Lupus Anticoagulant Assays
Questions Answered and to Be Answered

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Context.—Lupus anticoagulants are antibodies with heterogeneous specificities to phospholipids. They have been associated with clinical syndromes consisting of thrombosis and recurrent fetal loss.

Objective.—To address questions about the laboratory assay aspects of lupus anticoagulants. This review is intended for clinicians managing lupus anticoagulant testing in clinical laboratories.

Data Sources.—Published literature on lupus anticoagulants, with emphasis on laboratory assay methods.

Conclusions.—Although there are published criteria for confirming the presence of a lupus anticoagulant, there is no consensus on assay methods for lupus anticoagulant testing. The mixing study is a useful screening test for lupus anticoagulants, but it may have limited utility. Clinical context may necessitate the performance of factor assays in addition to lupus anticoagulant testing to rule out factor deficiency or factor-specific inhibitor. Additionally, the presence of different anticoagulants may affect the reliability of lupus anticoagulant assays. Lupus anticoagulants are an independent risk factor for thrombosis. It may be useful to use different assays when there is clinical suspicion for a lupus anticoagulant. When testing for lupus anticoagulants, clinicians must carefully consider the clinical context because factor assays may also be indicated.

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The term lupus anticoagulant was first coined in 1972. Since then the term has caused significant confusion. Most patients who test positive for lupus anticoagulant do not have systemic lupus erythematosus. In addition, in the absence of concurrent thrombocytopenia or factor deficiency or factor inhibitor, lupus anticoagulant per se is not a risk factor for bleeding. The anticoagulant term arises from the prolongation of phospholipid-dependent clotting assays, which results from lupus anticoagulant's antibody specificity to phospholipids. Moreover, anticoagulant is purely an in vitro phenomenon manifested as a prolongation of the activated partial thromboplastin time (aPTT). In the past, the Subcommittee for the Standardization of Lupus Anticoagulants of the International Society on Thrombosis and Haemostasis (ISTH) tried to change the name by sending a nomenclature survey. However, because of a lack of consensus, the name of lupus anticoagulant and its abbreviation (LA) were not changed.

Despite the commercially available assay kits for lupus anticoagulant, several studies have suggested suboptimal performance of the assays. In addition, interpreting the results of these assays is challenging for clinicians because of test nomenclature, heterogeneity of lupus anticoagulant, and the absence of one gold standard assay. However, there are important clinical implications of the presence of lupus anticoagulant. Lupus anticoagulant is a risk factor for venous or arterial thrombosis and recurrent pregnancy loss. One meta-analysis revealed that the odds ratio for venous thrombosis is 11.1 (95% confidence interval [CI], 3.81–32.3) with lupus anticoagulant compared with 3.21 (95% CI, 1.11–9.28) with anticardiolipin antibodies. Additionally, a multivariate analysis found that the odds ratio for venous and arterial thromboembolism is 4.4 (95% CI, 1.5–13.3) with lupus anticoagulant and 1.2 (94% CI, 0.5–2.7) with anticardiolipin antibodies. Compared with other risk factors, positivity for lupus anticoagulant and long-term elevation of factor VIII have the highest odds ratio for a first episode of venous thromboembolism in individuals with a thrombophilic defect compared with individuals without a defect. Thus, making a correct laboratory diagnosis of lupus anticoagulant as part of a thrombophilia evaluation may be important in determining the strength or duration of anticoagulant therapy in a patient with thrombosis. However, there is no consensus regarding assay methods for lupus anticoagulant, and many questions remain unanswered. This review addresses several laboratory aspects of lupus anticoagulant assays.

WHAT SPECIMEN PREPARATION IS REQUIRED?

Blood specimens should be collected in 3.2% sodium citrate tubes and centrifuged to generate platelet free plasma within 4 hours of collection; the residual platelet count in the plasma should be less than \(1 \times 10^{10}/\mu L\). Passenger platelets will provide phospholipids that may neutralize lupus anticoagulant and cause false-negative results. Residual platelets are more problematic if the specimen has been frozen and thawed because of the release of phospholipids. If the platelet count in the plasma is greater than or equal to \(1 \times 10^{10}/\mu L\), the specimen should be centrifuged again or filtered before testing or freezing. Of note, filtering plasma will cause the loss of large multi-
mers of von Willebrand factor, thus affecting von Willebrand factor level and the multimer pattern. Ultracentrifugation may be associated with platelet fragmentation, which may release phospholipids and cause false-negative lupus anticoagulant results.9

WHAT ARE THE UTILITY AND THE LIMITATIONS OF THE MIXING STUDIES?

If the prothrombin time (PT) or aPTT is prolonged, a mixing study with normal plasma is performed. Most commonly, an equal volume of patient plasma and normal plasma are mixed. However, there is neither a standard method for the mixing study nor standard interpretation of the results. Furthermore, it is not advisable to use pooled plasma from patient specimens with a normal PT and aPTT since normal PT and aPTT guarantee neither normal coagulation factor levels nor absence of lupus anticoagulant. The normal plasma should be verified as containing normal coagulation factor levels.10 Normal plasmas of this kind are available from several commercial manufacturers. However, there is a possibility that commercially available normal plasma may not be platelet free and thus would release phospholipids when it is lyophilized, causing false corrections in mixing studies. The plasma collection process may be unique to each manufacturer. The manufacturer most often provides information regarding its collection process in the form of a product information sheet.

In our laboratory, if the PT is prolonged beyond 2.0 seconds above the normal range, or if the aPTT is prolonged beyond 3.0 seconds above the normal range, we perform a mixing study. After 1:1 mixing, a prolonged aPTT is not typically shortened into the normal range in the presence of lupus anticoagulant. However, each laboratory should determine its own criteria for correction or no correction.2 To aid in the determination of the criteria, factor sensitivity studies should be performed for the PT and aPTT assays used in the laboratory. The factor sensitivity study is used to determine the level of one specific factor, all others being normal, at which the PT or aPTT will be prolonged. This information can be useful when interpreting results of mixing studies. For example, if a specimen contains 1% factor VIII and a mixing study is performed by using normal plasma that contains 100% factor VIII, then the resulting factor VIII level of the mixture will be 50.5%. If the aPTT assay being used is sensitive to a factor VIII level of less than 40%, then the mixing study should correct into the normal range. However, if the aPTT assay being used is sensitive to a factor VIII level less than 55%, then a mixing study sample with a factor VIII activity of 50.5% may not correct the aPTT into the normal range. In this latter case, the factor sensitivity of the assay should also be considered as a reason for the noncorrection of the mixing study. However, since the factor sensitivity varies with each factor, correction of the prolonged aPTT into the normal range should not be the only reason to rule out lupus anticoagulant. On the other hand, the interpreter of the mixing study should also be aware that a mild prolongation of the PT or aPTT due to a weak lupus anticoagulant might correct upon mixing despite the presence of the inhibitor.

The utility of a PT mixing study is primarily to screen for a factor VII inhibitor, which remains very rare. The PT mixing study may also screen for rare inhibitors to factors II, V, and X, since the PT is sensitive to low levels of these factors. The aPTT mixing study, while typically used to screen for inhibitors to the intrinsic pathway factors and lupus anticoagulant, may also be sensitive to inhibitors of the common pathway. Of note, the aPTT measurement should be performed both immediately after 1:1 mixing and after an incubation of 60 to 120 minutes. This is necessary because in addition to factor VIII inhibitors, up to 15% of lupus anticoagulant may be time dependent.11 Mixing study results do not confirm or exclude lupus anticoagulant. A mixing study should be used only as a screening test to guide the pursuit of factor deficiency versus circulating inhibitor. Mixing study results must be confirmed by direct factor assays or lupus anticoagulant assays. Even though 4:1 mixing and the use of the percentage correction method have been reported to increase the specificity of the mixing study,12,13 confirmatory testing must still be performed. Mixing study results alone should not be used to clear a patient for invasive procedures. For the preceding reasons, utility of the mixing study is limited.14 This may be especially true in pediatric patients, who may not have experienced significant hemostatic challenges (ie, to unmask factor deficiency). Even if the pediatric patient has lupus anticoagulant confirmed, it is safest to perform coagulation factor assays to rule out concurrent factor deficiency.15

WHAT ARE THE CRITERIA FOR LABORATORY TESTING FOR LUPUS ANTICOAGULANT?

There is no gold standard assay for lupus anticoagulant. Detection of lupus anticoagulant is based purely on different laboratory assays. According to the ISTH, the criteria for confirming the presence of lupus anticoagulant are the following15:16,17: (1) prolongation of a phospholipid-dependent clotting assay, (2) evidence of an inhibitor demonstrated by mixing studies, (3) confirmation of the phospholipid-dependent nature of the inhibitor, and (4) lack of specific inhibition of any one coagulation factor.

There are no criteria to define weak-positive lupus anticoagulant results versus strong-positive lupus anticoagulant results since the assays are not quantitative. Additionally, it is not known whether a positive lupus anticoagulant result by more than one confirmatory test poses a higher risk for thrombosis than does a positive lupus anticoagulant result by only one test. Because of the heterogeneity of lupus anticoagulant, some tests are more sensitive than other tests. Therefore, use of at least 2 types of assays for lupus anticoagulant is recommended.15

WHAT TESTS FOR LUPUS ANTICOAGULANT ARE WIDELY USED?

aPTT Sensitive to Lupus Anticoagulant

Some manufacturers offer an aPTT reagent containing a low amount of phospholipid, specifically designed as a screening test for lupus anticoagulant. However, in the setting of acute phase reaction (ie, elevated factor VIII levels), the lupus anticoagulant–sensitive aPTT may not be prolonged even if lupus anticoagulant is present. Therefore, if lupus anticoagulant is clinically suspected, further tests should be performed even if the lupus anticoagulant–sensitive aPTT result is normal. Only 46.4% of 28 patient samples that tested positive for lupus anticoagulant by the hexagonal phase phospholipid neutralization test showed a prolongation by the aPTT–lupus anticoagulant test result (a lupus anticoagulant–sensitive aPTT by Diagnostica Stago, Asnieres, France).14
**Dilute Russell’s Viper Venom Test**

Russell’s viper venom activates factor X in the patient’s plasma. Dilute Russell’s viper venom test (DRVVT) screen reagents contain a low amount of phospholipids. If the screening test is prolonged, a DRVVT mixing study should be performed. If the mixing study does not correct, then a DRVVT confirmatory test should be performed. DRVVT confirm reagent contains a higher amount of phospholipids, intended to neutralize lupus anticoagulant in the specimen. A ratio is derived from the screen clotting time divided by the confirmatory clotting time. If the ratio exceeds the established cutoff, then lupus anticoagulant is confirmed. In some laboratories, DRVVT screen and DRVVT confirmatory are performed simultaneously for convenience. A question may be raised: If the DRVVT screening is not prolonged, but the ratio of the DRVVT screening and confirmatory exceeds the established cutoff, is the result still positive for lupus anticoagulant? This would not satisfy step 1 of the ISTH recommendation because the screening test does not reveal prolongation of a phospholipid-dependent clotting assay. However, in our personal communications with 4 experts in coagulation, the answers were equivocal.

**Hexagonal Phase Phospholipid Neutralization Test**

A patient’s plasma is mixed with buffer (screening test) or hexagonal phase phosphatidyl ethanolamine (confirmatory test) to neutralize any lupus anticoagulant present. Then, the mixtures are incubated with normal plasma to correct any coagulation factor deficiency. The aPTT is measured in both mixtures. If the specimen contains lupus anticoagulant, the aPTT of the confirmatory test will be significantly shorter than that of the screening test. Therefore, this test is an integrated system that combines the aforementioned criteria for lupus anticoagulant steps 1 to 3 into a single test. Since it is based on the aPTT, an elevated factor VIII level as an acute phase reactant may shorten the screening test. A similar question may be raised: If the clotting time of hexagonal phase phospholipid screening test is not prolonged, but the difference between the confirmatory test and the screening test exceeds the established cutoff, is the result still positive for lupus anticoagulant? Again, the answers from experts were not in agreement.

**Platelet Neutralization Procedure**

The platelet neutralization procedure depends on the comparison of the aPTT or lupus anticoagulant–sensitive aPTT of the patient’s platelet-poor plasma to that of the patient’s plasma mixed with a platelet membrane preparation. In this assay, platelet membrane is used as a source of phospholipids. If the specimen contains lupus anticoagulant, then the clotting time may be substantially shortened in the patient’s platelet membrane–containing plasma compared with the patient’s platelet-poor plasma. When the platelet neutralization procedure was compared with the hexagonal phase phospholipid screening test, the former gave lower rates of detection than did the latter.

**Dilute Prothrombin Diagnostic Assay**

A new dilute PT assay (ACTICLOT, American Diagnostics Inc, Stamford, Conn) has recently become available. It uses a relipidated recombinant human tissue factor–based reagent in the presence of calcium to activate the tissue factor pathway. Similar to the DRVVT, the screening test contains a lower amount of phospholipids and the confirmatory test contains a higher amount. The clotting times of the screening assay are compared with those of the confirmatory assay. A ratio is derived from the screen clotting time divided by the confirmatory clotting time. If the ratio exceeds the established cutoff, then lupus anticoagulant is confirmed. This test may give higher sensitivity and specificity if it is combined with other lupus anticoagulant assays.

**WHEN ARE FACTOR ASSAYS NEEDED IN THE CONTEXT OF LUPUS ANTIICOAGULANT TESTING?**

Regardless of the presence of lupus anticoagulant, factor assays should be performed when the patient has bleeding symptoms or history, or when an invasive procedure is required. The antiprothrombin antibody specificity of lupus anticoagulant may result in a deficiency of prothrombin in some patients. Although the antiprothrombin does not neutralize the ability of prothrombin to be cleaved to thrombin, it might accelerate the clearance of prothrombin/antiprothrombin complexes when the antibody titer is high. Again, factor assays are also needed in most pediatric settings because bleeding tendency or symptoms may not yet be manifest as a result of young age.

To perform factor assays, a patient’s plasma is diluted to 1:10 and then mixed with an equal volume of plasma that is deficient in the factor of interest. Then, a PT or aPTT is measured depending on the indicated factor assay. Further dilution of the patient’s plasma to 1:20, 1:40, and 1:80 before mixing with factor-deficient plasma will result in dilution of the lupus anticoagulant. Thus, the lupus anticoagulant will interfere less with the PT or the aPTT assay of the higher dilutions (Table 1). This translates into a pattern of increasing percentage factor activity as the dilutions increase. A similar pattern may be seen in the presence of heparin or direct thrombin inhibitors. Additionally, the presence of a factor-specific inhibitor may falsely decrease the levels of other coagulation factors measured in vitro. For example, a factor VIII inhibitor may cause a false decrease in the measured factor IX, XI, or XII levels. In this case, performance of the factor assays at multiple dilutions may also reveal an inhibitor pattern (Table 2).

**HOW CAN A POSITIVE LUPUS ANTIICOAGULANT RESULT BE CONFIRMED IF THERE IS CONCURRENT EXISTENCE OF A FACTOR-SPECIFIC INHIBITOR?**

Factor V inhibitors are known to mimic lupus anticoagulant. Most patients with factor V inhibitors have anti-factor V antibodies that bind to the phospholipid-binding site of factor V in the second C-type domain of the protein. Therefore, in the presence of a factor V inhibitor, further testing, such as purifying immunoglobulin G, is needed to determine whether the result of the DRVVT or the hexagonal phase phospholipid screening test is a true-positive or a false-positive (Table 3). According to aforementioned criteria 4, there must be a lack of inhibition of any one coagulation factor to confirm the presence of lupus anticoagulant. Therefore, not only in the presence of a factor V inhibitor but also in the presence of a factor VIII or factor IX inhibitor, cautious interpretation is needed if the patient has concurrent lupus anticoagulant. If factor VIII or factor IX levels are low in the setting of positive results on lupus anticoagulant assays, further testing, such...
Table 1. Example of Positive Lupus Anticoagulant Result and a Possible Factor XI Inhibitor*

<table>
<thead>
<tr>
<th>Factor</th>
<th>1:10</th>
<th>1:20</th>
<th>1:40</th>
<th>1:80</th>
<th>1:160</th>
</tr>
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<tbody>
<tr>
<td>Factor VIII</td>
<td>11</td>
<td>16</td>
<td>30</td>
<td>51</td>
<td>79</td>
</tr>
<tr>
<td>Factor IX</td>
<td>5</td>
<td>8</td>
<td>14</td>
<td>41</td>
<td>54</td>
</tr>
<tr>
<td>Factor XI</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
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* Factor XI deficiency cannot be ruled out by this laboratory work-up. Other results were as follows: prothrombin time, 18.7 seconds; activated partial thromboplastin time, 66.4 seconds; 1:1 mixing at 0 minutes 37.7 seconds, and at 90 minutes 56.0 seconds. The dilute Russell’s viper venom test result was negative (screening and confirmatory ratio, 1:20), and the hexagonal phase phospholipid screening test result was positive (+30.3 seconds). The activated partial thromboplastin time 1:1 mixing did not correct into the normal range at 0 minutes or after incubation, suggesting circulating inhibitor. Lupus anticoagulant was confirmed by the hexagonal phase phospholipid screening test. Since this patient had bleeding symptoms, a factor assay was performed. With the specimen dilution, the levels of factors VIII and IX increased because of the inhibitor pattern. The values that hit a plateau were reported, not the average of all the values obtained. However, the factor XI level remained 1% with 4 different dilutions. The extrinsic coagulation factors measured, which are based on the prothrombin time, did not show any inhibitor pattern. Because of the bleeding symptoms, which started 2 years before, the patient underwent plasma exchange by using fresh frozen plasma as replacement fluid before and after a knee surgery 2 times each. However, the patient continued bleeding and, despite the repeated surgery, later died. In conclusion, the patient has a lupus anticoagulant and a possible factor XI inhibitor that caused factor XI deficiency. Demonstration of a low factor XI level by antigenic assay would be helpful to confirm that the factor XI deficiency is with a true factor XI inhibitor and not with an unusual case of selective lupus anticoagulant interference.

Table 2. Example of an Inhibitor Pattern in the Presence of Factor VIII Inhibitor in the Absence of Lupus Anticoagulant*

<table>
<thead>
<tr>
<th>Factor</th>
<th>1:10</th>
<th>1:20</th>
<th>1:40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VIII</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Factor IX</td>
<td>94</td>
<td>105</td>
<td>103</td>
</tr>
<tr>
<td>Factor XI</td>
<td>88</td>
<td>92</td>
<td>99</td>
</tr>
<tr>
<td>Factor XII</td>
<td>107</td>
<td>128</td>
<td>133</td>
</tr>
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</table>

* These findings were obtained in an 11-year-old boy with severe hemophilia A and a factor VIII inhibitor level of 9.9 Bethesda units. A lupus anticoagulant was not detected by either dilute Russell’s viper venom test or hexagonal phase phospholipid screening test. The inhibitor effect is caused by the interference of anti–factor VIII with the factor IX, factor XI, and factor XII assays.

as a chromogenic factor VIII assay (to distinguish whether the low factor VIII is due to a factor VIII inhibitor or a lupus anticoagulant) or an antigenic factor IX assay (to distinguish whether the low factor IX is due to a factor IX inhibitor or a lupus anticoagulant), may provide useful information.

**CAN LUPUS ANTICOAGULANT ASSAY BE PERFORMED CORRECTLY WHEN THE PATIENT IS RECEIVING AN ANTICOAGULANT?**

Accurate testing for lupus anticoagulant is compromised by warfarin. The criteria for positive lupus anticoagulant results using DRVVT or the hexagonal phase phospholipid screening test have not been validated in the presence of warfarin. Some DRVVT and the hexagonal phase phospholipid reagents can neutralize heparin and low-molecular-weight heparin up to a level exceeding the usual therapeutic range. Plasma that contains unfractionated heparin up to 1 U/mL will not interfere with most tests for lupus anticoagulant. However, a specimen with heparin in excess of 1 U/mL may result in a false-positive lupus anticoagulant result. Additionally, depending on the type of low-molecular-weight heparin, there may be interference with the DRVVT regardless of the concentration of heparin in the specimen. Thrombin inhibitors prolong the clotting assays and thus interfere with the DRVVT, but not the aPTT-lupus anticoagulant–based platelet neutralization procedure.

**HOW OFTEN SHOULD THE LUPUS ANTICOAGULANT ASSAY BE REPEATED IF THE RESULT IS POSITIVE?**

Although positive lupus anticoagulant results can be transient after infection, persistently positive lupus anticoagulant results are considered to indicate a high risk for recurrence of venous thrombosis in children and adults. Therefore, testing should be repeated. If the lupus anticoagulant result is repeatedly positive, after 6 weeks, then a diagnosis of antiphospholipid antibody syndrome should be considered, according to the Sapporo criteria. Although these criteria are intended to assemble a uniform patient group for clinical studies from different institutions, they were validated for clinical studies and give some clinical guidance as well. According to the most recent publication of the consensus statement, lupus anticoagulant should be present at 12 weeks from the initial finding in order to classify the condition as antiphospholipid antibody syndrome. The authors of this statement emphasized that the period of 12 weeks is an expert opinion and should be validated in the future. Experts have recommended indefinite anticoagulation in patients with antiphospholipid antibodies and thrombosis. However, there is no consensus as to whether anticoagulant therapy should be continued indefinitely despite the disappearance of lupus anticoagulant.
CONCLUDING THOUGHTS AND REMAINING QUESTIONS

Accurate assessment of lupus anticoagulant is important since it is a risk factor for thrombosis. A positive lupus anticoagulant result may change the length of coagulation for patients who have developed thrombosis. Unfortunately, the heterogeneity of lupus anticoagulant gives rise to the inability to establish a gold standard diagnostic assay; as a result, several different assays are used. The use of different tests requires the interpreter to be knowledgeable of the specific clinical and laboratory interferences and limitations of each assay.

The following is a limited list of questions that remain without definitive answers:

1. If the result of a screening test for lupus anticoagulant is normal but the confirmatory test result is positive, should the results be regarded as true-positive or false-positive?
2. How should lupus anticoagulant assay results be interpreted in the setting of acute phase reaction?
3. Should multiple (ie, >2) different confirmatory tests for lupus anticoagulant be routinely performed?
4. How soon and how often should the lupus anticoagulant assays be repeated when the initial assay result is positive or negative?
5. Are criteria needed to define weak-positive lupus anticoagulant results and strong-positive lupus anticoagulant results? Is a strong-positive lupus anticoagulant result clinically more significant than a weak-positive result?

References