The laboratory diagnosis of lupus anticoagulant in patients on oral anticoagulation

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Summary   Laboratory-specific cut-off lupus ratios (LR), above which a plasma is judged positive for lupus anticoagulant (LA), were established for both activated partial thromboplastin time-based and dilute Russell viper venom time-based methods. The validity of using these cut-off values to determine the presence of LA in patients on oral anticoagulation (OAC) was assessed. A cohort of 40 patients (23 male and 17 female), aged 22–84 years (mean 52 years) were tested for LA at the time of a thrombotic event. Repeated testing was performed after the same patients were treated with OAC (international normalized ratio 2.0–3.5). For 36 patients (90%), LA status was unchanged pre- and on-OAC. Thirteen of the 40 patients (32.5%) were positive for LA both pre- and on-OAC. Of the 27 patients negative for LA pre-OAC, 23 remained negative on-OAC. The four discordant results were interesting in that LA positivity was demonstrated only after the patient was stable on-OAC. In our cohort of 40 patients, there was a trend for LRs to decrease on-OAC, but this did not reach statistical significance. The subset (4) went against this trend and became positive after the thrombotic event.

Introduction

Antiphospholipid antibodies (APA) are a heterogenous group of autoantibodies directed against phospholipid bound to protein (McNeil *et al.*, 1990; Roubey, 1994). Routine laboratory testing for the presence of APA typically involves solid-phase immunoassays testing for the presence of anticardiolipin antibody (ACA) and coagulation testing for the presence of lupus anticoagulant (LA). 'Lupus anticoagulant' is the term that has evolved for the *in vitro* phenomenon of prolonging clotting times in tests where the clot end-point is phospholipid-dependent (Exner, 1995). Clinically, LA are associated with an increased risk of venous thromboembolism (VTE),

arterial thromboembolism and recurrent fetal loss (Hughes, 1993). The laboratory demonstration of LA is considered to have a stronger link to thrombotic events than ACA (Gali *et al.*, 2003), so this ideally requires a sensitive and specific test system to detect its presence. Laboratory investigations rely on the recommendations of Scientific and Standardization Committee of the International Society of Thrombosis and Haemostasis (Brandt *et al.*, 1995). Interlaboratory correlation of results remains suboptimal (Arnout *et al.*, 1999; Jennings *et al.*, 2002) because of the differing reagent sensitivity to LA inhibition, varying interpretation of data results and from the differing cut-off values used to demonstrate LA.

Oral anticoagulant (OAC) therapy causes a reduction in vitamin K-dependent clotting factors, prolonging clotting times in the laboratory tests for LA. This prolongation is potentially compounded by the inhibitory effect of LA. Some reports cast doubt on the reliability of testing anticoagulated blood (heparin or warfarin) for LA (Greaves *et al.*, 2000). However, while it is feasible to

postpone testing until a patient is off heparin, many clinicians desire to know the LA status of patients on OAC. As APA are associated with recurrent and atypical thromboembolic events (Proven *et al.*, 2004), the findings of an LA may influence clinical decision-making for patients on OAC treatment.

Opinion concerning the effect of OAC on laboratory tests for LA is conflicting. A 1 : 1 mix of test plasma with pooled normal plasma (PNP) aims to correct the deficiency state induced by OAC, but there is a belief that it does not completely correct in plasma from patients on more intense dose OAC, leading to false-positive diagnoses (Arnout, 2001). Mixing with PNP may neutralize weak LA leading to false-negative diagnoses (Thom *et al.*, 2003). No use of a prior mix with PNP is advocated in two studies. One is an in-house dilute Russell viper venom time (dRVVT) method and the reported sensitivity and specificity for LA in patients on OAC was 90% (Tripodi *et al.*, 2002). The other demonstrates the usefulness of a comparison between sensitive and insensitive activated partial thromboplastin time (APTT) reagents (Brancaccio *et al.*, 1997). These two studies focus purely on the ability of the confirmatory reagent to shorten clotting times in LA-positive plasmas.

Our laboratory also serves as a hospital (130 LA investigations/month) receiving frozen aliquots of plasma from other institutions. For consistency of approach to the laboratory diagnosis of LA, we employ a single process with all test plasmas. Based on the model of Jacobsen *et al.* (2000), all test plasmas are mixed 1 : 1 with the same PNP prior to assessing the difference in clotting times between low (screen) and high (confirm) concentration phospholipid tests. We have established our reagent and analyser specific cut-off lupus ratios (LR) for two differing methods. One is the APTT that initiates coagulation via contact factors, sequentially activating all clotting proteins except factor VII. The second, the dRVVT, initiates coagulation by activating factor X to factor Xa, involving just the common pathway.

Patients with unprovoked venous and arterial thrombosis are tested for LA as part of a thrombophilic screen at the time of an event. The aim of this study was to assess the validity of using our locally established LR in both an APTT-based method and a dRVVT-based method with patients at the time of thrombosis, and when anticoagulated with a vitamin-K antagonist. A cohort of 40 patients were tested for LA at the time of a thrombotic event (36 VTE/four stroke) and the same patients were tested for LA when they were stabilized on OAC. A lack of adequate clinical data precluded an assessment of the diagnosis of antiphospholipid syndrome.

Materials and methods

Clinical material

During the 6-month period, January to June 2004, 40 patients (23 male and 17 female; median age 53 years) presenting at an emergency department, were tested for LA at the time they presented with a thrombotic event (36 VTE, four stroke). None was on heparin (normal thrombin clotting time) and none had commenced OAC [International normalized ratio (INR) 0.9–1.12]. The same 40 patients were tested for LA after they were stabilized on OAC (INR 2.0–3.5). The time interval between pre-OAC and on-OAC was not standardized (1–12 weeks), but most were captured as outpatients approximately 3 weeks after initial testing.

Plasma from 55 healthy volunteers (26 male and 29 female; median age of 52 years) were used to establish the cut-off LR (2 SD above the mean) for each of the APTT and the dRVVT-based methods. The PNP used in mixing studies was obtained from a pool of 30 similar healthy volunteers.

Laboratory studies

Blood from both control and patients were collected by clean venepuncture, using 21 gauge needle, into a tube containing 0.109 M citrate in a ratio of nine parts blood to one part citrate. Platelet poor plasma (platelet count $<5 \times 10^9$/l) was prepared as previously described (Aboud & Ma, 1997). Aliquots of plasma were stored under liquid nitrogen until testing.

The LR (Jacobsen *et al.*, 2000) is the ratio of two clotting times for 1 : 1 mixes of patient's plasma and PNP, one using dilute phospholipid (screen) and the other using phospholipid-rich reagent (confirm), normalized by dividing by the corresponding ratio for undiluted PNP performed in the same assay.

$$LR = \frac{(\text{test plasma screen} + \text{PNP1} : 1)/(\text{test plasma confirm} + \text{PNP1} : 1)}{\text{PNP screen/PNP confirm}}.$$

Reagents used for the APTT-based method were Actin FSL (screen) and Actin FS (confirm), supplied by Dade Behring (Marburg, Germany). Reagents for the dRVVT-based method were La Screen and La Confirm from Life Therapeutics (Sydney, Australia). All tests were performed on the fully automated STA-R coagulation analyser from Diagnostica Stago (Paris, France). The clotting times in seconds were downloaded from the analyser to an excel file for calculation of the ratios.
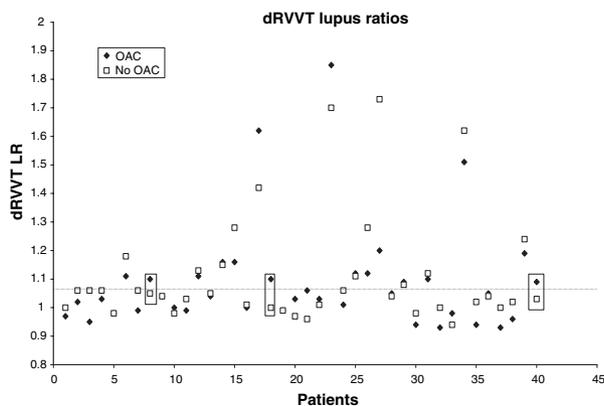
Figure 1. The dilute Russell viper venom time (dRVTT) lupus ratios (LRs) for 40 individual patients pre-oral anticoagulation (OAC; open square) and on-OAC (closed diamond). Ratios falling above 1.07 were judged positive for lupus anticoagulant (LA). Note for patients 5, 9 and 19, the LRs pre- and on-OAC are so similar that the symbols are superimposed. Presence/absence of LA was consistent pre- and on-OAC for all patients except for three (patients 8, 18 and 40) whose paired results are enclosed in boxes. All three were negative at the time of a thrombotic event, but became positive on OAC.

The clotting times for PNP in 20 consecutive assays provided data for between-assay precision (FS, 1.62%; FSL, 1.52%; La Screen, 0.91% and La Confirm, 0.88%). An LA-positive patient plasma was used as an abnormal
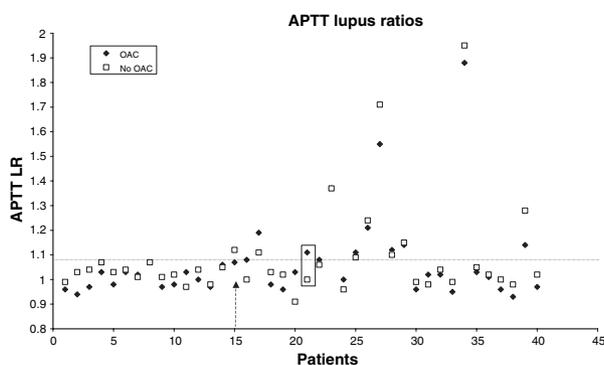

Figure 2. The activated partial thromboplastin time (APTT) lupus ratios (LR) for 40 individual patients pre-oral anticoagulation (OAC; open square) and on-OAC (closed diamond). Ratios falling above 1.08 were judged positive for lupus anticoagulant (LA). Note for patients 8 and 23, the LRs pre- and on-OAC are so similar that the symbols are superimposed. Presence/absence of LA was consistent pre- and on-OAC for all patients except for one (patient 21) whose paired results are enclosed in a box. This patient was negative at the time of a thrombotic event but became positive on OAC. Patient 15 appears anomaly, but the overall LA status pre-OAC and on-OAC remained unchanged as the LR was clearly positive in the dilute Russell viper venom time method (see Figure 1).

control and in 10 consecutive assays the between-assay precision was also acceptable (FS, 1.51%; FSL, 1.40; La Screen, 1.45% and La Confirm, 1.27). Test plasma was judged positive for LA if its LR exceeded the cut-off ratio in only one or both methods.

Statistical analysis

The LR pre-OAC and LR on-OAC for APTT and dRVVT-based methods were plotted for visual inspection (Figures 1 and 2). For the mean differences, 95% CI and *P*-values were calculated using statistical software from SPSS Inc. (Chicago, IL, USA). To assess the possibility of a systematic difference between LR pre-OAC and LR on-OAC in either of the dRVVT or the APTT-based method, a comparison between the difference and the mean (Bland & Altman, 1986) was used.

Results

The LR cut-off ratios (2 SD above mean) derived from 55 healthy volunteers were 1.07 for the dRVVT method and 1.08 for the APTT method. The LRs for the 40 patients pre- and on-OAC are shown in Figure 1 for the dRVVT method and in Figure 2 for the APTT method. Taking into account the 40 patient results for both the methods (Table 1), for 36 of 40 (90%) of the patients, the LA status was unchanged pre- and on-OAC. LA positivity pre- and on-OAC was diagnosed in 13 (32.5%) of the patient group. Of 27 patients negative at the time of thrombosis, 23 remained negative on OAC, leaving four discordant results. All four (10%) patients progressed from negative pre-OAC to positive on-OAC. These four patient plasmas became positive in one method only (three in dRVVT and one in APTT) (Table 2) and were close to cut-off values.

To assess the possibility of OAC systematically influencing the LR, we plotted the difference (LR pre-OAC – LR on-OAC) *vs.* the average of the two ratios. The scatter of points above and below zero is evident (Figure 3), but there is a trend towards more negative values, i.e. the LR on-OAC is less than the LR pre-OAC. This trend is reflected in both the methods, but it is not statistically significant

Table 1. LA status of 40 patients

	On-OAC	
Pre-OAC	Negative	Positive
Negative	23	4
Positive	0	13

LA, lupus anticoagulant; OAC, oral anticoagulant.

Table 2. Patient demographics of the four discordant results

	INR	dRVVT (>1.07)	APTT (>1.08)	LA status
38 year M				
PE	1.0	1.05	1.07	LA negative
	2.5	1.10	1.07	LA positive
74 year M				
PE	0.9	1.00	1.03	LA negative
	2.8	1.10	0.98	LA positive
48 year F				
PE	1.0	0.96	1.00	LA negative
	2.2	1.06	1.11	LA positive
59 year F				
PE	1.0	1.03	1.02	LA negative
	2.5	1.08	0.97	LA positive

PE, pulmonary embolism; INR, international normalized ratio; dRVVT, dilute Russel viper venom time; APTT, activated partial thromboplastin time; LA, lupus anticoagulant; M, male; F, female.
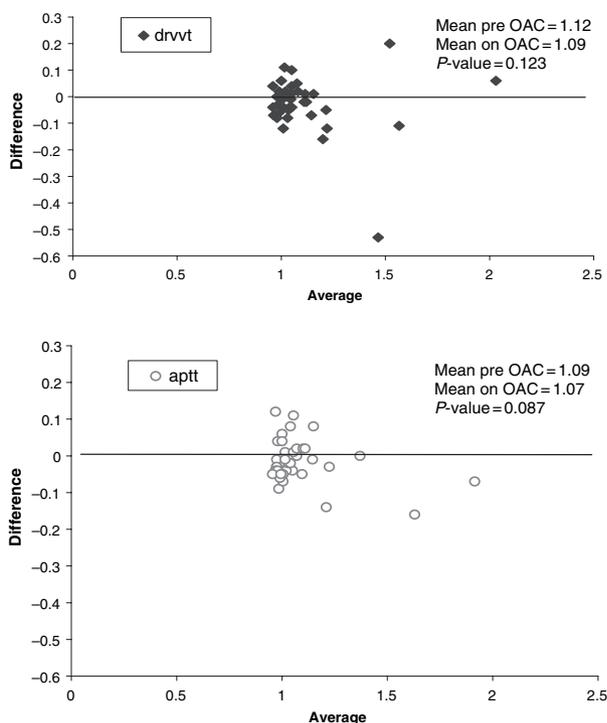

Figure 3. Scatter plots of the difference between pre-oral anticoagulation (OAC) and on-OAC lupus ratio (LR) *vs.* the average LR for each of the 40 patients. With both the methods there are more points below zero, indicating a trend towards the LR on-OAC being lower than the LR pre-OAC.

for either the dRVVT ($P = 0.123$) or the APTT method ($P = 0.087$). Our four discordant results were in the opposite direction, i.e. higher LRs on-OAC.

The time interval between pre- and on-OAC samples varied between 1 and 12 weeks. Plotting the mean LR *vs.* time showed no difference (data not shown) in LR over time for either assay.

Discussion

Laboratory tests for the presence of LA are, at best, a surrogate marker of an *in vivo* prothrombotic process with multifactorial (probably autoimmune) pathogenesis. (Greaves *et al.*, 2000; Proven *et al.*, 2004). However, demonstration of LA activity remains clinically useful, even when a patient is on OAC. Our study showed that using locally derived LR cut-off values for patients pre- and on-OAC, we observed 90% concordance of LA status.

Some of the ratios for the 40 thrombotic patients pre- and on-OAC (Figures 1 and 2) are well above the cut-off values and are clearly positive for LA. It is the ratio that clusters around the cut-off values that challenge the laboratory definition and clinical interpretation of LA positivity. Because of the heterogeneity of APA and the differing test systems in use, assessing the potency of LA activity is problematic. Indeed, the potency of clotting inhibition by LA *in vitro* does not necessarily correlate with the severity of clinical symptoms and there is concern over assays that fail to detect the weak LA (Jacobsen & Wisloff, 1996; Jennings *et al.*, 1997; Ames *et al.*, 2001; Thom *et al.*, 2003). All four discordant results became 'weakly' positive on OAC. The cut-off values are 2 SD above the mean of healthy donors. Our cut-off LR values (1.07 for dRVVT and 1.08 for APTT) are similar to Jacobsen's study (1.05 established with 120 blood donors, excluding two outliers and using their in-house APTT reagent). It is important to validate or re-establish cut-off values with a change of lot number of reagents as the laboratory judgement of LA status hinges on these values. Borderline values may create diagnostic uncertainty (Ames *et al.*, 2001), but the potential clinical significance of those just above our analyser/reagent-specific cut-offs cannot be ignored. All four patients in Table 2 were diagnosed with pulmonary embolism (PE) without other risk factors for thrombosis being identified. The nature of PE in patient 4 was recurrent and she had a positive ACA. Her LA status was positive on follow-up testing and she remained on OAC on the basis of her thrombophilia being associated with APA. Follow-up testing on the three other patients would have shed light on the permanent/transient nature of their LA status, but unfortunately this was not performed.

Assessment of the possible systematic influence of OAC on LR shows a trend towards ratios being lower when the

patients were on OAC. This perhaps confers more significance on our four discordant results that have higher ratios on OAC.

A limitation of this study is that the time interval between pre- and on-OAC was not standardized. Also, repeated testing for LA some months after commencement of OAC may have been enlightening regarding the transient or reproducible nature of the borderline-positive plasmas. We are at present prospectively examining patients pre- and on-OAC at several standardized intervals. There have been studies that have assessed the LA diagnosis in patients on OAC using plasma from patients with clearly defined APA (Brancaccio *et al.*, 1997; Tripodi *et al.*, 2002; Thom *et al.*, 2003). However, to our knowledge, testing the same cohort of thrombosis patients pre- and on-OAC is a novel approach to the investigation of the possible effect of OAC on the laboratory diagnosis of LA.

In conclusion, on the basis of this study, we feel it is valid to test patients on OAC using the principle of the LR of Jacobsen *et al.* (2000) with both the APTT and dRVVT-based assays. Whether the four discordant results represent a subset of these heterogeneous autoantibodies that are masked at the time of a thrombotic event by acute phase reactants (Arnout, 2001) or other factors is not evident from this study. However, this study does indicates that an initial negative finding ought not to preclude follow-up testing in patients considered at risk of having APA.

Acknowledgements

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