CLINICAL UTILITY OF PLASMA PROTEIN C MEASUREMENT

TEST
Plasma protein C, antigenic and functional activity measurement

BACKGROUND
Protein C is one of several known naturally occurring anticoagulants, which can be divided into three general categories: (1) The antithrombins, which inhibit the activity of thrombin and other serine proteases, including factors IXa, Xa, XIa, and XIIa; (2) Proteins C and S, both vitamin K dependent, which inactivate factors Va and Villa; and (3) The plasminogen-plasmin system which digests fibrin and inhibits the polymerization of fibrin during clot formation.

MECHANISM OF ACTION
Protein C was originally described as autoprothrombin Ila by Seegers in 1960 when he demonstrated that the protein, extracted and purified from blood clots, could act as an anticoagulant in vitro. This protein was later isolated by Stenflo in 1976. He designated it protein C and found that it had an amino acid sequence similar to other serine proteases, but lacked coagulant activity. Other workers soon demonstrated the anticoagulant properties of protein C and discovered that the physiologic mechanism of action was the inactivation of activated co-factors Va and Villa (10).

Protein C causes the proteolytic cleavage of factors Va and Villa. However, it must first be activated by thrombin. The rate of this activation is greatly accelerated by thrombomodulin, a cofactor present on the surface of endothelial cells (7). After binding to thrombomodulin, the activity of thrombin is altered such that fibrinogen cleaving activity is diminished and the ability to cleave an activation peptide from protein C is enhanced. With the cleavage of the activation peptide, the proteolytic domain is unblocked and protein C becomes capable of inactivating factors Va and Villa (10). Protein S, another vitamin K dependent anticoagulant, is a required cofactor for the inactivation of factor Va. Activated protein C can be neutralized in vivo and in vitro by forming stable complexes with protein C inhibitor (2, 18).

CLINICAL CORRELATES
Hereditary Protein C Abnormalities
The clinical conditions associated with an abnormality of protein C fall into several groups. The first was first recognized in 1981 when Griffin described a kindred in which affected individuals had the onset of thromboembolic events in their early twenties and protein C antigen and activity levels were 50% of normal or less (9). In this kindred, the clinical manifestations appeared to be transmitted as an autosomal dominant trait with affected heterozygotes. Subsequently, many similar kindreds have been described in which the clinical manifestations and pattern of inheritance are similar (3,4).

Treatment consists of lifelong warfarin therapy. However, treatment must be initiated with caution because skin necrosis in the early stages of oral anticoagulant therapy has been described in patients who are severely protein C deficient. The mechanism of necrosis is thought to be diffuse thrombosis of small venules caused by a further rapid decrease in the level of protein C, a vitamin K dependent protein. In these cases, concurrent anticoagulation with heparin may be warranted until a steady state decrease in the vitamin K dependent coagulation factors is reached (14).
A second phenotypic clinical pattern also was described in the early 1980s (16, 17). It is an autosomal disorder in which the homozygous state is characterized by the virtual absence of protein C and by fatal thrombosis in the neonatal period. Typically, an individual with this disorder dies in early infancy with venous thrombosis of large vessels and/or massive progressive bleeding into the skin, accompanied by thrombosis of capillaries and venules with necrosis of the target organ (12). Measurements of protein C antigen in the infant show undetectable levels. Levels in the parents are decreased to 50% of normal or less and reflect heterozygosity. The parents and other members of the pedigree who are found to have significantly decreased antigen levels are clinically unaffected. Thus, in this pattern clinical protein C deficiency is only manifest in the homozygote. In contrast to the clinical syndrome first described by Griffin, heterozygotes are unaffected. Untreated, affected infants die in early infancy. Some success has been reported with administration of protein C purified from factor IX concentrates (17) and warfarin administration (20).

Even rarer functional defects in the protein C molecule have also been described in patients who are prone to developing deep vein thromboses. In these individuals, the antigen level of protein C is normal, but the functional activity of the protein, as measured by in vitro activation studies, is low (4).

Acquired Protein C Abnormalities
Protein C levels may be abnormal for a variety of reasons other than a heritable deficiency. Other conditions associated with decreased protein C include oral anticoagulant therapy, liver disease, DIC, postsurgical state, neonatal period, L-asparaginase therapy, and plasma exchange (11, 8).

Range of Protein C Levels in Normals
It must be noted that a recent large study of protein C antigen levels in blood donors (13) found that protein C levels below 65% of normal, considered by most investigators to be predictive of heterozygosity (3, 8), are found in 1 in 60 healthy adults. Based on the normal curve predicted from log-transformed data of this population, Clouse and his coworkers predicted that the majority of these individuals had protein C levels at the lower end of the normal distribution and were not, in fact, heterozygotes. However, some individuals had protein C levels that were greater than 3.5 standard deviations below the mean (less than 55% of normal) and were considered to be heterozygous. A study of protein C levels in families of half of these individuals confirmed heterozygosity. Those with levels between 55-65% were considered to represent the low end of a continuous distribution of normal, though some overlap with true heterozygotes certainly occurs. Importantly, no associated risk of thrombosis was found in either of these populations. Though Clouse and coworkers could not distinguish between patients who were at the lower level of the normal distribution and some of the patients who were heterozygous for the deficiency, they estimated the prevalence of heterozygosity to be 1 in 200-300. This figure is consistent with the known prevalence of homozygous infants.

METHODS
ANTIGEN LEVEL - ENZYME-LINKED IMMUNOSORBENT ASSAY/ LIMITATIONS
Plasma protein C antigen can be quantitated by electroimmunoassay (EIA), radioimmunoassay (RIA), and enzyme linked immunosorbent assay (ELISA). The ELISA is presently being adopted in many laboratories because it is more sensitive than the EIA, which has a reported limit of detection ranging from 3-10% or 3 - 10 U/dl (12, 1), is faster, and does not require the use of radioactivity as does the RIA technique. Methods for ELISA quantification of protein C are described below.

The assay used at UCSF is a polyclonal assay manufactured by Diagnostica Stago. Reagents are
prepared as follows: Antibodies are obtained from rabbits immunized with purified protein C. From these purified antibodies, F(ab)2 fragments are prepared with which to coat the plastic microwells. The use of the F(ab)2 fragment is advantageous because, unlike the intact immunoglobulin, it is not subject to interference by the rheumatoid factor. In addition, the same intact antibodies are labelled with horseradish peroxidase (1).

Using these reagents, the protein C level can be determined with the immuno-enzymatic method (ELISA sandwich). Test plasma, at dilutions of 1:50 and 1:100, is added to the wells. The protein C in the plasma contacts and binds to the bound antibodies. Next, anti-protein C coupled with peroxidase is added and allowed to bind to the free remaining antigenic determinants of the bound protein C. Bound enzyme peroxidase is then detected by its activity over a predetermined time with the substrate orthophenylenediamine in the presence of hydrogen peroxide. The resulting color change, read as optical density at 492 nm, is a direct measure of the protein C in the plasma. (5).

The reference range given by Diagnostica Stago for their ASSERACHROM(R) immunoassay is 98.6 ± 17.6% for normals and 44±8.1% for congenitally deficient plasma. However, the normal range should be established for each laboratory. For patients on oral anticoagulant therapy an attempt is made to normalize the measurement by comparing the level to that of another vitamin K dependent protein, such as factor II or VII. Griffin found a mean ratio of protein C to prothrombin in normal individuals be to 1.25± 0.21 and these same ratios in three protein C deficient patients to be 0.48, 0.56, and 0.61 (9). Again, each laboratory should establish its own reference range. If the suspicion of protein C deficiency is high and the ratio confusing, the patient may be changed to heparin therapy and then tested for protein C.

Limitations include a decreased efficiency of detection of protein C antigen when complexed with the protein C inhibitor, estimated to be on the order of 50% for assays using polyclonal antibodies and 0% for assays using monoclonal antibodies. During treatment with vitamin K antagonists, the gamma carboxylation of the glutamyl residues is partially inhibited and results in the appearance of partially carboxylated and nondecarboxylated forms which lead to systematically lower values by ELISA when compared to measurements obtained using immunoelectrophoresis (2). In addition, it must be remembered that this assay does not give information about the activity of the molecules as do the functional assays. Therefore, a patient may have normal levels of protein C as antigenically determined and still have a protein C abnormality.

ANTICOAGULANT ACTIVITY ASSAY/LIMITATIONS

Two assays are presently commercially available. Both utilize a venom to activate protein C instead of the thromin-thrombomodulin activation as is reported to be used in many of the experimental studies. In the first, a chromogenic substrate is cleaved by activated protein C in the test plasma and then quantitated spectrophotometrically. The second is a clotting assay in which test plasma is mixed with protein C deficient plasma and then activated with the venom. The activated PTT is then measured and compared to controls with known levels of protein C. Reagent concentrations are generally adjusted so that a normal protein C level yields a PTT of 150 seconds and a level of 0 yields a PTT of 30 seconds (6, 10).

There are several in vitro limitations to these techniques. The chromogenic substrate technique is limited because it does not measure a physiologic endpoint and, therefore, the the results do not necessarily reflect in vivo activity. In fact, there is good evidence that the snake venom activator is capable of activating decarboxylated protein C molecules which can cleave a chromogenic substrate but
remain inactive in the APTT test (19). Patients undergoing anticoagulation therapy and a subset of those with recurrent DVTs may show such a discrepancy. The clotting assay results, though reflecting a more physiologic endpoint, may be less reproducible and adjustment of reagents difficult. In addition, the presence of a lupus anticoagulant has been reported to alter results (falsely normal)(15) as have high levels of heparin (>1IU/ml) (6).

INTERPRETATION
It follows that there are many pitfalls in interpreting abnormal protein C levels. With the exclusion of neonatal purpura fulminans, in which symptomatology is gross and the protein C level nearly undetectable, determining the clinical significance of an abnormal value may be difficult. First, acquired causes of protein C abnormalities must be ruled out. In addition, it must be remembered that there is an overlap between the lower values of normal and some heterozygous individuals. 1 in 60 normal subjects have protein C values consistent with heterozygosity, yet are not predisposed to thrombosis. Importantly, it appears that the majority of individuals who are heterozygous are of the phenotypic type that is not at an increased risk of thrombosis in the heterozygous state. Therefore, before beginning the patient on a lifetime course of anticoagulant therapy, the burden is on the clinician to justify the diagnosis of clinically significant protein C deficiency. In a patient with a suggestive history and abnormally low protein C level, a detailed family history and further testing of the kindred, if possible, is certainly warranted. In the absence of the supporting family history, the significance of the measurement alone must be questioned and other causes for recurrent thrombosis actively ruled out.

CONCLUSION
Interpretation of an abnormally low protein C must be tempered by the clinical setting. Other factors such as anticoagulant therapy, DIC, and liver disease must not be present. A detailed family history should be obtained and, when possible, measurements from affected family members obtained. The significance of the measurement itself, in the absence of symptoms or a family history, is negligible. Given the implications of the diagnosis of protein C deficiency, lifelong anticoagulant therapy, many investigators recommend more than one measurement.

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