The Use of the Dilute Russell Viper Venom Time for the Diagnosis of Lupus Anticoagulants

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We describe here a test for lupus anticoagulants based on a modified Russell viper venom time (RVVT), using limiting amounts of phospholipid and venom. We have studied 29 patients with a prolonged dilute RVVT. Five of the 29 had a normal activated partial thromboplastin time and three of 14 tested by the tissue thromboplastin inhibition test were normal. In 17 of 19 patients tested, the dilute RVVT was completely normal when ionophore-treated platelets were substituted for phospholipid; the remaining two patients, both with very long phospholipid-dependent dilute RVVT's, were nearly completely normalized. The dilute RVVT is not prolonged in the presence of antibodies to factors VIII, IX, or XI. Thus, the dilute RVVT appears to be a simple, reproducible, sensitive, and relatively specific method for the detection of lupus anticoagulants.

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Lupus anticoagulants are antibodies reactive against anionic phospholipids, thereby prolonging phospholipid-dependent coagulation tests.1-5 Though initially recognized in patients with systemic lupus erythematosus, similar inhibitors have been described in a variety of disorders and, occasionally, in apparently normal individuals.2,6-8 In recent years it has become clear that the presence of a lupus anticoagulant is a risk factor for thrombosis and recurrent spontaneous abortions.9-11 Nevertheless, the prevalence of lupus anticoagulants has been difficult to determine, since criteria for the diagnosis of this coagulation inhibitor have not been clearly established. Suspicion of a lupus anticoagulant is usually aroused by the finding of a prolonged activated partial thromboplastin time (aPTT) that is not corrected by addition of an equal volume of normal plasma. Corroboration is frequently sought by performance of the tissue thromboplastin inhibition test (TTI).12 However, the TTI can be negative in the presence of some lupus anticoagulants13,14 and has been reported to be positive in the presence of some factor VIII or factor IX antibodies, as well as in the presence of heparin.15 In order to avoid some of these problems, we have employed a modified Russell viper venom time, using diluted venom and a limiting concentration of the phospholipid reagent.16 We describe here details of the method, as well as some studies of its sensitivity and specificity compared to the TTI and the aPTT in the diagnosis of lupus anticoagulants.

MATERIALS AND METHODS

Coagulation tests. Blood was collected in one-tenth volume of 3.8% trisodium citrate. Platelet-poor plasma was obtained by centrifugation at 2,500 g for 15 minutes at room temperature. The prothrombin time, activated partial thromboplastin time and thrombin time, using tissue thromboplastin (General Diagnostics, Morris Plains, NJ), Thrombofax (Ortho Diagnostic Systems, Raritan, NJ), and Thrombin (Upjohn, Kalamazoo, Mich), respectively, were performed as described previously.17 The normal ranges for these tests in our laboratory are 12 to 15 seconds, 23 to 36 seconds, and 20 to 26 seconds, respectively. The tissue thromboplastin inhibition test (TTI) was performed according to the method of Schiefer and al.,3 using a 1:100 dilution of tissue thromboplastin. The titers of antibodies to specific coagulation factors were determined by the Bethesda Assay.3 All test results described here were performed in our laboratory.

The dilute Russell viper venom time (RVVT) was performed as described previously.35 Briefly, Russell viper venom (Burroughs Wellcome, Raleigh, NC) was reconstituted as suggested by the manufacturer and further diluted 1:200 in Tris-buffered saline (0.15 mol/L NaCl, 0.02 mol/L Tris, pH 7.5). The phospholipid reagent Thrombofax was diluted 1:8 in Tris-buffered saline. Other partial thromboplastins can be substituted for Thrombofax; however, the dilution of each reagent needs to be determined in the manner described for Thrombofax (see Results). The dilute RVVT was performed by incubating 0.1 mL of plasma, 0.1 mL of diluted Russell viper venom, and 0.1 mL diluted phospholipid for 30 seconds at 37 °C, after which 0.1 mL of 0.03 mol/L calcium chloride was added and the clotting time recorded. In experiments using platelets, 0.1 mL of calcium ionophore-treated platelets was substituted for phospholipid.

Preparation of washed, ionophore-treated platelets. Citrated blood was centrifuged at 150 g for 15 minutes at room temperature. The platelet-rich plasma supernatant was centrifuged at 1,500 g for ten minutes at room temperature and the platelets were washed twice by suspension and centrifugation in a buffer consisting of 0.15 mol/L NaCl, 0.02 mol/L Tris, 0.001 mol/L EDTA, 0.005 mol/L glucose, pH 7.5. The twice-washed platelets were resuspended at a concentration of 8 x 10^8/mL in the same buffer, but without EDTA. Activation was achieved by adding to 2 mL of platelet suspension 1 μL of a 5 mmol/L solution of calcium ionophore A23187 (Calbiochem, San Diego, Calif) in absolute ethanol (giving a final concentration of 2.5 μmol/L, and incubating for five minutes at room temperature. The ionophore-treated platelets were used immediately or frozen in aliquots at −70 °C for future use.

RESULTS

Effect of phospholipid concentration on the RVVT. Russell viper venom was diluted to give a clotting time in normal plasma of approximately 25 seconds. At this dilution, slightly better separation of lupus anticoagulant and normal plasma was achieved than at higher venom concentrations. Since lupus anticoagulants are antibodies with immunologic reactiv
ity towards anionic phospholipids, we wished to increase the test sensitivity further by using the minimal concentration of phospholipid reagent necessary for prothrombin activation. As can be seen in Fig 1, Thrombofax was slightly inhibitory when used undiluted, and was optimal at a dilution of 1:2 to 1:4, when tested in normal plasma. However, optimal separation of lupus anticoagulant from normal plasma was achieved at a Thrombofax dilution of 1:8 or greater. At this phospholipid concentration lupus anticoagulant patients were clearly abnormal, even though some had a normal RVVT with undiluted phospholipid reagent. The coefficient of variation of this test, determined by performing 20 replicates on a single normal plasma, was 0.8%. The normal range, determined on 14 fresh normal plasmas, was 26.2 ± 1.5 sec (± 2 SD). The normal range observed in our laboratory over a 3-year period, using fresh and frozen plasmas, was 25.6 ± 2.6 sec (± 2 SD). We consider clotting times of 30 seconds or greater (>3.8 SD above the mean) to be abnormal, and make a presumptive diagnosis of a lupus anticoagulant when an abnormal test is not corrected by addition of an equal volume of normal plasma. Using this test, we have diagnosed the presence of a lupus anticoagulant in 29 patients referred to us because of the suspicion of such an anticoagulant. Of the 29 patients, only 24 had a prolonged aPTT. Of the remaining five, two patients were on steroid therapy at the time of our study but had a prolonged aPTT previously; one patient had a chlorpromazine-related lupus syndrome with an antinuclear antibody titer of 1:320 and a rapid plasma reagin (RPR) titer of 1:16; the fourth patient had a history of recurrent abortions; and the fifth patient had systemic lupus erythematosus.

Substitution of platelets for phospholipid in the dilute RVVT. The effect of substituting calcium ionophore-activated platelets for the phospholipid reagent was investigated as follows. Ionophore-treated platelets were diluted to a concentration giving an RVVT of 23 to 28 seconds. The treated platelets are stable in suspension at least during the day they are prepared, and can be quick-frozen at least once and stored for 12 months or more at −70 °C without loss of activity. As can be seen in Fig 2, in 17 of 19 cases tested in this manner the RVVT was completely normal when performed with platelets rather than phospholipid. In the other two cases the RVVT was not completely normalized. These two patients had very prolonged dilute RVVTs and, in addition, one of the two was receiving oral anticoagulants. However, in these situations the prolonged test is normalized by the addition of an equal volume of normal plasma. In contrast, addition of an equal volume of normal plasma does not correct the dilute RVVT of lupus anticoagulant plasma. In fact, an occasional lupus anticoagulant plasma shows a further prolongation of the RVVT test on admixture with normal plasma (Fig 3), an effect previously referred to as the “lupus cofactor” phenomenon.

The dilute RVVT in coagulation factor deficiencies. The dilute RVVT is normal in plasma deficient in factors VII, VIII, IX, XI, or XII. Plasmas with factor V or factor X levels below 0.4 U/mL and plasmas from patients receiving oral anticoagulants give a prolonged dilute RVVT. However, in these situations the prolonged test is normalized by the addition of an equal volume of normal plasma (Fig 3). In contrast, addition of an equal volume of normal plasma does not correct the dilute RVVT of lupus anticoagulant plasma. In fact, an occasional lupus anticoagulant plasma shows a further prolongation of the RVVT test on admixture with normal plasma (Fig 3), an effect previously referred to as the “lupus cofactor” phenomenon.

The dilute RVVT in the presence of heparin or antibodies to coagulation factors. The presence of antibodies to factors VIII, IX, or XI does not prolong the dilute RVVT (Table 1).
Table 2. Effect of Factor V Antibody on the Dilute RVVT

<table>
<thead>
<tr>
<th>Factor V Antibody Concentration (Bethesda U/mL)</th>
<th>Dilute RVVT Phospholipid (seconds)</th>
<th>Platelets (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>663.0</td>
<td>68.9</td>
<td>59.2</td>
</tr>
<tr>
<td>66.3</td>
<td>66.9</td>
<td>57.8</td>
</tr>
<tr>
<td>6.63</td>
<td>61.9</td>
<td>56.7</td>
</tr>
<tr>
<td>0.663</td>
<td>38.5</td>
<td>40.1</td>
</tr>
<tr>
<td>0.066</td>
<td>32.3</td>
<td>29.2</td>
</tr>
<tr>
<td>0</td>
<td>25.4</td>
<td>26.9</td>
</tr>
</tbody>
</table>

was also normal in the TTI test. Thus, although the correlation between results of all three tests was good, the dilute RVVT appears to be the most sensitive of the three.

DISCUSSION

A number of different tests have been proposed and used for the diagnosis of lupus anticoagulants, reflecting the difficulty in reaching a consensus concerning their definition and mechanism of action.\textsuperscript{3,8,39,40} It is now clear that lupus anticoagulants have immunologic reactivity towards anionic phospholipids and thereby prolong phospholipid-dependent coagulation tests. Nevertheless, phospholipid-dependent tests may show variable sensitivity and/or specificity toward lupus anticoagulants for several reasons. First, there are no standards for phospholipid reagents used in coagulation tests, and the quantity of phosphatidylserine present in such reagents, for example, has been shown to affect their sensitivity toward lupus anticoagulants.\textsuperscript{41,42} One test\textsuperscript{38} does not utilize any added phospholipid, presumably relying on phospholipid originating from the plasma or the "platelet dust."\textsuperscript{43,44} Second, the physical state of the phospholipid differs in different tests: the aPTT and the RVVT utilize

Fig 4. Effect of heparin on the aPTT and the dilute RVVT of three normal plasmas. Open symbols, aPTT; closed symbols, dilute RVVT.
phospholipid in micellar form, whereas the TTI and the prothrombin time (PT) make use of tissue thromboplastin, in which the phospholipid is protein-bound. Third, the tests vary in their response to deficiencies of clotting factors and to antibodies to clotting factors. For instance, antibodies to factor VIII may prolong the aPTT, but not the RVVT. Finally, there appear to be variations in test sensitivity related to the immunoglobulin class of the lupus anticoagulant; for example, as we have shown, some IgM anticoagulants do not prolong the TTI, although they do prolong other tests. As a result, the incidence of lupus anticoagulants has not been clearly established, since patients may be positive in one test and not another.

Strategies to increase the sensitivity of tests have taken two forms. Since lupus anticoagulant activity is less evident in the presence of platelets, the use of platelet-free plasma has been advocated. Second, as in our procedure, several tests make use of limiting concentrations of phospholipids to increase their sensitivity. We have chosen the RVVT as our primary test for the diagnosis of lupus anticoagulants, since it is unaffected by the presence of antibodies to factors VIII, IX, or XI, and have further increased the sensitivity of the test by using limiting concentrations of both phospholipid and venom. This test is easy to perform and highly reproducible. Nevertheless, it is not entirely specific for lupus anticoagulants. Coagulation factor deficiencies can prolong the dilute RVVT, but these can be easily excluded by repeating the test on a mixture of normal and patient plasma. The presence of an antibody to factor V, an extremely rare event, also prolongs this test. However, in one factor V antibody patient whom we had the opportunity to study, substitution of ionophore-treated platelets for the phospholipid reagent did not correct the test, unlike the findings with lupus anticoagulants. Furthermore, the presence of a factor V antibody is associated with a marked prolongation of the PT, a rare occurrence with lupus anticoagulants. Nevertheless, definitive exclusion of a factor V antibody requires specific measurement of factor V inhibition in a mixture of patient and normal plasma.

Finally, therapeutic concentrations of heparin prolong the dilute RVVT, although to a somewhat lesser extent than the aPTT (Fig 4). Heparin effects can be ruled out by measurement of the thrombin time, since this test is normal in the presence of a lupus anticoagulant.

The significance of the presence of a lupus anticoagulant does not lie in its association with bleeding, which rarely, if ever, occurs as a result of the lupus anticoagulant alone, but rather in the fact that approximately 25% to 30% of patients with this antibody have a history of thromboembolic phenomena, or repeated spontaneous abortions. The presence of anticardiolipin antibodies, as measured by any one of several immunologic techniques, has also been identified as a risk factor for thrombosis. However, the relationship between these two measurements has not been evaluated adequately. It is clear, for example, that some types of "anticardiolipin" antibodies are not associated with an increased thrombotic risk. For example, positive serologic tests in patients with syphilis are due to reactivity of patient serum with cardiolipin, yet patients with syphilis do not have an increased prevalence of lupus anticoagulants or of thromboembolic disease.

For an adequate understanding of the biologic role of antibodies reactive with anionic phospholipids, it will be necessary to standardize both types of tests. The dilute RVVT which we describe here is simple and highly useful as a screening diagnostic test for further studies.

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