Laboratory Testing for Lupus Anticoagulants: A Review of Issues Affecting Results

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Background: Lupus anticoagulants (LA) are a heterogeneous class of immunoglobulins. Persistent LA positivity is a risk factor for the occurrence and recurrence of venous/arterial thromboembolism and/or pregnancy morbidity and qualifies the patient for anticoagulation therapy. The laboratory diagnosis for LA that is used for crucial decision-making about the optimal duration of the therapy rests entirely on diagnostic criteria. These criteria are based on the prolongation of phospholipid-dependent tests not corrected upon mixing patient and normal plasmas, with confirmation provided by the evidence that the anticoagulant is directed against proteins bound to negatively charged phospholipids.

Aims: This article reviews issues related to the diagnosis of LA, including the effect of preanalytical variables, choice of tests, results interpretation of screening, mixing and confirmation procedures, patients to be investigated, and transmission of results. Unresolved issues and future direction for research on laboratory diagnosis are also discussed.

Methods: Search of PubMed with the key term “lupus anticoagulant” plus articles and unpublished data known to the author.

Results and Conclusions: The preanalytical variables (i.e., plasma preparation and storage before analysis) as well as the diagnostic steps to detect LA present potential problems that undermine the process of making a correct diagnosis. A truly specific test for LA detection is badly needed, but its development may require understanding of the mechanisms associated with the occurrence of clinical events. Until then, clinical laboratories should rely on the existing procedures, which must be applied with caution and awareness of the many issues that may affect their results.

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Lupus anticoagulants (LA) are a heterogeneous class of immunoglobulins that may develop spontaneously or as a consequence of autoimmune diseases. They bind to proteins such as β2-glycoprotein I (β2-GPI), prothrombin, or others in complex with negatively charged phospholipids and thus prolong phospholipid-dependent coagulation tests. Persistently positive LA tests when associated with the presence of a recent history of vascular thrombosis or pregnancy morbidity are a requisite to define the antiphospholipid syndrome and are stronger risk factors for thrombosis than anticardiolipin antibodies.

Furthermore, persistent positivity for LA in the face of a recent history of vascular thrombosis is a strong risk factor for recurrence and qualifies the patient for long-term oral anticoagulation therapy. Long-term anticoagulation is not devoid of hemorrhagic risk, and the accuracy of the laboratory diagnosis of LA plays a crucial role in making decisions on whether and how long anticoagulation should be undertaken.

Several surveys performed during the last 10 years have shown variable performance of clinical laboratories with respect to sensitivity and specificity of LA tests. The rates of false-positive and false-negative detections remain relatively high. The former are of particular concern because they qualify the patients for long and unnecessary oral anticoagulant treatment. Some of the reasons behind the poor performance have been elucidated, and efforts should be made to raise awareness among clinical laboratories. This article reviews the most important issues related to LA detection, such as the effect of preanalytical variables, the choice of tests (screening and confirmation), the interpretation of results, and the
transmission of diagnostic information to clinicians. Another relevant feature of the laboratory diagnosis of the antiphospholipid syndrome, the search for the presence of solid-phase antiphospholipid antibodies (2), is not discussed in this report. The observations that form the basis of the present overview are derived from personal experience and from an extensive search of the literature indexed by MedLine PubMed with the key term “lupus anticoagulant”.

PREANALYTICAL VARIABLES
Adequate plasma preparation for LA testing is an essential prerequisite for reliable diagnosis. For many years residual platelets in plasma have been known to affect phospholipid-dependent coagulation tests (12, 13) through the exposure of platelet membranes of anionic phospholipids that quench LA activity. This effect leads to shorter coagulation time and is particularly evident, especially in test plasmas that undergo freezing and thawing before analysis. The effect is more pronounced with reagents whose phospholipid content is relatively low (12) and may be greater with the kaolin clotting time (KCT) than with the dilute Russell viper venom test (dRVVT) (14). According to Sletnes et al. (12), the numbers of residual platelets in plasma after centrifugation of whole blood may be as high as 16 × 10⁷/L at 3000g and is 0 only after filtration. The effect on the shortening of coagulation time, which may be expected by the above numbers of residual platelets after freezing and thawing, is on average 7% with standard phospholipid concentrations, but may be as high as 33% in more diluted preparations (12). Recently, Chantarangkul et al. (15) investigated the effect of centrifugation at 2500g vs filtration through 0.22-μm cellulose acetate filters on 42 plasma samples from patients who tested positive for LA (15). Both series of samples were tested after freezing and thawing by using silica clotting time (SCT) at low and high phospholipid concentrations and the activated partial thromboplastin time (APTT) with and without hexagonal (II) phase phospholipids. Both SCT and APTT were 100% diagnostic for LA even when the test plasmas were not filtered, but a trend toward decreased diagnostic capacity was observed for nonfiltered plasmas (Figs. 1 and 2), suggesting that weak LAs may be lost during freezing and thawing of nonfiltered plasmas. The above information forms the basis for the recommendation (16) to render plasmas for LA testing platelet-free (<10 × 10⁹/L), especially if they are stored frozen for delayed testing. Either filtration through 0.22-μm cellulose acetate filters or double centrifugation of plasmas before freezing may be suitable procedures. It should be noted, however, that evidence has been reported that filtration may cause loss of such high-molecular-weight coagulation proteins as von Willebrand factor and consequently factor VIII (17). This loss may give rise to an artificial prolongation of those coagulation tests (namely the APTT) that are particularly responsive to factor VIII.

CHOICE OF TESTS
No specific test for LA is available and therefore its detection is still based on the diagnostic criteria set by the Scientific and Standardization Committee, Subcommittee on antiphospholipid antibodies/lupus anticoagulant of the International Society on Thrombosis and Hemostasis (16). These criteria require (a) prolongation of 1 or more phospholipid-dependent tests beyond the upper limit of the reference interval; (b) evidence that the above prolongation is due to the presence of an inhibitor and not to a deficiency of 1 or more coagulation factors; and (c) direction of the inhibitor against negatively charged phospholipids in complex with proteins and not against a specific coagulation factor (16). The above evidence may be provided by screening, mixing, and confirmatory testing procedures, respectively.

Screening tests. In theory, any of the tests that are dependent on phospholipids are suitable to screen patients for LA. Historically the APTT and the KCT (18) have been the tests of choice, but it is well known that their sensitivity to LA varies according to the composition of the reagents (4). For the APTT in particular, the varied sensitivity displayed by commercial preparations (19, 20) is probably due to the class and concentration of phospholipids (21). It has been reported that the use of reagents rich in phosphatidyl serine abolishes the LA effect on the APTT.

Fig. 1. Results of testing for 42 plasmas from LA-positive patients. Testing was performed with SCT on filtered (through 0.22-μm cellulose acetate filters) and nonfiltered plasmas. Results are reported as percentage correction of coagulation times obtained at low and high concentrations of phospholipids. The greater the percentage correction the more likely the presence of LA. Horizontal bars, median values; filled columns, cutoff values. Reprinted with permission from Chantarangkul et al. (15).
dRVVT, first described for the detection of LA in 1986 (26), is based on the activation of factor X by a fraction of the venom derived from the Russell viper in combination with dilute phospholipids. dRVVT is widely used in clinical laboratories and is believed to be specific for detecting LA in those patients at high risk for developing thrombosis. Galli et al. (27) attempted to define 2 clinical profiles with respect to the risk of thrombosis and abnormalities of either KCT or dRVVT. Retrospective and prospective studies have shown that patients who presented with the dRVVT profile had a higher risk of thrombosis than those with the KCT profile (27). This evidence is corroborated by Pengo et al. (28), who found that the dRVVT was more sensitive than the KCT to detect LA activity associated with anti-β2-GPI antibodies. These observations are in line with those of de Laat et al. (29) who found that β2-GPI-dependent LAs are stronger risk factors for venous thromboembolism (odds ratio 42.3; 95% CI 9.9–194.3) than β2-GPI-independent LAs (1.6; 0.8–3.9). Despite these facts, it should be realized that not all the dRVVT commercial preparations behave in the same manner (4, 30) and therefore the above results and conclusions cannot be generalized.

Another screening test used variably over the years to detect LA is the dilute prothrombin time test. According to Arnout et al. (31) the diagnostic capacity of the dilute prothrombin time test may be improved by using a recombinant thromboplastin.

In spite of the vast array of phospholipid-dependent tests that can be used to screen patients, it should be realized that no single test is 100% sensitive to LA; the consensus is that at least 2 tests preferably with different assay principles should be used (16). The evidence so far accumulated would suggest that 1 of the 2 should be the dRVVT and the other may be based on the intrinsic pathway of coagulation (APTT, KCT, or SCT). If the choice is the APTT, it is important to adopt a sensitive reagent, although some authors contend that a combination of 2 APTTs, 1 sensitive and the other insensitive to LA, may give better detection capability (21, 32). One positive test only may suffice to diagnose LA provided that its positivity is documented on 2 occasions 12 weeks apart (2).

Mixing test. The mixing test is based on the rationale that the mixture of equal amounts of plasma from the index patient and from a healthy individual may considerably shorten the prolonged patient coagulation time if it is due to a deficiency of 1 or more coagulation factors. Conversely, the mixture does not correct the prolonged coagulation time when it is due to the presence of an inhibitor. Although very simple in principle and reliable in many instances the mixing test has drawbacks, among them the quality of the normal plasma used for mixing and the difficult interpretation of results. Normal plasmas used for mixing tests should be prepared by pooling equal amounts of plasmas from healthy individuals. The final
preparation should be made with sufficient numbers of individual donations to ensure normal concentrations for all coagulation factors (usually 30 donations are adequate) and must be platelet-free. This material can be quick-frozen and is stable for months if stored at −70 °C. Commercial lyophilized preparations may be suitable alternatives to homemade preparations if they fulfill the above requirements.

The interpretation of results for the mixing test deserves special attention. Current guidelines do not state exactly what to do, and the operator may be uncertain on how to make a decision. It is advisable to establish solid consensus criteria (within-laboratory) for interpretation (see below) and to use them with consistency over time. There are essentially 3 criteria to decide whether the mixture does or does not correct the prolonged coagulation time observed in the patient-only test plasma. The 1st criterion assumes that there is correction when the coagulation time of the mixture falls within the reference interval. This criterion may be valid, especially when grossly prolonged coagulation times are being investigated, but may give erroneous results with weak inhibitors. The application of this criterion requires that the reference interval be established for the specific test in the laboratory where the test is used because there are reports that clearly show a coagulometer-dependent effect (33). Furthermore, to minimize day-to-day variability it is advisable to express results as ratio (i.e., patient-to-normal coagulation time) (33).

The 2nd criterion to interpret mixing tests is more demanding, but is probably more important than the 1st. It assumes that the coagulation time of the mixture of test and normal plasma does not correct if it is longer (>2 SDs from the mean) than that of a normal plasma mixed with various non-LA plasmas (i.e., plasmas from patients with coagulation factor deficiencies).

The 3rd criterion calls for the calculation of the index of circulating anticoagulant (ICA) according to Rosner et al. (34) defined as

\[ ICA = \left( \frac{CT_{\text{mix}} - CT_{\text{NPP}}}{CT_{\text{patient}}} \right) \times 100, \]

where CT is the coagulation time and NPP is the normal pooled plasma. By definition, the greater the ICA the more likely the presence of an inhibitor. This criterion is probably the most robust, but requires that each laboratory determine its own cutoff value to interpret results. Whatever the choice, it should be realized that the interpretation of mixing results may be difficult when the patient coagulation time is only slightly prolonged. Furthermore, false-negative results are likely to be obtained for weak LA on account of the dilution effect brought about by the mixing procedure.

Confirmatory tests. The rationale for the use of confirmatory tests is that increasing the concentration of phospholipids in the test system neutralizes the effect of LA and shortens the prolonged coagulation time if it is due to the presence of LA. Conversely, the coagulation time remains prolonged if it is due to the presence of an inhibitor directed against 1 of the coagulation factors. Many confirmatory procedures are available. In some, aged platelets may be used in combination with the APTT or the dRVVT as a convenient source of phospholipids after cycles of freezing and thawing (35). Other procedures use synthetic phospholipids, either bilayer or hexagonal (II) phase, in combination with the dRVVT, APTT, or SCT (36, 37). Although very simple in principle and reliable in many instances, the confirmatory tests have drawbacks. First, heparin-containing plasmas may occasionally behave like LA (4, 11). This problem may occur although some commercial reagents include in their formulation such antiheparin substances as polybrene or heparinase. It is advisable, therefore, to rule out the presence of heparin by performing thrombin time tests or antifactor Xa assays whenever unexplained prolonged coagulation times are observed, especially for samples that are collected outside the responsibility of the testing laboratory. Second, anti-factor antibody-containing plasmas (hemophiliacs and others) may occasionally behave like LA (23, 24). Although this drawback is difficult to circumvent, scrutiny of the clinical history (hemorrhagic in patients with anti-factor antibodies) (38) may give valuable clues on the cause of the abnormal tests (Table 1). The criteria to judge whether the prolonged coagulation time does or does not normalize upon increasing the phospholipid concentration can be essentially the same as those suggested above for the interpretation of the mixing test.

Integrated tests to detect LA. Integrated tests include screening and confirmation in a single procedure. Such tests consist of testing the plasmas under investigation twice by means of the APTT (36), SCT (25), or dRVVT (37) performed in parallel at low (test1) and high (test2) phospholipid concentrations. In principle, these tests do not require performance of the mixing test and the results may be interpreted according to the specific cutoff values by calculating either the percentage correction [(test1 − test2)/test1 × 100] (25) or the LA ratio (test1/test2) (39).

**Table 1. Inhibitors of coagulation and hemorrhage (38).**

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<tr>
<th>Inhibitors usually associated with hemorrhage</th>
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<tr>
<td>Antifactor VIII, II, IX, X, XI</td>
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<tr>
<td>Anti-Willebrand factor</td>
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<tr>
<td>Antifibrinogen/fibrin</td>
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<tr>
<td>Heparin-like anticoagulants</td>
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<tr>
<td>Inhibitor variably associated with hemorrhage (from trivial to life-threatening)</td>
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<tr>
<td>Anti-factor V</td>
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<tr>
<td>Inhibitors usually not associated with hemorrhage</td>
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<tr>
<td>LA (with the possible exception of an association with hypoprothrombinemia)</td>
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<tr>
<td>Anti-factor XII</td>
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Both the percentage correction and the LA ratio may benefit from normalization of results against a normal pooled plasma run in parallel with the test plasmas. Some of the above tests are designed to measure the coagulation times on a mixture of patient and normal plasma (36, 39). The integrated tests are more convenient and less time-consuming than conventional procedures. Numbers of observations suggest that they are as reliable as the conventional procedures, but their value in detecting LA should be thoroughly investigated by large collaborative studies including many patients. Undoubtedly they represent a leap toward standardization.

PATIENTS TO BE INVESTIGATED AND TRANSMISSION OF RESULTS

Indications to search for LA are the occurrence of (accidentally found) prolongation of the APTT without known etiology, patients with venous and/or arterial thrombosis occurring at a young age (<50 years), thrombosis at unusual sites (cerebral, mesenteric) or associated with autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis, autoimmune thrombocytopenia, autoimmune hemolytic anemia), or pregnancy complications (2). Generalized searches on asymptomatic individuals or categories of patients other than the above are highly discouraged to avoid the risk of false-positive results that are relatively common on account of the poor specificity of the testing procedures.

Once the patient has been identified as positive for LA, it is imperative that testing be repeated on a 2nd occasion 12 weeks later (2). Transmission of results to clinicians should include analytical results and a comment on whether the results are compatible with the presence/absence of LA. Provision of these comments is highly encouraged because clinicians may not be aware of the significance of all the complex testing procedures carried out by the laboratory.

UNRESOLVED ISSUES

Patients on vitamin K antagonists. Detecting LA in patients on vitamin K antagonists may be challenging for the clinical laboratory because the prolonged coagulation times induced by coumarin congeners may superimpose the prolonged coagulation times induced by the presence of LA, thus making difficult the interpretation of screening, mixing, and confirmatory tests. For this reason, the revised guidelines on the antiphospholipid syndrome recommend to postpone the laboratory investigation until discontinuation of treatment (2). Alternatively, the diagnostic procedure may be carried out on a mixture of equal portions of patient and normal plasmas (2). Other possible approaches include the performance of such integrated tests as the APTT, SCT, or dRVVT at low and high phospholipid concentrations (36, 37) or tests that use snake venoms such as the textarin/ecarin coagulation times (40). The above procedures have proven reliable in the hands of their proponents, but should be evaluated in large cohorts of patients.

Quantification of LA. Whether high-titer LAs are stronger risk factors than low-titer LAs for thrombosis and pregnancy morbidity is not precisely known, but this information would be useful to stratify patients according to risk. On the other hand, such an evaluation would require clinical studies that are not possible until quantification of the LA is feasible. Few have tackled this issue, which remains essentially unresolved. One possibility would be to use plasma samples enriched with monoclonal antibodies raised against anti-β2-GPI antibodies that possess LA activity (41), or plasmas enriched with IgG purified from patients testing positive for LA (4). Both venues need to be explored by well-planned collaborative studies.

Differentiation between antiprothrombin- and anti-β2-GPI-dependent LA activity. LA may be classified into 2 broad categories in which the LA activity depends either on the presence of β2-GPI (42) or prothrombin (43). Accumulating evidence suggests that the 1st category of LA is associated more strongly than the 2nd with risk of thromboembolic events (29, 44). Differentiation between the 2 categories may therefore be beneficial to assess the risk of thrombosis. In principle, this differentiation can be obtained by solid-phase detection of the relevant antibodies (anti-β2-GPI or anti-prothrombin), but these assays are not standardized. Recently, 2 different methods have been described that can differentiate between the LA activity dependent on β2-GPI or prothrombin. One method is based on the interaction of β2-GPI and cardiolipin. The authors designed a confirmatory assay in which an LA-sensitive APTT assay is used with or without cardiolipin as a source of phospholipids. Shortening of the prolonged APTT is a strong indication that the prolongation is due to the presence of β2-GPI-dependent LA activity (45). The other method exploits the property of calcium chloride to enhance the binding of β2-GPI to phospholipids (46). The authors designed confirmatory assays for which dilute prothrombin time or dRVVT tests are used at high (10 mmol/L) or low (5 mmol/L) concentrations of calcium chloride, thus causing a further prolongation of the coagulation time only with the plasmas from patients in whom LA activity is associated with anti-β2-GPI antibodies (46). Both methods are promising tools to differentiate LA

<table>
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<th>Table 2. Main mechanisms of thrombosis in the antiphospholipid syndrome.</th>
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<td>Impairment of the protein C pathway</td>
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<tr>
<td>Inhibition of tissue factor pathway inhibitor</td>
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<tr>
<td>Displacement of Annexin V shield from the endothelium</td>
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<tr>
<td>Activation of platelets and endothelial cells</td>
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<tr>
<td>Induction of tissue factor exposure/synthesis</td>
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<tr>
<td>Impairment of fibrinolysis</td>
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<tr>
<td>Increase of plasminogen activator inhibitor-1</td>
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<td>Induction of apoptosis</td>
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Clinical Chemistry 53, No. 9, 2007 1633
according to the risk of thrombosis, but should be investigated in prospective clinical studies.

Conclusions and Future Directions

The LA, once regarded as a laboratory nuisance, gained considerable interest among clinicians and laboratory workers on the basis of the preliminary observation of Bowie et al. (47) that its positivity may be associated with the occurrence of thrombosis. Numbers of reports and metaanalyses have subsequently delineated the relative risk of thrombosis as well as of pregnancy morbidity associated with LA [see Refs. (1) and (3) for review], and the search for LA is now one of the major commitments for the hemostasis clinical laboratory. Despite this fact, testing procedures, although refined over the last 20 years, remain essentially unchanged and still rely on a set of diagnostic criteria. A truly specific and undisputed test is badly needed and may be developed only after we understand the mechanisms associated with the occurrence of clinical events (48, 49). The candidate mechanisms that received attention in the past are listed in Table 2. Among these, the most plausible seems to be the impairment of the protein C pathway (50). Accordingly, anti-β2-GPI antibodies with LA activity may identify patients at increased risk of thrombosis and would suggest an acquired activated protein C resistance as a possible explanation of the pathogenesis of the thromboembolic events (50). Perhaps the refinement of the phenotypic test for activated protein C resistance might be a venue to be explored to design a test suitable to detect the effect of LA on the protein C pathway. The evaluation of other mechanisms (48, 49) may also be instrumental in helping the design of newer and more specific testing procedures for LA. Until then, clinical laboratories should rely on the existing procedures, which must be applied with caution and awareness of the many issues that may affect their results.

Grant support/funding: None declared.
Financial disclosure: None declared.

References


