Comparison of the Native Prothrombin Antigen and the Prothrombin Time for Monitoring Oral Anticoagulant Therapy

By Bruce Furie, Howard A. Liebman, Rita A. Blanchard, Michael S. Coleman, Steven F. Kruger, and Barbara C. Furie

We have measured the fully carboxylated (native) prothrombin antigen and the undercarboxylated (abnormal) prothrombin antigen in patients treated with sodium warfarin using specific immunoassays to evaluate a new approach for monitoring oral anticoagulant therapy. Plasma and serum samples (391) were assayed for the prothrombin time, native prothrombin antigen, and abnormal prothrombin antigen. The results were correlated with the presence of bleeding or thromboembolic complications at the time of phlebotomy. The native prothrombin antigen correlated with the occurrence of complications in 95% of samples. Of 13 samples from patients with bleeding complications, 13/13 (100%) had a native prothrombin of 12 μg/mL or lower. Of seven samples from patients with thromboembolic complications, 6/7 (86%) had a native prothrombin of 24 μg/mL or greater. By comparison, a prothrombin time index of 1.5 to 2.5, 1.5 to 2.2, 1.5 to 2.0, or 1.3 to 1.8 identified 6/20 (30%), 9/20 (45%), 11/20 (55%), or 12/20 (60%) patients at risk, respectively. Although the prothrombin time index did correlate with the presence of bleeding complications, the native prothrombin antigen correlated closely with the presence of bleeding and thromboembolic complications. According to these results, the native prothrombin antigen, maintained in a range of 12 to 24 μg/mL by regular adjustment of the warfarin dosage, may be associated with a reduced risk of complications due to excessive or insufficient warfarin therapy. On the basis of these preliminary data, we recommend that the native prothrombin antigen be considered to monitor warfarin therapy.

SODIUM WARFARIN (Coumadin) is a widely used oral anticoagulant. This drug functions as a vitamin K antagonist and lowers the plasma activity of the vitamin K-dependent blood coagulation proteins factor X, factor IX, factor VII, and prothrombin. Warfarin is commonly employed to treat patients who have developed thromboembolic disease and are prone to recurrences or to treat patients for disorders known to be associated with thromboembolic complications. Although warfarin is highly efficacious, its toxic-therapeutic ratio is narrow. In current practice, the dosage of warfarin administered is titrated by monitoring the prothrombin time.3,4 Despite careful attention to the prothrombin time measurements, 10% to 20% of patients treated with warfarin develop a bleeding complication attributed to warfarin therapy or a thromboembolic complication due to inadequate warfarin therapy.6-11 Although the British Comparative Thromboplastin has been used to lower the incidence of bleeding complications,3,5,10 the efficacy of this method for treating thrombotic tendencies is unproven. Because of the attendant risks of oral anticoagulants, therapy is often curtailed or avoided because the potential benefits may not outweigh the risks. For these reasons, we have evaluated a new method to assess the optimal dosage of warfarin to assure adequate antithrombotic therapy, while minimizing the hemorrhagic complications of oral anticoagulant therapy.

After the synthesis of a prothrombin precursor in the liver, specific glutamic acid residues near the NH2-terminal domain are carboxylated by a vitamin K-dependent carboxylase to yield γ-carboxyglutamic acid.1,2 The native prothrombin, as secreted into the plasma, contains ten γ-carboxyglutamatic acid residues. In the absence of vitamin K or in the presence of vitamin K antagonists, an undercarboxylated prothrombin, known as abnormal prothrombin, circulates in the blood.13 In contrast to native prothrombin, abnormal prothrombin lacks clotting activity, does not bind to calcium, and does not bind to membrane surfaces.14-17 We have developed specific immunoassays for each of these prothrombin species, native prothrombin and abnormal prothrombin.18,19 Using these assays, we have demonstrated that abnormal prothrombin is not a component of normal blood, but appears in patients with vitamin K deficiency,18 acute hepatitis,19 cirrhosis,18 and primary hepatocellular carcinoma.20 Abnormal prothrombin and native prothrombin can be quantitated in the blood of patients treated with sodium warfarin.18,19 The purpose of the

From the Division of Hematology-Oncology, Department of Medicine, New England Medical Center and Tufts University School of Medicine, Boston.

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Address reprint requests to Dr Bruce Furie, Division of Hematology-Oncology, New England Medical Center, 171 Harrison Ave, Boston, MA 02111.
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current investigation was to determine whether measurement of these species might detect those patients at risk for the complications of oral anticoagulant therapy. Because the prothrombin time is conventionally employed to monitor warfarin therapy and is successful in predicting most bleeding complications, our study was designed to evaluate samples from patients treated with warfarin and monitored with the prothrombin time. Assays of the abnormal prothrombin and native prothrombin antigen were compared to the prothrombin time and correlated with the clinical complications of warfarin therapy. We have evaluated 391 samples obtained from patients treated with warfarin. Using these specific prothrombin assays, we have shown that the native prothrombin antigen was outside of the range of 12 to 24 μg/mL in 95% of samples from patients who developed warfarin-related complications.

MATERIALS AND METHODS
Abnormal prothrombin was purified from plasma obtained from patients receiving long-term sodium warfarin therapy. Human prothrombin was prepared from fresh frozen plasma. Abnormal prothrombin and prothrombin migrated as single bands on gel electrophoresis in dodecyl sulfate. Both proteins were labeled using the solid-phase lactoperoxidase method and Na125I (New England Nuclear, Boston) or chloramine T.

Antibodies to the prothrombin–Ca(II) complex were isolated from rabbit antiprothrombin antiserum; these antibodies bind to native (fully carboxylated) prothrombin but do not bind to abnormal (acarboxy or descarboxy) prothrombin. Antibodies specific for abnormal prothrombin were obtained from rabbit antiabnormal prothrombin antiserum. Abnormal prothrombin and native prothrombin in either plasma or serum were measured using a competition radioimmunometric assay.

Blood samples anticoagulated in citrate or serum samples were obtained from 133 patients treated with sodium warfarin. The indications for warfarin therapy included deep venous thrombosis (23%), pulmonary embolism (9%), prosthetic heart valve (24%), transient ischemic attack (TIA) or cerebral vascular accident (CVA) (8%), atrial fibrillation associated with valvular heart disease (21%), synthetic grafts (5%), ventricular aneurysm (1%), peripheral venous thrombosis (2%), and embolization (1%), and miscellaneous (6%). The prothrombin time, the abnormal prothrombin antigen, and the native prothrombin antigen were determined in each sample. The clinical events at the time the samples were obtained were determined by us. Bleeding complications were documented by clinical examination, while thrombotic complications were documented by angiography, venography, or ventilation-perfusion lung scanning. The warfarin dosages of all patients were adjusted by their physicians on the basis of the prothrombin time. The prothrombin time was performed using Thromboplastin-C (Dade, Aquada, PR) or Simplastin (General Diagnostics, Morris Plains, NJ) reagents. The prothrombin time index is the ratio of the patient prothrombin time to the control prothrombin time.

Patient data were maintained and analyzed on a DEC VAX-11/730 computer using the CLINFO System of the Clinical Study Unit at New England Medical Center. Consent was obtained from participants under guidelines established by the Human Investigation Review Committee at the New England Medical Center.

RESULTS
The native prothrombin antigen (Fig 1A) and the abnormal prothrombin antigen (Fig 1B) were compared with the prothrombin time index, the ratio of the patient prothrombin time to the control prothrombin time. The native prothrombin antigen, which correlates precisely with the prothrombin activity, shows a complex relationship with the prothrombin time index. The abnormal prothrombin antigen shows a linear relationship with the prothrombin time index, but a correlation coefficient of .71.

A summary of the results is tabulated in Table 1. Abnormal prothrombin is not present in blood samples from normal subjects but appears in all blood samples from patients treated with sodium warfarin. The abnormal prothrombin antigen concentration did not correlate well with the presence of bleeding or thromboembolic complications of warfarin therapy. By contrast, the native prothrombin antigen concentration was lower in the blood samples associated with bleeding complications than in the blood samples associated with thromboembolic complications. Based on this observation, a detailed analysis of the correlation of the native prothrombin antigen concentration and clinical complications of warfarin therapy was performed. A parallel analysis of the prothrombin time index was also performed for purposes of direct comparison.

Bleeding Complications
Thirteen samples were obtained from patients at the time of bleeding complications. A summary of the clinical features of patients with bleeding complications is tabulated in Table 2. These patients demonstrated the common types of bleeding problems seen with warfarin: mucosal bleeding, retroperitoneal bleeding, hematuria, intracranial hemorrhage, purpura. Some of the complications were life-threatening, some were serious enough to warrant hospitalization, and all required a modification of oral anticoagulant therapy.

A histogram showing the distribution of the native prothrombin antigen in all samples in the study is shown in Fig 2 (open bars). The native prothrombin ranged from 1.5 to 173 μg/mL, with a mean of 26.4 μg/mL and a median value of 28. The native prothrombin antigen concentrations in samples associated with bleeding complications are also shown as a histogram in Fig 2 (solid bars). Low native prothrombin antigen levels were associated with hemorrhagic complications.

The bleeding incidence was defined as the ratio of the number of samples associated with bleeding complications to the total number of samples for a given...
native prothrombin antigen concentration. Our data indicate that no bleeding was associated with samples in which the native prothrombin was in excess of 12 μg/mL. As shown in Fig 2 (inset), the bleeding incidence (solid bars) correlates closely with the native prothrombin antigen. Native prothrombin antigens of 0 to 3 μg/mL, 3 to 6 μg/mL, 6 to 9 μg/mL, and 9 to 12 μg/mL yielded bleeding incidences of 1.0, 0.5, 0.13, and 0.02, respectively. These results indicate two important points: (1) the lower the native prothrombin antigen, the greater the incidence of bleeding complications; and (2) samples with low native prothrombin levels are not necessarily associated with bleeding complications.

A similar analysis was performed comparing bleeding complications and the prothrombin time index. A histogram showing the distribution of the native prothrombin antigen in all samples in the study is presented in Fig 2 (open bars). The native prothrombin antigen concentrations in samples associated with thromboembolic complications are also shown in the histogram in Fig 2 (hatched bars). As shown in Fig 2 (inset), the thromboembolic incidence (hatched bars) rises with the native prothrombin concentration. A parallel analysis was performed comparing thromboembolic complications and the prothrombin time index. A histogram showing the distribution of the prothrombin time index in all samples is shown in Fig 3 (open bars). The prothrombin time indices of samples associated with thromboembolic complications are also shown on the histogram (hatched bars). There is a poor correlation between the prothrombin time index and the incidence of thromboembolic complications. When the therapeutic range of the prothrombin time index is defined as 1.5 to 2.5, 1.5 to 2.2, 1.5 to 2.0, or 1.3 to 1.8, it is observed that 6, 6, 6, or 7 samples, respectively, associated with thromboembolic complications had a prothrombin time index above the lower limit of the therapeutic range and were not identifiable at risk by the prothrombin time.

On the basis of these results, we conclude that bleeding complications and thromboembolic manifestations of an underlying disorder correlate with native prothrombin antigen concentration, also shown as a histogram in Fig 3 (solid bars). It can be noted that 8, 5, 3, and 1 samples were associated with bleeding complications when the prothrombin time index was in a therapeutic range defined by 1.5 to 2.5, 1.5 to 2.2, 1.5 to 2.0, and 1.3 to 1.8, respectively.

Thromboembolic Complications

Seven samples were obtained from patients at the time of thromboembolic complications. A summary of the clinical features of these patients and their relevant laboratory data is shown in Table 3. Pulmonary embolism, recurrent deep venous thrombosis, embolic stroke, and arterial thrombosis characterized these complications. All but a single sample had a native prothrombin antigen concentration equal to or greater than 24 μg/mL.

A histogram showing the distribution of the native prothrombin antigen in all samples in the study is presented in Fig 2 (open bars). The native prothrombin antigen concentrations in samples associated with thromboembolic complications are also shown in the histogram in Fig 2 (hatched bars). As shown in Fig 2 (inset), the thromboembolic incidence (hatched bars) rises with the native prothrombin concentration. A parallel analysis was performed comparing thromboembolic complications and the prothrombin time index. A histogram showing the distribution of the prothrombin time index in all samples is shown in Fig 3 (open bars). The prothrombin time indices of samples associated with thromboembolic complications are also shown on the histogram (hatched bars). There is a poor correlation between the prothrombin time index and the incidence of thromboembolic complications. When the therapeutic range of the prothrombin time index is defined as 1.5 to 2.5, 1.5 to 2.2, 1.5 to 2.0, or 1.3 to 1.8, it is observed that 6, 6, 6, or 7 samples, respectively, associated with thromboembolic complications had a prothrombin time index above the lower limit of the therapeutic range and were not identifiable at risk by the prothrombin time.

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Table 1. Native Prothrombin, Abnormal Prothrombin, and Prothrombin Time Index in Subpopulations of Patients Treated With Warfarin

<table>
<thead>
<tr>
<th></th>
<th>Native Prothrombin (μg/mL)*</th>
<th>Abnormal Prothrombin (μg/mL)</th>
<th>Prothrombin Time Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patient samples</td>
<td>26.4 (1.5-173)</td>
<td>36 (0-144)</td>
<td>1.88 (1-8.4)</td>
</tr>
<tr>
<td>Patients with complications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bleeding (n = 13)</td>
<td>6.2 (1.5-12)</td>
<td>68.7 (28-144)</td>
<td>2.75 (1.8-5.3)</td>
</tr>
<tr>
<td>Thrombotic (n = 7)</td>
<td>30.6 (14-45)</td>
<td>75.2 (32-118)</td>
<td>2.0 (1.4-3.1)</td>
</tr>
<tr>
<td>Normal subjects</td>
<td>108 ± 19</td>
<td>0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Mean (range).
prothrombin antigen concentrations of 12 μg/mL or less or native prothrombin antigen concentrations of 24 μg/mL or more, respectively. We suggest that optimal anticoagulation might be obtained if the native prothrombin were maintained between 12 and 24 μg/mL. No similar range of the prothrombin time index can be identified in which few complications are seen. As shown in Table 4, 13 samples with prothrombin time indices within a therapeutic range of 1.5 to 2.5 (N = 286) were associated with complications (complication incidence 0.049); 11 samples with prothrombin time indices within a therapeutic range of 1.5 to 2.2 (N = 254) were associated with complications (complication incidence 0.045); ten samples with prothrombin time indices within a therapeutic range of 1.5 to 2.0 (N = 209) were associated with complications (complication incidence 0.045); eight samples with prothrombin time indices within a therapeutic range of 1.3 to 1.8 (N = 163) were associated with complications (complication index 0.049). Our data indicate that bleeding complications can be reduced with the use of less intense warfarin therapy. These conclusions are similar to those of Hull et al, who observed bleeding in 2/47 (4%) patients and thrombosis in 1/47 (2%) patients treated with less intensive therapy.10 In contrast, only one sample with a native prothrombin level between 12 and 24 μg/mL was associated with a complication. The
complication incidence of samples with the native prothrombin antigen in this range was 0.0063. These results indicate that the native prothrombin antigen correlates with bleeding and thromboembolism about eightfold more closely than does the prothrombin time index.

**DISCUSSION**

The control of the dosage of most drugs is, at best, limited to the determination of serum drug levels. Sodium warfarin and related vitamin K antagonists are unique in that the dosage schedule is determined by monitoring the biologic effect of the drug on blood coagulation. This has been particularly important for warfarin because of its narrow toxic-therapeutic range and its frequent association with bleeding complications. The prothrombin time has remained the method of choice for the evaluation of the manifestations of warfarin on blood coagulation. Although complications occur despite rigid control of the prothrombin time, the prothrombin time has been clearly shown to offer benefits in the control of oral anticoagulant therapy.4,10

Considerable efforts have been made to improve the prothrombin time and its reliability in predicting complications related to oral anticoagulant therapy.5,5 Unfortunately, few clinical studies have dealt simultaneously with bleeding and thrombotic complications, a necessity for optimizing warfarin dosage schedules. As with most complex biologic tests, variability in the prothrombin time relates to the components of the assay system: thromboplastin, phospholipids. Thromboplastin reagents are not chemically defined and vary in potency, composition, and stability, depending on the species and the organ from which this reagent is isolated. Standardization of the assay remains a continued problem: a prothrombin time or prothrombin time index performed on a sample in one laboratory may yield a different value in another laboratory. For these reasons, patients monitored with tests based on the British Comparative Thromboplastin receive significantly less warfarin than patients monitored with Simplastin.23,24 Furthermore, blood samples must be carefully obtained, properly anticoagulated, and the prothrombin time promptly performed. Gralnick and others have shown that the storage of plasma samples at room temperature and the use of glass tubes can cause an erroneous prothrombin time value.25 For these reasons—the intrinsic problems of a biologic assay—we have measured a vitamin K-dependent blood coagulation protein whose concentration correlates with the complications of anticoagulant therapy with vitamin K antagonists.

Using specific immunoassays that quantitate native prothrombin despite the presence of abnormal prothrombin and quantitate abnormal prothrombin despite the presence of native prothrombin, we have compared these antigen concentrations with the pro-
thrombin time to evaluate their correlation with the incidence of both bleeding and thromboembolic complications in patients treated with warfarin. Our results indicate that the abnormal prothrombin antigen does not correlate with the bleeding and thromboembolic complications associated with warfarin therapy. We might have anticipated this result, as abnormal prothrombin is biologically inert and does not interfere with blood coagulation.

The concentration of native prothrombin, an antigen that correlates precisely with the biologic coagulant activity of prothrombin, is, however, closely associated with the incidence of bleeding and thrombosis. Our data indicate that 100% of samples associated with hemorrhagic complications had native prothrombin antigens of 12 \( \mu \text{g/mL} \) or less. Furthermore, the incidence of bleeding increased dramatically as the native prothrombin level in samples fell below 12 \( \mu \text{g/mL} \). Of the samples studied, 86% of those associated with thromboembolic complications had native prothrombin antigens of 24 \( \mu \text{g/mL} \) or greater. However, many samples contained native prothrombin levels in excess of 24 \( \mu \text{g/mL} \) either because the patients from which they were obtained were inadequately anticoagulated or because they had recently initiated warfarin therapy. Because the incidence of thromboembolic complications in these patients is low even in the absence of warfarin therapy, it is not surprising that the incidence of thromboembolic complications in warfarin-treated patients with native prothrombin antigens in excess of 24 \( \mu \text{g/mL} \) is also low. Our results, obtained using the immunological assay for the prothrombin antigen, confirm the findings of Sise et al, who found that the prothrombin coagulant activity correlated with warfarin-related complications.

Because of the proven efficacy of the prothrombin time in monitoring oral anticoagulant therapy, we have not been able to compare directly the use of the native prothrombin antigen and the prothrombin time in two separate patient populations. Rather, we have measured the native prothrombin antigen in blood samples obtained from a warfarin-treated population monitored with the prothrombin time. In the 13 samples associated with bleeding complications and the seven samples associated with thromboembolic disorders, only 30% to 60% of these samples were demonstrated at risk by the prothrombin time, while 95% were demonstrated at risk by the native prothrombin antigen. On this basis, our data suggest that the incidence of complications in well-monitored patients might be reduced significantly if the native prothrombin antigen assay were substituted for the prothrombin time in monitoring oral anticoagulant therapy (Table 4). In addition to its closer correlation with untoward complications, the native prothrombin antigen can be measured by precise immunoassays using prothrombin standards available to all laboratories. Because of the stability of the antigen, it can be measured in serum, obviating the need for meticulous handling of plasma samples. Although this work has been performed with polyclonal rabbit anti-prothrombin:Ca(II) antibodies, the preparation of monoclonal antibodies specific for native prothrombin provides the necessary immunochemicals available in adequate quantity to allow the configuration of an immunoassay that is rapid, inexpensive, and accurate.

On the basis of the current preliminary data and its analysis, we propose that measurement of the native prothrombin antigen be considered to monitor warfarin therapy. A direct clinical trial comparing the immunologic assay and the prothrombin time will be required to determine the efficacy of this assay in optimizing warfarin dosage.

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