RAPID COMMUNICATION

Use of a Generally Applicable Tissue Factor–Dependent Factor V Assay to Detect Activated Protein C–Resistant Factor Va in Patients Receiving Warfarin and in Patients With a Lupus Anticoagulant

By Dzung The Le, John H. Griffin, Judith S. Greengard, Vibhas Mujumdar, and Samuel I. Rapaport

The original activated partial thromboplastin time-based assay for activated protein C (APC)-resistant factor Va (FVα) requires carefully prepared fresh plasma and cannot be used in patients receiving warfarin or in patients with antiphospholipid antibodies. A new test is described here that circumvents these limitations and distinguishes without overlap heterozygotes for APC-resistant FVα from persons with normal FV. A diluted test plasma is incubated with an FV-deficient substrate plasma and tissue factor and then clotted with Ca2+ or Ca2+ plus APC. Test results are independent of the FV level or the dilution of the test plasma used. Of 39 controls, 37 gave normal results. Two controls (5%) gave results indicative of APC resistant FVα and on DNA analysis were found to be heterozygous for FV R506Q. Twenty of 21 randomly selected patients receiving warfarin gave normal results. In the single patient with abnormal results, heterozygous FV R506Q was confirmed by DNA analysis. Two of 15 patients with protein S deficiency and 5 of 29 patients with a lupus anticoagulant had abnormal results. APC resistance caused by FV R506Q was confirmed in the five of these seven patients available for DNA analysis. APC-resistant FVα was also detected in 10 of 21 (48%) stored plasma from unrelated patients with venous thrombosis and negative earlier evaluation for a lupus anticoagulant or a deficiency of protein C, protein S, or antithrombin, which confirms a high incidence of this defect among patients with venous thrombosis.

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IN 1993, Dahllbeck et al. described a previously unrecognized mechanism for familial thromboembolic disease characterized by a failure of activated protein C (APC) to prolong appropriately the activated partial thromboplastin time (APTT) of plasma. Inadequate APC-induced prolongation of the APTT was subsequently found in remarkably high prevalence in cohorts of patients with thromboembolic disease and family studies established its autosomal dominant mode of transmission. Soon thereafter, Dahllbeck and Hildebrandt reported that APC resistance stemmed from an abnormality of the factor V (FV) molecule involving its role as an APC cofactor.

Bertina et al., Greengard et al., and Voorberg et al. first identified the molecular basis for the abnormality. The phenotype of APC resistance was shown to be associated with heterozygosity or homozygosity for a single point mutation in the FV gene that resulted in the substitution of arginine at amino acid residue S06 with glutamine (FV R506Q). This R506Q mutation prevents APC from cleaving a peptide bond at Arg-506 that is required to inactivate factor Va (FVα). Because APC-resistant FVα appears to be the most prevalent cause of inherited thrombophilia, a reliable, rapid coagulation test that can be used in a variety of circumstances is needed to evaluate a patient with a personal or family history of thrombosis. To date, detection of APC resistance has been based on obtaining the ratio of the clotting time of the APTT test when plasma is clotted with calcium ions plus APC to the clotting time of the APTT test when plasma is clotted with calcium ions alone (APC ratio). The test is considered positive if the APC ratio of a test sample falls below the range established for a control population.

With increasing use of this test it became apparent that careful standardization was of utmost importance to obtain consistent and reproducible results. For example, it is reported that activation of platelets during the preparation of platelet-poor plasma and the use of frozen and thawed plasma can alter test results. Moreover, the test cannot be used on plasma from two important groups of patients: patients taking oral anticoagulants and patients with a lupus anticoagulant.

We describe here a new coagulation test for detecting APC-resistant FVα that is based on a one-stage tissue factor–dependent FV assay. The test requires no special handling of plasma and can be performed reliably on frozen and thawed plasma. Data are provided on its use in several groups of patients, including patients receiving warfarin and patients with a lupus anticoagulant.

MATERIALS AND METHODS

Test plasma samples. Four and one-half milliliters of venous blood was collected into a siliconized BD Vacutainer tube (Becton Dickinson, Rutherford, NJ) containing 0.5 mL of 3.2% sodium citrate. Platelet-poor plasma was prepared by centrifugation at 3,000 rpm for 15 minutes at room temperature. Plasma samples were kept frozen in capped plastic tubes at -70°C to -80°C until tested. Plasma was obtained from the following sources: (1) 39 asymptomatic volunteers from the staff of the Laboratory Service of UCSD Medical Center and the staff of a research laboratory; (2) 21 randomly selected patients receiving oral anticoagulant therapy with warfarin; (3) 29 patients with evidence of plasma lupus anticoagulant activity as determined from a combination of (a) a prolonged APTT that failed to correct on a 1:1 mix with normal plasma, (b) increasing apparent coagulant activity with increasing dilution of the plasma in assays for factors VIII, IX, XI, and XII, and (c) an abnormal dilute Russell's viper venom test; (4) 15 patients with known free
protein S deficiency; and (5) 21 patients with history of thrombosis and no evidence for a lupus anticoagulant or deficiency in antithrombin, protein C, or protein S. The plasma samples had been stored frozen for periods varying from several days to several years.

### Pooled normal reference plasma

This plasma was prepared by pooling plasma obtained from venous blood collected in one-tenth volume of a buffered citrate anticoagulant (0.06 mol/L sodium citrate plus 0.04 mol/L citric acid) from between 15 and 20 healthy donors. It was stored in small aliquots at −70°C. The prothrombin time and APTT of the plasma from each donor was within 2 standard deviations of the mean of the individual plasma samples from the donors used for the routine control pooled reference plasma of the Special Coagulation Laboratory of the UCSD Medical Center.

The tissue factor–dependent FV assay for APC resistance. The assay is based on measuring the clotting time of a diluted test plasma sample in a one-stage FV assay that is activated with tissue factor and clotted with CaCl₂ alone and with CaCl₂ plus APC. Test plasmas were diluted 1/20, 1/40, and 1/80 in Tris-buffered saline, pH 7.5, containing 0.1% bovine serum albumin (TBS/BSA) and kept on ice. For each plasma dilution, four clotting cuvettes were prepared, each containing 40 μL of the diluted sample, 40 μL of reconstituted rabbit brain thromboplastin (Sigma Diagnostic, St Louis, MO), and 40 μL of reconstituted human immunoadsorbed FV-deficient plasma (Baxter, Miami, FL). After incubation for 3 minutes at 37°C, clotting was initiated in two cuvettes with 40 μL of 30 mmol/L CaCl₂ in TBS/BSA that had been warmed to 37°C and in two cuvettes with 40 μL of warmed CaCl₂ containing 2.5 μg/mL APC (Enzyme Research Laboratories, South Bend, IN). Clotting times were determined with an ST-4 semi-automated coagulometer (Diagnostica Stago, Parsippany, NJ) and recorded as the average of the duplicate samples. For each test sample, the assay was performed initially at a 1/40 dilution, and then at a second dilution, either 1/20 or 1/80, as decided by the clotting time of the 1/40 dilution. When abnormal results were obtained, the test was performed at all three dilutions. On each day the assay was performed, reference curves for each assay condition were prepared as log/log plots of clotting times against 1/10 to 1/80 dilutions in TBS/BSA of the pooled normal reference plasma (see Results).

Detection of FV R506Q mutation by DNA analysis. A lymphocyte-rich layer was obtained from 10 mL of venous blood collected in acid citrate dextrose by standard Hypaque gradient technique and stored as a cell pellet at −20°C until used. Genomic DNA was extracted from the cell pellet with the DNA Extraction Kit (Biosynthesis, Lewisville, TX) according to the manufacturer’s instructions.

The 267-bp fragment of FV containing the coding sequence for the amino acid Arg-506 was obtained by a modification of the polymerase chain reaction (PCR) method of Bertina et al.1 The 100-μL PCR reaction mixture contained ~1 μg DNA, 20 pmol of each of the two primers, PR-6967 and PR-99020, and 20 μL of reconstituted rabbit brain thromboplastin (Sigma Diagnostic, St Louis, MO) and 20 μL of reconstituted human immunoadsorbed FV-deficient plasma (Biolabs, Beverly, MA) according to the manufacturer’s instructions. The whole digest was then subjected to electrophoresis on a 2% agarose gel.6

### RESULTS

#### Standardization of the analysis of test results.

Figure 1A provides an example of the reference curves obtained for the tissue factor–dependent FV assay (see Materials and Methods). As seen in this primary plot, the slope of the reference curve for the assay performed with APC was greater than the slope of the assay performed with CaCl₂ alone. Therefore, the log of the difference in clotting times between the FV assay with and without APC was plotted against the log of dilutions of pooled normal reference plasma. Figure 1B provides an example of this secondary plot, which illustrates that the difference in clotting times increases from about 5 seconds to about 20 seconds as the dilution of the pooled normal reference plasma increases, i.e., as the concentration of FV in the test plasma decreases.

Therefore, the following procedure was adopted to obtain a test result independent of both the basal level of FV in the test plasma and the dilution of the test plasma used in the assay:

1. The clotting time of a dilution of a test plasma in the factor V assay without APC, denoted as Y on the y-axis of Fig 1A, was located on the reference curve of that assay [step (a) of Fig 1A] and converted into an equivalent dilution of pooled normal reference plasma, denoted as X on the abscissa of Fig 1A [step (b) of Fig 1A].

2. The value for this equivalent dilution of pooled normal reference plasma was then located on the abscissa of the secondary plot (denoted as X on Fig 1B). This was then used [step (c) of Fig 1B] to delineate the clotting time difference caused by APC that would be expected for this dilution of a normal test sample [eg, (Δ) of Fig 1B]. In a heterozygote for APC-resistant FVα, this difference should be unequivocally shorter than expected for normal plasma [eg, (■) of Fig 1B].

A single batch of APC, which was stored in small aliquots at −80°C and thawed only once, was used to obtain the data presented here. The pooled normal plasma reference curves, prepared each day the assay was performed, yielded very similar primary and secondary plots. Therefore, for clarity of presentation of data, the clotting times obtained for primary and secondary plots on 10 different days were used to construct mean primary and secondary plots by linear regression. The results obtained with test samples on different days were analyzed in relation to these mean primary and secondary plots. In no instance did the use of these mean plots lead to a different interpretation of a test result than that derived from the use of same-day reference plots.

**Test results from control subjects.** The clotting time differences obtained on performing FV assays with and without APC on two or three dilutions of each of the plasma samples obtained from 39 control subjects are illustrated in Fig 2A. The clotting time differences obtained for 37 of the 39 control subjects are closely distributed around the regression line of the mean secondary plot. In contrast, plasma from two individuals yielded markedly shorter clotting time differ-
ences than expected for normal plasma [Fig 2A, (■) and (○)]. Both of these individuals are in their thirties with no history of health problems. Subsequent DNA analysis confirmed that both of these subjects are heterozygotes for the FV R506Q mutation (Fig 2B). As can be seen in Fig 2B, Mnl I digestion of the normal DNA fragment yields a single
Table 1. Clotting Time Differences Obtained in the FV Assay for Two Homozygotes for APC-Resistant FV

<table>
<thead>
<tr>
<th>Clotting Time Difference(s)*</th>
<th>1/20</th>
<th>1/40</th>
<th>1/80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal plasma</td>
<td>+7.0</td>
<td>-11.0</td>
<td>+19.5</td>
</tr>
<tr>
<td>Homozygote 1</td>
<td>-2.5</td>
<td>-2.5</td>
<td>-2.0</td>
</tr>
<tr>
<td>Homozygote 2</td>
<td>-3.0</td>
<td>-2.5</td>
<td>-0.5</td>
</tr>
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* The clotting time in the FV assay obtained without APC was subtracted from the clotting time in the FV assay obtained with APC.

band of 163 bp (lane 3), whereas Mnl I digestion of the DNA fragment from a FV R506Q heterozygote yields an additional band of 200 bp resulting from the loss of an Mnl I restriction site in the mutant FV molecule (lanes 1 and 2).

**Effect of heparin on test results.** Heparin, in a final concentration of 0.5 U/mL, was added to plasma from two of the normal control subjects and to plasma from the two above-described heterozygotes for the FV R506Q mutation. Clotting time differences at a 1/40 dilution of plasma from the two normal subjects were as follows: for plasma not containing heparin, 13.6 seconds and 14.5 seconds; for plasma containing heparin, 14.0 seconds and 16.0 seconds. The corresponding values for the two heterozygote subjects were 6.6 seconds and 8.3 seconds, and 7.9 seconds and 7.4 seconds, respectively. Thus, this plasma concentration of exogenous heparin, which exceeds the plasma level recom-

**Effect of multiple cycles of freeze thawing on test results.** On one occasion fresh plasma from a normal control subject and plasma from a heterozygote for APC-resistant FV discovered in our study of normal subjects (see Fig 2) were tested before and after five cycles of freeze thawing. Clotting time differences at a 1/40 dilution were as follows: for the normal subject, 29 seconds before freeze thawing and 32 seconds after freeze thawing; for the heterozygote, 11 seconds before freeze thawing and 11 seconds after freeze thawing. The longer clotting time differences obtained in this experiment for both the normal subject and the heterozygote subject than in the experiment with heparin cited above stemmed from the use of a different FV-deficient substrate plasma (see Discussion).

**Test results from patients treated with warfarin.** The clotting time differences obtained in the tissue factor–dependent FV assay for APC resistance with dilutions of plasma from 21 randomly selected patients receiving warfarin are shown in Fig 3. In 20 of the 21 patients, these values were closely distributed around the regression line of the mean secondary plot. Dilutions of plasma from a single patient, who had experienced several episodes of deep vein thrombosis, yielded much shorter clotting time differences than expected for normal plasma. Subsequent DNA analysis of a blood sample from this patient showed heterozygosity for the FV R506Q mutation (data not shown).

**Test results from patients with protein S deficiency.** Stored plasma samples were available from 15 patients who had been found to have a deficiency of free protein S antigen by the Special Coagulation Laboratory at UCSD Medical Center. Plasma from 13 of these patients gave normal test results, whereas plasma from two patients yielded markedly shorter clotting time differences than expected for normal
plasma (Fig 4). In one of these patients, warfarin had been temporarily discontinued at the time the first sample was obtained and had been started again at the time a second sample was obtained. The data from both samples are shown in Fig 4 and provide further evidence that therapy with warfarin does not affect the tissue factor-dependent FV assay for APC resistance. Subsequent DNA analysis in this patient confirmed heterozygosity for the FV R506Q mutation (data not shown).

Test results from patients with a lupus anticoagulant. Twenty-nine patients with plasma lupus anticoagulant activity were tested for APC-resistant FVAs (Fig 5). In plasma from 24 patients the clotting time differences were similar to those expected for normal plasma. Twelve of these 24 patients had a history of thrombosis. In plasma from five patients, the clotting time differences were markedly shorter than those expected for normal plasma. Four of these patients had a history of thrombosis, and at least two were receiving oral anticoagulant therapy with warfarin when tested. The patient without a history of thrombosis was over 90 years old when the discovery of a lupus anticoagulant on a routine preoperative evaluation led us to test the plasma for APC resistance. Blood samples were available for DNA analysis from this patient and from two of the APC-resistant patients with a history of thrombosis. All three patients were found to be heterozygotes for the FV R506Q mutation (data not shown).

DISCUSSION

Hereditary deficiency of protein C, protein S, or antithrombin have been reported to account collectively for about 5% to 20% of younger adult patients with venous thromboembolic disease. In contrast, a poor anticoagulant response to APC, a newly discovered cause for hereditary thrombophilia, was identified in 33% of 104 patients with venous thrombotic disease in Sweden, 21% of 301 patients with venous thrombotic disease in The Netherlands, over 50% of 25 selected venous thrombotic patients in the United States, and 20% of 30 patients with juvenile or recurrent stroke in Austria. Moreover, a prevalence of between 2% and 7% of APC resistance was reported in control populations studied in Sweden, The Netherlands, and Austria.

The test used in the above studies to detect APC resistance requires meticulous preparation of the plasma sample and may be unreliable if samples have been frozen and thawed. Moreover, it cannot be used in two important patient populations: patients already receiving oral anticoagulant therapy because of a thrombosis and patients who had a history of significant thrombosis and earlier negative tests for a lupus anticoagulant or a deficiency of protein C, protein S, or antithrombin. In 10 of these plasmas, markedly shorter clotting time differences were found in response to APC than that expected for normal plasma (Fig 6). Four of these patients were available for DNA analysis and all four were found to be heterozygotes for the FV R506Q mutation (data not shown).
The response to APC was consistent with APC resistant Ne. Heterozygous APC did not differ from normal; no earlier identified cause for an increased risk of thrombosis. Secondary plot of results from: and no earlier identified cause for an increased risk of thrombosis. This is illustrated by comparing the data of the experiment with heparin and the experiment with multiple cycles of freeze thawing (see Results), which were performed with different batches of substrate. Therefore, in standardizing the assay it is useful to obtain a large quantity of a single batch of FV-deficient substrate.

A batch of commercially obtained APC should be tested to select an optimum concentration yielding differences in clotting times of dilutions of a pooled normal reference plasma similar to those illustrated in Fig 1, A and B. Small aliquots of a stock solution of APC can be stored frozen, but should be thawed only once and diluted to proper concentration just before use. Small aliquots of plasma from a heterozygote for FV R506Q can be stored and used as a positive control.

The data presented in Figs 2 through 6 are summarized in Fig 7. No overlap is seen between over 100 samples yielding normal results and 21 samples from heterozygotes for APC-resistant FVa. Under our conditions, which yielded

With the discovery that APC resistance stems from an abnormality of FVa preventing the inactivation of FVa by APC, it was possible to design a test based on the difference in clotting times of a diluted test sample in a tissue factor–dependent FV assay when clotting is initiated with calcium alone or calcium plus APC. This assay has three major advantages. First, the use of tissue factor as an activator eliminates any effect of APC on factor VIIIa and makes the test specific for the effect of APC on FVa. Second, the FV-deficient substrate provides an optimum, constant concentration of the other factors needed for the tissue factor–dependent pathway of coagulation. This allows the test to be performed on plasma from patients receiving an oral anticoagulant (Fig 3). Third, the high dilution of the test plasma eliminates any effect on clotting times due to a lupus anticoagulant (Fig 5) or to the administration of heparin.

Frozen and thawed platelet-poor plasma prepared by routine techniques was found to yield clotting time differences that clearly distinguished normal subjects from heterozygotes for APC-resistant FVa. Moreover, although the data are limited, it would appear that the test can distinguish between heterozygotes and homozygotes for APC-resistant FVa. Although we studied only two homozygotes, plasma from these patients consistently yielded a paradoxical, slight shortening of the clotting time of the FV assay performed with APC (Table 1). The mechanism for this shortening is unknown but may be related to the fact that APC inactivating FVa cleaves two peptide bonds in the molecule,21,22

Human immunoabsorbed FV-deficient plasma from a single source (Baxter) was used for the data presented here. Equally reliable data were also obtained when a different commercial source of FV-deficient plasma (Diagnostica Stago) or immunoabsorbed FV-deficient plasma that we prepared ourselves were used (data not shown). Thus, any sensitive FV-deficient substrate can be used, although the actual clotting times with and without APC, and therefore clotting time differences, may vary with different substrate preparations. This is illustrated by comparing the data of the experiment with heparin and the experiment with multiple cycles of freeze thawing (see Results), which were performed with different batches of substrate. Therefore, in standardizing the assay it is useful to obtain a large quantity of a single batch of FV-deficient substrate.

A batch of commercially obtained APC should be tested to select an optimum concentration yielding differences in clotting times of dilutions of a pooled normal reference plasma similar to those illustrated in Fig 1, A and B. Small aliquots of a stock solution of APC can be stored frozen, but should be thawed only once and diluted to proper concentration just before use. Small aliquots of plasma from a heterozygote for FV R506Q can be stored and used as a positive control.

The data presented in Figs 2 through 6 are summarized in Fig 7. No overlap is seen between over 100 samples yielding normal results and 21 samples from heterozygotes for APC-resistant FVa. Under our conditions, which yielded
clotting time differences of at least 7 seconds for a 1/20 dilution and at least 15 seconds for a 1/80 dilution of a pooled normal reference plasma, samples from heterozygotes normalized to equivalent dilutions (see Results) yielded clotting time differences of less than 3.5 seconds and less than 7 seconds. Although this provides guidelines for scoring normal and abnormal test results under our conditions, for the reasons cited earlier each laboratory should establish its own guidelines.

Five percent (2 of 39 persons) of our asymptomatic control subjects were found to have APC-resistant FVa (Fig 2). Although our control subjects were of diverse ethnic backgrounds and our data are limited, this prevalence is similar to that reported earlier in more homogeneous control populations. Both of our positive control subjects with FV R506Q were white.

Because the FV-deficient substrate supplies the necessary vitamin K–dependent clotting factors for the assay, the test was expected to be independent of the activity of these factors in a test sample. This expectation was confirmed when normal test results were found in all but 1 of 21 randomly selected patients receiving oral anticoagulants (Fig 3). In the single patient giving an abnormal test result, subsequent DNA analysis confirmed the presence of the FV R506Q mutation. Moreover, in an additional patient with the DNA-confirmed FV R506Q mutation, similar abnormal test results were obtained on plasma samples when the patient was on or off warfarin (Fig 4).

By similar logic, the protein S provided by the FV-deficient substrate plasma should eliminate the possibility that the protein S activity of a diluted test plasma would influence test results. Thus, the abnormal test results in 2 of 15 patients with previously established free protein S deficiency (Fig 4) are taken as evidence of coexistent APC-resistant FVa. As noted earlier, in the one protein S–deficient patient in whom it could be performed, DNA analysis confirmed the presence of the FV R506Q mutation. Coexistence of APC-resistant FVa with protein C deficiency has recently been reported to increase substantially the risk for thrombosis over that caused by either condition alone. It is reasonable to assume that this may also apply to the coexistence of APC-resistant FVa and protein S deficiency. It would appear that identifying one cause for hereditary thrombophilia does not eliminate the need to look for further causes.

Plasma antiphospholipid antibodies (lupus anticoagulant antibodies, anticardiolipin antibodies) are associated with an increased risk for thrombosis, which may be related, at least in part, to these antibodies blocking phospholipid from serving as a cofactor for APC. The existence of APC-resistant FVa could be a particularly deleterious defect in this circumstance. A major advantage of the test described here is that the high dilution of the test sample and the use of tissue factor as activator permits one to test for APC-resistant FVa in plasma containing a lupus anticoagulant. It is noteworthy that 5 of 29 patients with a lupus anticoagulant had test results indicative of heterozygosity for APC-resistant FVa. Subsequent DNA analysis of three patients available for testing confirmed heterozygosity for the FV R506Q mutation. Four of these five APC-resistant lupus anticoagulant patients had experienced thrombosis in contrast to 12 of the 24 patients with plasma lupus anticoagulant activity and normal test results. Thus, it may be prudent to test for APC-resistant FVa in all patients with antiphospholipid antibodies because the evidence is growing that the presence in a person of multiple risk factors compounds the predisposition to clinical manifestations of thrombosis.

The discovery of APC-resistant FVa in 10 of 21 stored plasma samples from patients with thrombosis in whom other causes for thrombophilia had been ruled out (Fig 6) adds to a growing evidence that APC-resistant FVa may be the most common hereditary cause for thrombophilia. This highlights the need for a relatively simple but reliable coagulation test for APC-resistant FVa that can be performed on fresh or stored plasma from all persons in whom it may be indicated. The test described here meets these criteria.

ACKNOWLEDGMENT

The authors thank Kathleen Donnelly for her assistance in obtaining plasma samples from patients and donors and Li Wang for performing the DNA analysis.

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