plasmas. However, combined thromboplastin reagents were not evaluated in this study. Robert *et al*.[24] compared INR values obtained with 8 thromboplastins, inclusive of both reagents adopted in our study, in patients with and without LA. When INR values were calculated by extrapolation of a calibration curve constructed by plotting prothrombin times against assigned INR values of calibrated lyophilized plasmas, there was no significant difference between INR values obtained with 7 reagents, but one recombinant thromboplastin did overestimate INR values in LA patients. The authors concluded that the INR system is valid in the monitoring of oral anticoagulation for LA patients, provided the LA-sensitive reagents are identified and discarded.[24] The results of these two studies are contradictory with respect to the suitability of one recombinant thromboplastin (Innovin) for monitoring oral anticoagulation in LA patients; in addition, only a single INR determination was carried out for each patient, which may have hampered the identification of significant differences between thromboplastin reagents. In view of their reduced factor V content, lyophilized plasmas, such as those used for local INR calibration, behave differently with combined and with plain or recombinant thromboplastin reagents.[42,43] Attribution of a single INR value to these calibrator plasmas, irrespective of the type of thromboplastin reagent, may bias interpretation of the results of *calibrated* INR determinations.

Because plain thromboplastin reagents are more commonly used than combined reagents, our findings provide an explanation of the higher intensity of anticoagulation recommended for patients with the APA syndrome. In two of the retrospective studies supporting this recommendation, prothrombin time tests were performed with various thromboplastins[10] or with rabbit brain thromboplastins from different sources.[9] No information about the thromboplastin reagent adopted is given in the study which failed to observe recurrence of venous thromboembolism while patients with LA were maintained at a INR between 2.0 and 3.0.[13] The thromboplastin reagents adopted are also not mentioned in two other studies,[44,45] which questioned the indication for high intensity anticoagulation in patients with antiphospholipid antibodies (± LA) and venous thromboembolism. However, the approximate recurrence rate of thromboembolism was 4.4 and 7.4 per 100 patient-years in patients whose INR maintained between 2.0 and 3.0 in studies by Prandoni *et al.*[44] and by Rance *et al.*[45] Because the percentage of patients with LA in retrospective management studies ranged from 26% to 85%[9,10,16,17,45] it is unknown whether the supposedly higher intensity of anticoagulation is required by all patients with antiphospholipid antibodies or by the subgroup of patients with LA. Interestingly, the anti-β_2-GPI and the anti-cardiolipin IgG titers were both positively associated with overestimation of INR values with the recombinant reagent in our series of LA patients. β_2-GPI has been identified as a major, but not the only, target for the activity of LA in phospholipid-dependent clotting assays.[46-49]

There is concern about an increased bleeding risk when recommending life-long high intensity oral anticoagulation to patients with the APA syndrome.[44,45,50,51] The demonstration of assay-dependency of INR values in LA patients is relevant to the management of thrombosis in the APA syndrome with respect to both safety and efficacy of oral anticoagulant treatment. Notwithstanding the relatively short follow-up time, it is not surprising that the prevalence of recurrence of thrombosis in our series (0.1-6.5 per 100 patient-years) is similar to that reported for patients

kept at an INR ≥ 3.0 (0-2.6 per 100 patient-years) and lower than that in patients kept at an INR < 3.0 (17-31 per 100 patient-years) in the study by Khamashta *et al*.[10] More importantly, prothrombin time testing at high plasma dilution – as occurs with all commercially available combined reagents – may avoid unnecessary excess anticoagulation in a high proportion of patients. We did not observe major bleeding complications in any of our patients. All 29 patients who experiencing bleeding complications (severe in 7) in the study by Khamasta *et al*.[10] had an INR of 3.0 or higher at the time of bleeding, while 2 of 19 patients whose INR values were maintained between 2.5 and 4.0 with a combined thromboplastin reagent (Thrombotest) suffered a major bleeding complication in another study.[16] If we had kept our patients at INR values >3.0 by monitoring treatment with the recombinant reagent, at least 6 patients would have been unnecessarily exposed to an increased risk of bleeding.

Most recently, the *Duration of Anticoagulation Study Group* has provided prospective evidence that patients with anti-cardiolipin IgG antibodies require prolonged anticoagulation to avoid recurrences of venous thromboembolism.[52] While on oral anticoagulant treatment targeted at an INR between 2.0 and 2.85, the recurrence rate increased with the anticardiolipin antibody titer, but was not significantly different in patients with (1.32 per 100 patient-years) or without (0.6 per 100 patient-years) anti-cardiolipin antibodies. It is noteworthy that the two thromboplastin reagents adopted in this study were both combined thromboplastins.

Future prospective studies evaluating management of thrombosis in the APA syndrome[53] should take into account the assay system-dependency of INR values in patients with lupus anticoagulants. At present, patients with LA requiring oral anticoagulant treatment should be monitored with prothrombin time systems using a high plasma dilution and their target INR should be between 2.0 and 3.0 irrespective of the presence of anticoagulant antibodies.[26]

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All the authors listed gave substantial contributions to the conception and design of the study. PDV, LC and ADA analyzed and interpreted the data; PDV and ADA wrote the manuscript. The remaining authors are listed in alphabetical order. All the authors approved the final version of this manuscript

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Disclosures

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