Congenital Resistance to Activated Protein C in Patients with Lupus Anticoagulants: Evaluation of Two Functional Assays

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Summary

The R506Q mutation ("Factor V Leiden") is responsible for the resistance to activated Protein C (aPCR), that is evaluated by coagulation tests. Such tests cannot be used in patients with lupus anticoagulants (LAs), due to the interfering effect exerted by these antibodies on "in vitro" phospholipid-dependent coagulation tests. For this reason, assays have been developed to evaluate aPCR that are insensitive to the presence of LA antibodies. We evaluated two such coagulation tests in the plasma of 82 consecutive patients with LAs. By polymerase chain reaction 3 patients (3.6%) were found heterozygous for the R506Q mutation. aPCR was evaluated by two clotting assays, proposed to be "insensitive" to the presence of LAs: 1. aPCR-tissue factor-based assay, using Factor V deficient plasma and 1:40 diluted test plasma; 2. aPCR-dRVVT-based assay with highly concentrated phospholipids. Their interassay coefficient of variation was 28% and 6.2%, respectively. Compared to the polymerase chain reaction analysis, the 2 tests displayed the following characteristics: sensitivity 67% vs 100%, specificity 92% vs 96%, positive predictive value 25% vs 50%, negative predictive value 99% vs 100%, respectively. Among LA patients without the R506Q mutation, 5 scored positive in the aPCR-tissue factor-based assay, 2 in the aPCR-dRVVT-based assay and another one in both assays. Our findings suggest that the aPCR-dRVVT-based test is more reliable and sensitive than the aPCR-tissue factor-based one to the R506Q mutation in patients with LAs. Both assays, when negative, make unlikely the presence of the R506Q mutation. Polymerase chain reaction analysis remains, however, to be performed when either test is positive.

Introduction

Resistance to activated protein C (aPCR) is defined as a poor anticoagulant response to the addition of activated protein C to plasma in phospholipid-dependent coagulation tests (1). It is commonly due to the mutation of Arg 506 to Gln in the Factor V gene (FV R506Q, the "Factor V Leiden") (2): the mutated molecule is normally activated by thrombin, but is resistant to inactivation by activated protein C (3). Factor V Leiden represents the most common hereditary risk factor for venous, though not arterial, thrombosis (4). Its prevalence in patients with familial venous thromboembolic history is 15-20% (5). Up to 15% of the general population from caucasian origin is a carrier of the FV Leiden (6).

Lupus anticoagulants (LAs) are acquired inhibitors of coagulation that belong, together with anticardiolipin antibodies, to the family of antiphospholipid antibodies (7). Since they affect the coagulation times of the phospholipid-dependent coagulation tests, the assays commonly used to evaluate aPCR in plasma do not give reliable results in the presence of these inhibitors. This effect may be overcome by prediluting the test plasma in Factor V-deficient plasma or by increasing the concentration of the phospholipid reagent. Several assays based on either principle have been reported to give reliable results in LA-positive patients (8-13). The number of cases analyzed by each of these studies was, however, too small to draw definite conclusions about the performance of these "insensitive" assays. Aim of the present study was to evaluate the sensitivity and specificity of two assays based on either principle in a large group of patients with LAs identified according to their FV Leiden status.

Patients, Materials and Methods

Patients

The study enrolled 82 consecutive patients with LAs regularly seen at the Division of Hematology of the Ospedali Riuniti of Bergamo and at the Hemophilia and Thrombosis Center "A. Bianchi Bonomi", IRCCS, Maggiore Hospital and University of Milano, Italy. They were 27 males and 55 females, aged 13-70 years. Fifteen of them suffered from autoimmune diseases: Systemic Lupus Erythematosus (SLE)/SLE-like disease (n = 9), Basedow’s disease (n = 2), autoimmune hemolytic anemia (n = 2), Sjogren’s syndrome (n = 1), Addison’s disease (n = 1). One patient suffered from non-Hodgkin’s lymphoma and another one of breast cancer. Eight patients (10%) were thrombocytopenic (platelet count <150 × 109/l) at the time of the study. History of thrombosis was positive in 50 patients (62%): 33 of them suffered from at least one episode of deep vein thrombosis and/or pulmonary embolism, 12 cases from arterial thrombosis only, whereas the remaining 5 patients experienced both venous and arterial thrombosis. Twelve patients (22%) experienced at least 2 spontaneous miscarriages. Twenty-five patients (30%) were on oral anticoagulation at the time of the study.

Diagnosis of LAs

Venous blood was collected in plastic tubes containing 1/10th volume of 3.8% sodium citrate and centrifuged at 2,500 g for 20 min to obtain platelet-poor plasma. Plasma was divided in small aliquots and stored at –70 °C until use. The revised criteria proposed by the SSC Subcommittee for Standardization of Lupus Anticoagulants were used for the diagnosis of the phospholipid-dependent inhibitors of coagulation (14). In particular, the following assays were used: activated partial thromboplastin time (aPTT), Thrombofax, Ortho, Raritan, NJ), performed according to the manufacturers’s instructions; KCT, carried out according to Exner and coworkers (15); dRVVT, performed according to Thiaagarajan and coworkers (16). Each test was performed in duplicate on a manual coagulometer (Clot-Timer 202A, Mechrolab, Heller Laboratories,
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Santa Rosa, Ca) and the mean values of the two determinations were used. Ratios were calculated by dividing the clotting time of patient’s plasma by that of normal pooled plasma, obtained from 20 normal, apparently healthy, subjects: values exceeding 1.2 were considered abnormal. In this case, the assays were repeated in the 1:1 mixture of patient’s with normal pooled plasma. The phospholipid-dependent nature of the inhibitor was confirmed by the shortening of the clotting time of at least one of the abnormal tests performed in the presence of an excess of exogenous phospholipids (14). In case of oral anticoagulation, the Textratin/Ecarin test (17) was also used. At the time of the study all patients’ plasma displayed LA activity.

Measurement of Anticardiolipin and Antiprothrombin Antibodies

IgG and IgM anticardiolipin antibodies were measured essentially according to the ELISA procedure described by Loizou and coworkers (18). At the time of the study increased levels of IgG and/or IgM anticardiolipin antibodies were found in 74 patients (90%). IgG and IgM antiprothrombin antibodies were measured as previously described (19). At the time of the study increased levels of IgG and/or IgM antiprothrombin antibodies were found in 73 patients (89%).

Assessment of aPCR

The aPCR-tissue factor-based test was performed essentially according to Le et al. (8). Briefly, sample plasma was 1:40 diluted in tris-buffered saline (pH 7.4) containing 0.1% bovine serum albumin (TBS/BSA) (Sigma, St. Louis, MO). Forty μl of this mixture were incubated with 40 μl of FV-deficient plasma (prepared by immunoadsorption methods from normal plasma; Diagnostica Stago, Asnieres, France) and 40 μl of brain rabbit thromboplastin (Sigma) for 3 min at 37°C. Subsequently, 40 μl of 30 mM CaCl2 containing either 2.5 μg/ml aPC (Enzyme Research Laboratories, South Bend, IN) or TBS/BSA were added and clotting time recorded. Results were expressed as the difference of the coagulation time in the presence of exogenous aPC minus that obtained in its absence: values below 11 s were considered abnormal. This cut-off value was obtained by performing the assay in the plasma of 20 normal control subjects. Since approximately 30% of the patients were on oral anticoagulant treatment at the time of the study, we checked the effect of this therapy by performing the test in the plasmas of 12 orally anticoagulated patients without LAs: those without the mutation (n = 6) gave differences above 11 s, whereas the carriers of the R506Q mutation (n = 6) gave differences below 11 s in all cases.

The aPCR-dRVVT-based assay (PCI test kit, kindly provided by Dr. T. Exner, Gradipore, Sidney, Australia) was performed according to the manufacturer’s instructions. Ratios were obtained by dividing the coagulation time in the presence of aPC by that obtained in its absence. Results were expressed as normalized ratio, which was calculated by dividing the ratio of patient’s plasma with that of normal pooled plasma. Values below 0.80 were considered abnormal. This cut-off value was obtained by performing the assay in the plasma of 20 normal control subjects. In case of oral anticoagulant treatment this test was performed in the 1:1 mixture of patient’s with normal pooled plasma. Under these conditions, the assay was repeated in the plasma of 12 orally anticoagulated patients without LAs: those without the mutation (n = 6) gave normal ratios, whereas the carriers of the R506Q mutation (n = 6) gave ratios below 0.8 in all cases.

Assays were performed by the mechanical coagulometer Clot-Timer 202A (Mechrolab).

Assessment of the R506Q (“Factor V Leiden”) Mutation

Genomic DNA was prepared from citrated blood by standard procedures. Mutant Factor V was detected by amplification of the Factor V gene by polymerase chain reaction and digestion of the fragment with MnlI (2). Three out of the 82 patients (3.6%) were found heterozygous.

Statistical Analysis

Specificity, sensitivity, positive and negative predictive values were calculated by means of 2 × 2 tables (see Table 1a and b). The inter-assay coefficient of variation was calculated by dividing the standard deviation by the mean of the results of normal pooled plasma obtained in 10 separate sessions of experiments.

Results

aPCR was analyzed in the plasma of 82 consecutive patients with LAs by means of 2 tests that are considered relatively “insensitive” to the presence of phospholipid-dependent inhibitors of coagulation. Their comparison with the polymerase chain reaction for the R506Q mutation are shown in Table 1a and 1b. In particular, the aPCR-tissue factor-based test identified 2 out of the 3 heterozygous patients, whereas the aPCR-dRVVT-based assay scored positive in all the 3 cases. Among LA patients without the R506Q mutation, 5 scored positive in the former assay, 2 in the latter test and another one in both assays. In these 8 patients no correlations were found between the abnormality of either test and the levels of anticardiolipin or antiprothrombin antibodies, or the degree of prolongation of the coagulation tests used to detect LA (data not shown).

The performance of the 2 assays compared to the results of the polymerase chain reaction analysis for the R506Q mutation is reported in Table 2. The 2 assays differed in terms of inter-assay coefficient of variation and sensitivity; conversely, they had similarly good specificities and negative predictive values, and displayed poor positive predictive values.

The results of the functional assays were also analyzed according to the history of deep vein thrombosis and/or pulmonary embolism suffered from the patients in the study (38 out of 82, 46%). The prevalence was 100% in the heterozygous carriers of the R506Q mutation (3 out of 3 patients), 45% in the group of patients with both normal functional assays (32 out of 71 cases) and 37% among those with either functional test abnormal (3 out of 8 patients).

Table 1 Comparison of two “insensitive” assays for aPC resistance with the R506Q mutation in 82 patients with LAs

<table>
<thead>
<tr>
<th>R506Q mutation</th>
<th>Tissue factor-based assay</th>
<th>dRVVT-based assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterozygous</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Normal</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 2 Performance of two assays for aPCR evaluation in 82 patients with LAs

<table>
<thead>
<tr>
<th>aPCR Test</th>
<th>Inter-assay CV (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Predictive value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tissue factor-based test</td>
<td>28</td>
<td>67</td>
<td>92</td>
<td>99</td>
</tr>
<tr>
<td>dRVVT-based test</td>
<td>6</td>
<td>100</td>
<td>96</td>
<td>100</td>
</tr>
</tbody>
</table>
Discussion

The choice of the proper laboratory methodology is crucial to the understanding of congenital aPCR in LA-positive plasmas, due to the “in vitro” interference of these inhibitors with the phospholipid-dependent coagulation tests commonly used to evaluate aPCR. In the present paper, we studied the performance of 2 assays reported to be “insensitive” to the effect of LAs in a number of patients with phospholipid-dependent inhibitors of coagulation large enough to establish their sensitivity, specificity, positive and negative predictive value in comparison with the results of the polymerase chain reaction for the R506Q mutation. The aPCR-tissue factor-based test was basically a prothrombin time, carried out with test sample highly diluted in FV deficient plasma, whereas the aPCR-dRVVT-based one was a dRVVT performed with concentrated phospholipids: their specificity and negative predictive values were high, even though only the latter test was 100% sensitive. The lower sensitivity of the aPCR-tissue factor-based test must be viewed in the light of the small number of patients carrying the R506Q mutation, whose prevalence (3.6%) is similar to that reported for the Italian general population (2.4%) (20). The aPCR-tissue factor-based test showed a higher inter-assay coefficient of variation than the aPCR-dRVVT-based assay: the use of different batches of FV deficient plasma – as occurred in the present study – could explain, at least in part, this finding. Moreover, the FV-deficient plasma used in this study has been reported to contain low levels of protein S (21): this also must be taken into account when interpreting the results. The different performance of the 2 assays may also be due to differences in terms of concentration as well as composition of the phospholipid reagent mixtures, which may greatly influence their sensitivity to the presence of LA inhibitors (13). Finally, each individual LA antibody has peculiar affinity and avidity properties that may also affect the results of the assay.

The positive predictive value of both assays was remarkably low, because they tested positive in 8 plasma samples without the R506Q mutation. Several reasons may account for this finding. Firstly, one cannot exclude the possibility that, due to the known polymorphism of FV molecule (21), mutations other than the R506Q could be revealed in these 8 cases by one of the two assays. Another possibility to take into account is the interference of oral anticoagulant treatment on these assays, since approximately 30% of LA-positive patients enrolled in this study were under warfarin at the time of the study. In this respect, the effect of oral anticoagulation was ruled out, since in patients receiving warfarin the aPCR-dRVVT-based assay was performed in the 1:1 mixture of test plasma with normal pooled plasma, whereas in the other test vitamin K-dependent coagulation factors were provided by the FV deficient plasma. Under these conditions, we had demonstrated that oral anticoagulation did not affect the results of the 2 assays (see Methods). Furthermore, the abnormality of either test in these 8 plasmas could be due to acquired aPCR. Also this possibility seems unlikely, because the prevalence of acquired aPCR in the present series of patients (10%) appears too low when compared to that recently reported in a large group of LA-positive patients (approximately 60%) (22), who were studied by means of a relatively complex two-step coagulation system that allowed LA inhibitors to express their interference with the anticoagulant activity of endogenously activated protein C (23). Conversely, “insensitive” aPCR assays are specifically designed to overcome the presence of phospholipid-dependent inhibitors of coagulation, and are, in general, unsuitable to study acquired aPCR in LA-positive plasmas. This idea is indirectly suggested also by the lack of correlation between the prevalence of thrombosis in patients without the R506Q mutation and the results of the functional assays for aPCR. We neither can exclude the possibility that this overcoming effect is not totally reached in a minority of cases. In this respect, 2 groups of investigators recently reported a correlation between the abnormality of functional tests to detect aPCR and the levels of anticardiolipin or anti-prothrombin antibodies (25), or the degree of prolongation of the coagulation tests used to identify LA (26). At variance with these data, we failed to find such a correlation in the 8 patients displaying aPCR in the absence of the R506Q mutation.

In conclusion, the aPCR-dRVVT based test is more sensitive and more reproducible than the aPCR-tissue factor-based one to the R506Q mutation in patients with LAs. Both assays, when negative, make very unlikely the presence of this mutation. Since these assays scored positive in about 10% of the cases without the R506Q mutation, polymerase chain reaction analysis remains to be performed when either test is positive.

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References


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