The Diagnostic Value of the Fibrinogen/Fibrin Fragment E Antigen Assay in Clinically Suspected Deep Vein Thrombosis

By Ariel Zielinsky, Jack Hirsh, Gunta Straumanis, Cedric J. Carter, Michael Gent, David L. Sackett, Russell Hull, John G. Kelton, Peter Powers, and Alexander G. Turpie

We have evaluated the fibrinogen/fibrin fragment E antigen assay as a diagnostic test in patients with clinically suspected venous thrombosis by comparing the results of this assay with venography in 272 patients. The result of the fragment E antigen assay was elevated in 79 of 80 patients with positive venograms for recent venous thrombosis (sensitivity 99%) and within the normal range in 161 of 192 patients with normal venograms (specificity 84%). The fragment E assay was also evaluated in 130 medical and surgical controls without evidence of venous thrombosis by leg scanning and the test was found to be relatively nonspecific. However, in the patient group under study, a correct clinical diagnosis of no thrombosis, based on a normal fragment E result, was made in 161 of 162 cases (negative predictive value 99%). Therefore, a normal test result effectively excludes a diagnosis of venous thrombosis in clinically symptomatic patients. The assay, as currently performed, is technically demanding and takes 24 hr to complete. Therefore, it will have to be simplified before it can be applied to clinical practice.

The diagnosis of venous thrombosis remains a difficult practical problem. Clinical diagnosis is nonspecific and ascending venography, the reference method, is invasive and may be painful. Impedance plethysmography (IPG) and Doppler ultrasonography are sensitive and specific methods for the diagnosis of proximal vein thrombosis but are insensitive for calf vein thrombosis. This shortcoming can be overcome by adding 125I-fibrinogen leg scanning (LS) to IPG, but a positive LS result may not become evident for up to 72 hr after injection. Although this delay may not constitute an important limitation in hospitalized patients, it adds to the inconvenience and prolongs the uncertainty of the diagnostic process in outpatients.

In an attempt to simplify and speed up the diagnostic process, a number of blood tests have been evaluated for their ability to either confirm or exclude venous thrombosis. Assay of circulating fibrin monomer complexes, measured either by protamine sulphate or ethanol gelation, are relatively insensitive and nonspecific for the diagnosis of venous thrombosis. Standard assays for serum fibrin/fibrinogen degradation products (FDP) are more sensitive but are relatively nonspecific and exhibit considerable overlap between patients with and without thrombosis. In a recent study using the staphylococcal clumping assay for FDP, Gurewich et al. reported a relatively high sensitivity of the assay for the diagnosis of acute venous thrombosis.

Fibrinopeptide A assay (FPA), which is a measure of in vivo thrombin formation, has been shown to be highly sensitive to symptomatic acute venous thrombosis, but it too is relatively nonspecific, technically difficult, and requires meticulous blood sampling technique.

Recently, Gordon and associates reported promising results using a radioimmunoassay for fragment E antigen (FgE), a terminal product derived from proteolysis of fibrinogen or fibrin by plasmin. This radioimmunoassay is able to detect 2.5 ng/ml of FgE and is therefore at least 10 times more sensitive than the most sensitive assay (a staphylococcal clumping assay) for FDP and approximately 100 times more sensitive than the hemoglutination inhibition assay for FDP.

It appears inevitable that FgE assays and other tests that are highly sensitive to intravascular fibrin formation or its dissolution will be nonspecific for venous thrombosis due to their inability to differentiate it from other forms of intravascular fibrin deposition that frequently occurs in sick hospitalized patients. Nonetheless, because of its high sensitivity, we investigated the FgE radioimmunoassay to determine if we could establish a range of test results that could be used to exclude a diagnosis of deep vein thrombosis.

MATERIALS AND METHODS

Patients

Between June 1978 and March 1980, 304 consecutive patients with clinically suspected venous thrombosis were referred to the inpatient or outpatient diagnostic service of the Hamilton and District Thromboembolism Program. Sixty-one developed symptoms while hospitalized for some other disorder and were referred to as inpatients, while 243 developed symptoms of venous thrombosis outside the hospital and were referred as outpatients. Blood was
taken for the FgE assay prior to venography and anticoagulant therapy in all patients. Two-hundred and fifty-four of these patients were in a study evaluating IPG and LS in which venography was carried out on the third day after presentation (or earlier if either LS or IPG became positive) and, in these patients, we also obtained blood samples on the day of presentation and on as many as three occasions before venography. In calculating sensitivity and specificity, however, only the first blood sample was used for comparison with venography.

Controls

Normal controls. Sixty-eight healthy ambulatory volunteers who were free of symptoms or signs suggesting venous thrombosis were studied to obtain a disease-free range for the results of the FgE assay. Their ages ranged from 18 to 52 and the male/female ratio was 4.5.

Hospital controls. The effects of comorbid conditions on the specificity of the test were determined in 113 hospitalized medical and surgical patients who were selected because they were regular venography. All of these patients were asymptomatic, underwent daily LS, and had a negative LS for at least 48 hr after the blood sample was taken for FgE. Ninety-five of these hospital controls were medical patients with a variety of diagnoses including infection, diverse inflammatory conditions, stroke, neoplasms, and liver disease. Eighteen of the hospital controls were surgical patients who had undergone major abdominal surgery or hip surgery. Blood samples for fragment E assay were taken preoperatively, and on every other day postoperatively among surgical controls and a single blood specimen was obtained from medical controls.

Post venography and post leg scanning controls. To determine whether the diagnostic procedures have any effect on the FgE assay, samples for FgE were taken before, 30 min and 24 hr after venography on 10 patients. In addition, 5 patients had blood samples taken before, 30 min and 24 hr after injection with 125I-labeled fibrinogen.

Venography

Venography was performed by the method of Rabinov and Paulin at two hospitals and by a method previously described by Gallus et al. The venographic results were interpreted independently by two or more observers who were blind to these patients' other test results and were classified as follows:

(A) Consistent with venous thrombosis. An intraluminal filling defect was present on all films. Venograms positive for recent venous thrombosis were further subdivided into proximal vein thrombosis or calf vein thrombosis.

(B) Consistent with previous disease, but not diagnostic of recent venous thrombosis. Nonfilling of the venous segment was present in all films and persisted despite reinjection and was associated with collaterals. If an intraluminal filling defect was present in addition to these abnormalities, the venogram was classified as recent venous thrombosis.

(C) Inadequate for interpretation. Nonvisualization of deep veins in the absence of collaterals or in the absence of an intraluminal filling defect which persisted despite reinjection.

(D) Negative for venous thrombosis.

Laboratory Studies

All the FgE determinations were performed by the same laboratory personnel who had no knowledge of the patients' findings on clinical examination, IPG, LS, or venography.

Blood samples were taken into 0.129 M buffered citrate and 10 mg/ml of e-aminocaproic acid (Amicar, Lederle) to prevent in vitro proteolysis. Plasma was separated by centrifugation (10 min at 2500 g, at 4°C) and then stored at −20°C prior to assay. Serum was obtained from 0.5 ml of plasma clotted at room temperature with a (1:1 volume) of a solution containing calcium chloride 0.025 M, e-aminocaproic acid 0.1 M, and bovine thrombin (Parke, Davis and Company, Scarborough, Ontario) 100 NIH units (final concentration). The serum was removed after 30 min of adding thrombin to the plasma.

FgE Assay

Fragment E was prepared from human fibrinogen (Kabi, Grade L, Stockholm, Sweden) by enzymatic cleavage with streptokinase (streptase, Hoechst Canada Inc., Montreal) using 5000 U for 50 ml of fibrinogen (5 mg/ml). Digestion at 37°C was terminated after 24 hr by adding 500 U/ml of Trasylol. A volume of 15 ml (1G’s) protein of the 24 hr digest was fractionated on a Sephadex G 200 column that measured 2.6 × 80 cm. Three peaks were obtained and the fractions of the second peak containing the greatest concentration of protein were pooled. This pool was concentrated and further purified on acrylamide gel electrophoresis using glycine Tris-buffer, pH 8.4, and acrylamide concentration of 2.5 g in 35 ml of Tris-glycine buffer (5% methylene bis-acrylamide in 95% acrylamide monomer). The acrylamide gel was stained for protein and the major band identified. Unstained gels were aligned with the stained gel and the section of the gels corresponding to the major band were cut out, crushed in a tissue grinder, and the protein extracted with 0.15 M saline. The fragment E antigen was identified by immune electrophoresis using anti-E antibody (Behring).

The purified FgE (20 μg/ml) was used for standardization and iodination. FgE was labeled with 500 μCi of 125I by the chloramine-T procedure. The reaction mixture was purified on a cellulose column (Whatman CF 11).

An adult sheep was immunized with FgE by an initial intramuscular injection of 150 μg of purified peptide homogenized in complete Freund's adjuvant followed by booster injections at 10 wk intervals. Sera were harvested after the second boost injection and monitored by double diffusion in gel against the immunizing antigen. Ouchterlony-type immunodiffusion and immunoelectrophoresis revealed only a single band of reactivity.

In the radioimmunoassay procedure, doubling dilutions (1/5 and 1/10) of standard FgE and serum were set up in duplicate in a volume of 100 μl assay diluted 1% bovine serum albumin in phosphate-buffered 0.01 M saline, pH 7.8. The standard FgE was measured using Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, Ontario, Canada).

One-hundred microliters of labeled FgE was added to each tube, followed by 100 μl of anti-FgE antibody (usually 1/15,000 dilution). The final incubation volume was 300 μl.

Control tubes in duplicate were set up with each standard curve, containing labeled peptide and buffer (zero blank) or labeled peptide, antibody, and buffer (zero standard). After overnight incubation at room temperature, antibody-bound and free moieties were separated by the addition of 100 μl of rabbit anti-sheep gamma globulin (Antibodies Inc.; tenfold dilution Davis, Ca.) and 100 μl polyethylene glycol. After mixing the contents, fractions were separated by centrifugation at 3500 g for 30 min. The supernatant (free) fraction was aspirated and the precipitated (bound) fraction counted. The working range of the standard curve was adjusted to 2.5–100 ng/ml. The threshold of detection of the test was 2.5 ng/ml, and it had a long-term precision (CV) within and between runs of 10%. The overall duration of the test was 24 hr.
RESULTS

Range in Healthy Volunteers

The mean FgE level was 132 ± 51 ng/ml (SD) with a range of 35–290 ng/ml in 68 healthy volunteers. An elevated FgE level was defined as a value equal or above 270 ng/ml (mean ± 2 SD).

Patients Symptomatic for Venous Thrombosis

Of the 304 consecutive patients referred with clinically symptomatic venous thrombosis, 32 patients were excluded from the analysis; 7 of these had an inadequate venogram and 25 had venograms that were compatible with previous disease but not diagnostic of recent venous thrombosis. The decision to exclude these patients from the analysis was made without knowledge of the FgE results. In 23 patients, the FgE result was within the normal range, and in 9 patients, the result was elevated.

The results of FgE assay were compared with venography in 272 patients whose venograms were either negative or diagnostic of recent venous thrombosis. One-hundred and seventeen of the 272 patients were males and 155 were females. Their mean age was 55. Eighty percent were outpatients and 20% were inpatients.

One-hundred and ninety-two (71%) had no evidence of venous thrombosis by venography and 80

<table>
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<tr>
<th>Fragment E Result</th>
<th>Positive Venogram</th>
<th>Negative Venogram</th>
<th>Totals</th>
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<tbody>
<tr>
<td>Positive</td>
<td>79</td>
<td>31</td>
<td>110</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>161</td>
<td>162</td>
</tr>
<tr>
<td>Totals</td>
<td>80</td>
<td>192</td>
<td>272</td>
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</tbody>
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Sensitivity: 79/80 = 99%.
Specificity: 161/192 = 84%.
Positive predictive value: 79/110 = 72%.
Negative predictive value: 161/162 = 99%.

(29%) had recent venous thrombosis (Fig. 1). Of the latter, 55 had proximal vein thrombosis and 25 isolated calf vein thrombosis.

Table 1 shows the sensitivity and specificity of the FgE assay in the detection of symptomatic venous thrombosis.

The FgE assay was within the normal range on the day of referral in 161 of 192 patients with negative venograms (specificity 84%). One-hundred and sixty-two patients had a FgE result in the normal range on the day of referral and 161 of these had no evidence of venous thrombosis on venography (negative predictive value 99%). The test was repeated on at least one more occasion in 111 patients in whom the results were initially in the normal range. In only three patients did the result exceed the upper limit of normal.

One-hundred and ten patients had an elevated FgE result and in 79 of these cases, venography showed venous thrombosis (positive predictive value 72%). In 39 of the 79 patients with an elevated value, the FgE level was above 1000 ng/ml (1 μg/ml), and in these no further dilutions were performed.

The FgE result was elevated in 79 of 80 patients with recent venous thrombosis (sensitivity 99%). It was elevated in all 55 patients with proximal vein thrombosis and in 24 of the 25 patients with calf vein thrombosis. The only patient with FgE level below 270 ng/ml had unilateral calf vein thrombosis that was limited to the soleal plexus. Fourteen of the 79 patients with recent venous thrombosis had undergone operations within 3 wk prior to presentation.

The FgE assay was within the normal range, and therefore of potential clinical value, in 144 of 166 (87%) outpatients who were negative by venography and in 17 of 26 (65%) of hospitalized patients without evidence of thrombosis of venography (Table 2).

Hospital Controls

The FgE results in 95 medical patients with no evidence of venous thrombosis by LS is shown in Fig. 2. Thirty-eight of the 95 patients had FgE levels above 270 ng/ml. Figure 3 shows the FgE results in 18
surgical controls with no evidence of venous thrombosis by LS. Seven of these had fragment E levels above 270 ng/ml preoperatively and most had fragment E results above 270 ng/ml for up to 15 days postoperatively.

<table>
<thead>
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<th>Positive Venogram</th>
<th>Negative Venogram</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>52</td>
<td>52</td>
<td>74</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>144</td>
<td>144</td>
</tr>
<tr>
<td>Totals</td>
<td>52</td>
<td>166</td>
<td>218</td>
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Table 2. Fragment E Results in Outpatients and Inpatients

**DISCUSSION**

A diagnostic test can be clinically valuable for two different reasons. It can be helpful in establishing a given diagnosis; that is, have a high positive predictive value so that virtually all patients with positive test results have the disease of interest. Alternatively, it can be helpful in excluding a given diagnosis; that is, have a high negative predictive value so that virtually all patients with negative test results are free of the

### The Effect of Contrast Media and 125I-Labeled Fibrinogen on the Fragment E Level

Ten patients with normal venograms were studied before, 20 min and 24 hr postvenography. FgE results were 280 ± 90 ng/ml, 270 ± 105 ng/ml, and 250 ± 80 ng/ml, respectively. The FgE levels performed on blood from 5 patients taken before, 30 min and 24 hr after injection of 125I-labeled fibrinogen for LS were 334 ± 105 ng/ml, 379 ± 124, and 350 ± 132 ng/ml, respectively (p > 0.1).
disease. The prerequisites for a high positive predictive value are high specificity, plus a relatively high prevalence; when prevalence falls, so too does positive predictive value.\textsuperscript{15} The prerequisite for high negative predictive value is high sensitivity; moreover, when prevalence falls, negative predictive value rises.

Our study has documented that the FgE determination has a very high negative predictive value (99%); as a result, when the test is in the normal range, deep vein thrombosis can be effectively excluded. To determine its potential clinical utility, the FgE assay was evaluated in a number of different groups; healthy controls to provide a disease-free range for the test, patients with clinically suspected venous thrombosis who were also investigated by venography to determine sensitivity, specificity, and predictive values of the test, and patients with other diseases to determine the effect of a variety of comorbid conditions on the specificity of the test. Because this latter group of patients are prone to silent venous thrombosis, it was necessary to use fibrinogen LS and to only include those whose LS test was negative.

The FgE assay proved to be highly sensitive to acute venous thrombosis, being positive in 79 of the 80 symptomatic patients with clinical and venographic evidence of recent thrombosis. Forty-nine percent of these patients had levels below 1 µg/ml and, therefore, were below the threshold of detection that can be consistently obtained with other tests for fibrin/fibrinogen degradation products.

As predicted, the FgE assay is relatively nonspecific, since it was frequently abnormal in both sick medical patients and postoperative surgical patients who had negative LS. The usefulness of this assay lies, therefore, in obtaining a negative result in a clinically symptomatic patient. In such patients, a correct clinical decision of “no thrombosis,” based on negative FgE result, was made in 161 of 162 cases.

Seventy-two percent of patients referred with clinically suspected venous thrombosis failed to have the diagnosis confirmed venographically. This reflects both the nonspecificity of clinical diagnosis and the relatively low prevalence of venous thrombosis in outpatients who made up approximately 80% of the study group.

The result of the FgE assay is less subject to falsely positive results due to inadequate blood taking than the fibrinopeptide A assay but suffers the same disadvantage of being a radioimmunoassay which, as currently performed, takes 24 hr to complete. If these limitations can be overcome without a marked loss of sensitivity, it should provide a clinically useful means for excluding venous thrombosis, particularly in ambulatory patients because they are less likely to have comorbid conditions that produce falsely positive results. However, if the test result is positive, another more specific test such as venography or a combination of IPG and LS would have to be performed to establish a diagnosis of venous thrombosis.

REFERENCES

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