Acquired Deficiencies of Protein S

Protein S Activity during Oral Anticoagulation, in Liver Disease, and in Disseminated Intravascular Coagulation

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Abstract

Protein S is a vitamin K-dependent plasma protein which serves as the cofactor for activated protein C. Protein S circulates in both an active, free form and in an inactive complex with C4b-binding protein. To elucidate the role of protein S in disease states and during oral anticoagulation, we developed a functional assay for protein S that permits evaluation of the distribution of protein S between free and bound forms and permits determination of the specific activity of the free protein S. In liver disease, free protein S antigen is moderately reduced and the free protein S has significantly reduced specific activity. In disseminated intravascular coagulation, reduced protein S activity occurs due to a redistribution of protein S to the inactive bound form. During warfarin anticoagulation, reduction of free protein S antigen and the appearance of forms with abnormal electrophoretic mobility significantly decrease protein S activity. After the initiation of warfarin, the apparent half-life of protein S is 42.5 h. In patients with thromboembolic disease, transient protein S deficiency occurs due to redistribution to the complexed form. Caution should be exercised in diagnosing protein S deficiency in such patients by use of functional assays.

Introduction

Protein S is a vitamin K-dependent glycoprotein which serves as a cofactor for the expression of the anticoagulant and profibrinolytic activities of activated protein C (1, 2). Protein S binds to phospholipid surfaces and increases the affinity of activated protein C for synthetic membranes by forming a 1:1 stoichiometric complex (3). Coordinate binding of protein S and activated protein C onto biological membranes has been described in platelet (4) as well as in endothelial cell systems (5), although the ratio of protein S to activated protein C appears to be much greater than 1 on these cellular surfaces.

Potential insights into the regulation of the protein C anticoagulant pathway can be gained by examining the known interactions of protein S with other plasma proteins, and from a knowledge of its synthesis and compartmentalization. At the cellular level, protein S is synthesized in the liver and by endothelium and stored in platelet α-granules, from which it can be released in functional form during platelet activation (6–8). The ability to compartmentalize protein S may be an important regulatory mechanism, since protein S activity appears to be modulated in plasma by other factors. Protein S circulates in plasma in at least two forms: as free protein S and in complex with C4b-binding protein (C4bBP),[1] an inhibitor of the classical complement pathway (9). Whereas the significance of the protein S–C4bBP complex in the complement pathway remains unknown, it has been established that, upon binding to C4bBP, protein S loses its ability to function as a cofactor for the anticoagulant activity of activated protein C (10, 11). Thus, the concentration of free protein S, rather than total protein S, is relevant for the regulation of the protein C anticoagulant pathway.

The presence of two distinct forms of protein S in plasma (free and complexed) complicates its measurement in clinical samples. Both immunologic and functional methods for protein S determination have been described, which have permitted the identification of protein S deficiency in patients with recurrent thrombotic diseases (12–14). In principle, the functional assay for protein S is a necessary adjunct to immunologic measurements to be certain of the protein S status of the patient. Although a functional assay for protein S has been previously developed, interpretation and the general utility of the assay are compromised since factors other than free protein S levels influence the results (10). For example, plasma from patients with liver disease or on oral anticoagulants appears to be extremely sensitive to the anticoagulant effect of activated protein C. This leads to overestimations of the protein S activity.

Accurate assessment of the protein C anticoagulant pathway in clinical samples requires the determination of both protein C anticoagulant activity and protein S activity. To better understand the role of these proteins in thrombotic disease, we undertook an analysis of the protein C/protein S anticoagulant pathway in a series of clinical conditions where major changes in the components of the system might be anticipated (liver disease, oral anticoagulation, disseminated intravascular coagulation [DIC]). For the assessment of the activity of protein S in these conditions, we have developed a

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1. *Abbreviations used in this paper:* C4bBP, complement component 46-binding protein; DIC, disseminated intravascular coagulation; HPS-2, anti-human protein S antibody; MOPS, 3-[*N*-morpholino]-propanesulfonic acid; PEG, polyethylene glycol.

new functional assay which is based on selective immunoadsorption and elution of free protein S. Application of this assay has allowed the determination of the functional half-life of protein S and the demonstration that multiple mechanisms contribute to decreased protein S activity in common clinical situations.

Methods

Reagents. Tris (Tris(hydroxymethyl)aminomethane) (Trizma Base or Sigma 7–9), 3-[*N*-morpholino]propanesulfonic acid (MOPS), heparin sodium salt from porcine intestinal mucosa (140 U/mg), Lubrol Px, rabbit brain cephalin, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO); benzamidine hydrochloride hydrate was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Protein isolation. Bovine thrombin was isolated after activation of the purified prothrombin with purified factor Xa, Va, phospholipid, and calcium (15). The other coagulation factors and inhibitors used in this study were prepared by the methods cited: bovine Xa (16), human factor Xa (17), bovine antithrombin III (18), rabbit lung thrombomodulin (19), human protein C and activated protein C (20), and human C4bBP (21). Initially, protein S was purified as described (10). In the later portions of the work, after elution of the vitamin K-dependent proteins from the 10 × 60-cm QAE Sephadex column, the eluate was made 1 mM in diisopropylfluorophosphate (DFP) and 10,000 U of heparin was added. The eluate was then adsorbed with 50 ml of immobilized IgG murine anti–human protein S antibody (designated HPS-2) which had been linked to Affigen-10 at 5 mg/ml gel. After gentle mixing at room temperature for 90 min, the gel was allowed to settle for 30 min and the supernatant collected by syphoning. The resin was packed in a 2.5 × 40-cm column and washed with 0.4 M NaCl in 20 mM Tris buffer, pH 7.5, until the adsorption at 280 nm was < 0.005. The column was then washed with 500 ml 0.1 M NaCl in the same buffer and the bound protein S was eluted with 1 mM 2-(*N*-morpholino)ethane sulfonic acid (MES), pH 6.0.

Monoclonal antibody preparation. Balb/c mice (Jackson Labs, Bar Harbor, ME) were immunized i.p. three times with 100 μg of human protein S per injection at 2-wk intervals. After a 2-mo rest, they were then injected with 50 μg of antigen and fusion was performed 3 d later. A myeloma cell line (P3-X-63-Ag8-653, 1.5×10^7 viable cells) was mixed with 1.7×10^8 viable spleen cells from one animal and fused by standard procedures using polyethylene glycol (PEG) 1500. After selection in hypoxanthine/aminopterin/thymidine (HAT) medium, culture supernates were tested for binding activity to protein S. During selection, positive clones were tested with an ELISA on Costar 96-well plates coated with a 1 μg/ml solution of protein S. The ELISA employed horseradish peroxidase-conjugated rabbit anti–mouse Ig (Boehringer Mannheim, Indianapolis, IN) to detect protein S-bound antibodies. Positive clones were resubcloned two times by limit dilution. For production of ascites fluid, $\sim 5 \times 10^6$ hybridoma cells were injected per Balb/c mouse 2 wks after Pristane treatment. The cell line finally selected (HPS-2) has subsequently been subcloned three additional times.

Monoclonal antibody characterization and isolation. HPS-2 bound free protein S and thrombin-cleaved protein S equivalently when analyzed by ELISA. The monoclonal antibody is a IgG1 with κ light chain.

Ascites fluid was diluted 1:1 with H_2O, then precipitated by the addition of equal volumes of saturated NH_4SO_4 at 4°C for 60 min. The precipitate was harvested by centrifugation at 20,000 *g* for 30 min, dissolved, and desalted on a Sephadex G75 column into 0.027M Tris-PO_4, pH 6.3, before chromatography on a QAE Sephadex Q50 column (1 ml resin/ml ascites), equilibrated in the above buffer. The column was developed with a linear gradient from 0 to 0.2 M NaCl (total volume = five times the column volume) over ∼ 8 h. The HPS-2 was precipitated with 50% NH_4SO_4, the pellet was harvested by centrifugation, resuspended in a minimum volume of 0.1 M NaCl, 1 mM MOPS, pH 7.5, before further purification on a Sephadex G-200 column, equilibrated in this buffer. HPS-2 was then coupled to Affigel-10 (Bio-Rad Laboratories, Richmond, CA), in 0.1 M NaCl, 0.1 M MOPS, pH 7.5, for 12 h at 4°C to give a final concentration of bound antibody of 5 mg/ml Affigel-10.

The cross-reactivity of the monoclonal antibody with the epitopes of other vitamin K-dependent proteins was examined as follows: plasma immunoadsorbed with the immobilized HPS-2 and the HPS-2 eluates were assayed for prothrombin antigen, factor IX antigen, and protein C antigen by rocket immunoelectrophoresis with appropriate goat polyclonal antibodies. No decrease in the plasma levels of prothrombin, factor IX, or protein C was observed after immunoadsorption except that resulting from dilution. No significant prolongation in the activated partial thromboplastin time or the prothrombin time was observed following immunoadsorption. No protein C, factor IX, or prothrombin was detected in the HPS-2 eluate.

Immunologic methods. Protein S was quantitated using a radioimmunoassay (22) under conditions that favor dissociation of the complexed protein S (23). Crossed immunoelectrophoresis was conducted as previously described (12). Protein S-depleted plasma was prepared by immunoadsorption (10). C4bBP was measured with an ELISA method (22). Goat IgG to specific proteins was prepared as previously described (20).

Functional protein S assay. Since only free protein S contributes to the activated protein C cofactor activity of plasma, selective isolation of free protein S is desirable for activity measurements. This was achieved by the selection of a murine monoclonal antibody (HPS-2) which binds both intact and thrombin-inactivated protein S but does not interact with protein S complexed with C4bBP. This was demonstrated by crossed-immunoelectrophoresis of heparinized-citrated normal plasma before and after incubation with the immobilized monoclonal antibody (Fig. 1, *left [inset]*). Crossed-immunoelectrophoresis of plasma protein S in EDTA reveals two arcs showing immunologic identity. The slow-migrating component comigrates with the protein S–C4bBP complex, whereas the fast migrating arc comigrates with free protein S. Incubation of plasma with the immobilized HPS-2 antibody for 30 min leads to the selective loss of the fast migrating component. This is paralleled by loss of protein S activity in the supernatant as measured with the protein S functional assay (10), (Fig. 1, *left*). Only the fast-migrating component is eluted from the antibody, under the conditions described below, (Fig. 1, *left [inset]*). A potential problem that might occur would be the dissociation of protein S from the complex with C4bBP during the incubation with the immobilized antibody, which would lead to an overestimation of the free protein S levels. Dahlback reported a K_d of 0.7×10^{-7} M for the C4bBP–protein S interaction in plasma and provided evidence for a very low rate of dissociation of the purified C4bBP–protein S complex at 22°C ($K_{-1} = 6.1 \times 10^{-5}$) s^{-1} (24). To evaluate the extent of dissociation during the assay (see below) when performed at room temperature, the time course of protein S antigen depletion was measured. Protein S antigen in the HPS-2 supernatants decreased rapidly during the first 30 min of incubation, remaining essentially constant thereafter, (Fig. 1, *right*). Protein S antigen and activity in the HPS-2 eluates also increased rapidly during the first 30 min; after this time, there was only slight further increase. Failure to detect residual protein S activity in the HPS-2 supernatant after 30 min incubation (Fig. 1, *left*) suggested that incubation of plasma with immobilized HPS-2 for 30 min allows quantitative adsorption of the free plasma protein S. The time course also suggested that protein S from the complex contributed very little (< 10%) to the antigen recovered in the HPS-2 eluate. Independent support for this working hypothesis was provided by comparative measurements of protein S antigen in the HPS-2 eluate and in the supernatant of plasma treated with PEG 8000. Protein S complexed to C4bBP is precipitated at 4°C when PEG 8000 is added to a final concentration of 3.75% (12). Free protein S is not precipitated under these conditions and may be quantitated by immunoelectrophoresis. Virtually identical estimates of free plasma protein S were obtained on different occasions when the antigen in the HPS-2 eluate was com-

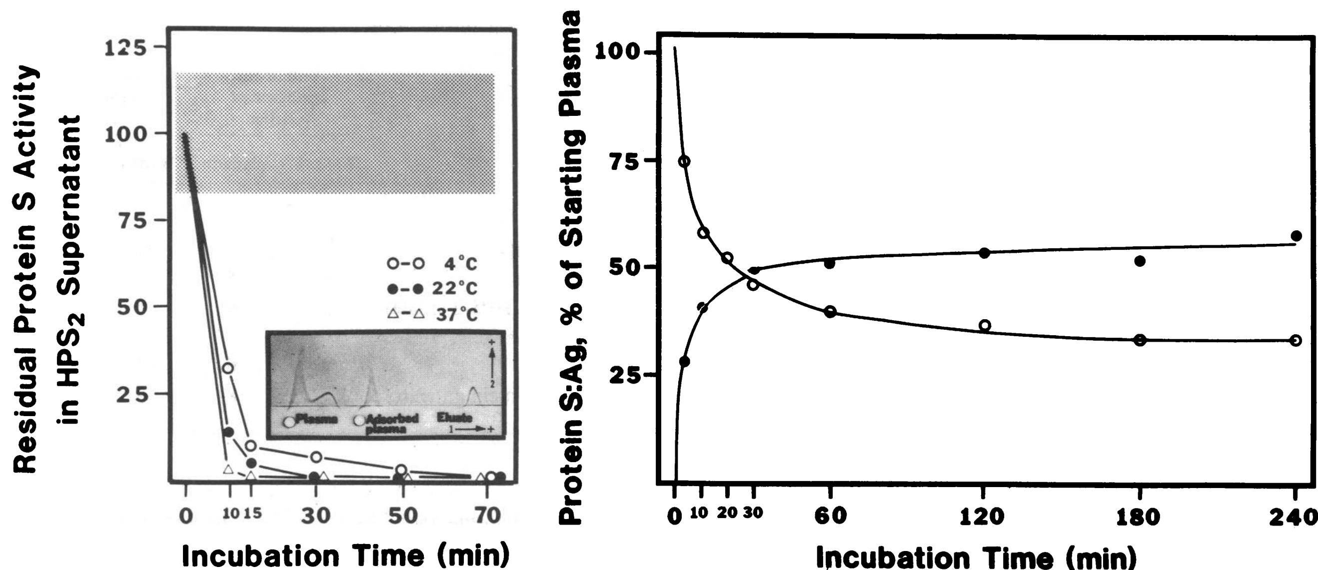

Figure 1. (*Left*) Recovery of protein S activity in immunoadsorbed plasma. Normal pooled heparinized citrated plasma (1.5 ml) was incubated with 150 μl of packed HPS-2 Affigel beads (antibody concentration 5 mg/ml of packed gel). At the indicated times, 200-μl aliquots were removed and, after centrifugation, 50 μl of the supernatant was tested for plasma protein S residual activity (10). Incubations were conducted at 4°C, 22°C (room temperature), and 37°C. The shaded area represents the range of plasma protein S residual activity observed upon incubation of plasma with corresponding amounts of either Affigel-10 beads alone (five determinations) or of Affigel-10 beads coupled to a monoclonal anti–protein C antibody at a concentration of 5 mg/ml gel (five determinations). (*Inset*) Crossed immunoelectrophoresis pattern of the starting normal plasma, of the plasma supernatant after 30 min of incubation with the immobilized HPS-2 antibody (adsorbed plasma) and of the corresponding protein S eluted from the monoclonal antibody at low ionic strength. Crossed immunoelectrophoresis was conducted as described in Methods and the anodes of the two electrophoresis runs are marked with plus signs. (*Right*) Protein S antigen levels were measured in HPS-2 eluates (•) and supernatants (○) of plasma (1-ml aliquots) incubated with packed HPS-2 Affigel beads (100 μl) at room temperature. At the indicated times, the samples were processed as for the functional assay and the radioimmunoassay was performed as described in Methods on the HPS-2 eluates and the corresponding HPS-2 supernates. Protein S antigen levels are expressed as percentage of a calibration curve constructed with dilutions of the starting plasma. The protein S activity recovered in the HPS-2 eluates paralleled the changes of protein S antigen (data not shown).

pared to that in the PEG supernatants (see below, Fig. 6). Moreover, no residual protein S was seen when the HPS-2 adsorbed plasma was treated with PEG 8000 and the supernatant was subjected to Laurell rocket immunoelectrophoresis under conditions previously described (12).

Recovery of protein S in the immunoadsorption step was followed by radioimmunoassay. The HPS-2 eluate contained 41%±7% (mean±1 SD, plasma samples from 16 normal subjects) and 53%±9% of the protein S remained in the adsorbed plasma. These values are similar to the distribution of complexed vs. free protein S observed by Dahlback (24) in normal human plasma. There was no detectable C4bBP in the HPS-2 eluate (< 1% plasma levels) and no detectable decrease in the HPS-2–adsorbed plasma (98%±8%). This was further evidence that only free protein S can be recovered from normal plasma by the monoclonal antibody immunoadsorption.

Based on these findings, a standardized procedure was developed for protein S isolation from clinical plasma samples. 1 ml of platelet-poor plasma, obtained by centrifuging blood anticoagulated with 0.13 M sodium citrate, pH 5.9, (9:1 vol/vol) was heparinized by the addition of 100 μl of a buffer containing 1.0 M NaCl, 0.5 M Tris-HCl, 0.05 M benzamidine-HCl, 60 U/ml heparin, pH 7.5. The heparinized plasma was then incubated with 100 μl of packed HPS-2 Affigel-10 beads on a rocking table at room temperature. After 30 min, the beads were removed by centrifugation for 30 s in a Beckman Microfuge B (Beckman Instruments, Inc., Fullerton, CA) and, after removal of the HPS-2 adsorbed plasma, were washed four times by resuspension in a buffer containing 0.10 NaCl, 0.02 M Tris-HCl, pH 7.5, and 5 mM benzamidine HCl followed by centrifugation. The beads were then washed a fifth time with 1.5 ml 0.005 M NaCl, 0.015 M Tris-HCl, pH 7.5, and the supernatant removed. Protein S was then eluted by mixing the beads in 1 ml of distilled water containing 0.1% Lubrol Px and 5 mg/ml BSA. (The BSA stock solution had been extensively dialyzed against distilled water.) The hypotonic elution was performed at room temperature for 60 min on a rocking table. This concentration of Lubrol Px had no influence on the clotting time in the assay described below.

The protein S activity of the HPS-2 eluate was measured in a modified one-stage factor Xa clotting assay. The assay was performed in the following manner: to 100 μl of protein S depleted plasma at 37°C was added 100 μl of elution buffer, 100 μl of cephalin, 20 μl of activated protein C (5 μg/ml, in 0.1 M NaCl, 0.02 M Tris HCl, pH 7.5, 1 mg/ml BSA), 100 μl of 25 mM $CaCl_2$, and 100 μl of purified bovine factor Xa. The bovine factor Xa was diluted into 0.1 M NaCl, 0.02 M Tris-HCl, 1 mg/ml BSA, pH 7.5, to give a 30-s clotting time (≈ 4 ng/ml, final concentration in the assay). The protein S immunoadsorbed from normal pooled plasma (HPS-2 eluate, 100 μl) was then substituted for the elution buffer. This level of protein S gives a 23–25-s prolongation of the clotting time. A standard curve was prepared using serial dilutions of the eluted protein S. Triplicate determinations were run for each test sample and converted to protein S concentration (% of activity in normal plasma) (Fig. 2, *left*). A linear relationship was obtained between the percent concentration of protein S and the prolongation of the clotting time. When human factor Xa is substituted for bovine factor Xa, identical results are obtained. To determine the sensitivity of the assay, the normal pooled plasma was serially diluted into protein S depleted plasma. Each dilution was then processed separately and assayed for activated protein C cofactor activity as described above (Fig. 2, *right*). The intercept of the curve obtained from four

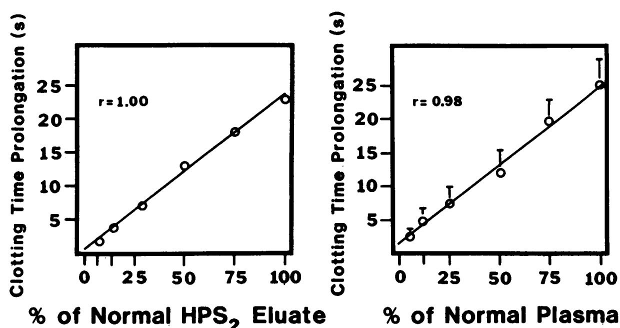

Figure 2. Activated protein C cofactor activity of protein S isolated from normal plasma. (*Left*) 1 ml of pooled normal plasma was processed as described in Methods and the eluted protein S was diluted up to a 1:8 dilution in the distilled water containing 0.1% Lubrol Px and 5 mg/ml BSA. The Xa one-stage clotting assay was modified as described in Methods for the measurement of activated protein C cofactor activity. The prolongation of the clotting time (seconds) is plotted over the protein S concentration (100% = undiluted HPS_2 eluate). (*Right*) 1-ml mixtures of normal pooled plasma with protein S depleted plasma (6.25%, 12.5%, 25%, 50%, 75%, 100%, normal pooled plasma concentrations) were independently processed in the functional assay as described in Methods. The prolongation of the clotting time (seconds) is plotted over the concentration of normal pooled plasma in the starting mixtures. Mean and SD of four separate experiments are shown; regression and correlation coefficient calculated on pooled data from the four experiments. The intercept of the curves on the x-axis is not significantly different from zero.

separate experiments on the x-axis is not significantly different from zero. The detection limit of the assay is 5% of normal plasma, corresponding to 1.5–3-s prolongations in the clotting assay. Using the new functional protein S assay we have measured protein S functional activity and free protein S antigen levels after (*a*) three cycles of freezing and thawing the starting plasma, (*b*) after storage of the plasma at 4°C, 25°C, and 37°C for 4 h prior to assay, (*c*) with the immunoadsorption step carried out at 4°C, 25°C, and 37°C, and (*d*) with the following anticoagulants in the plasma: EDTA, citrate alone, and citrate with heparin. None of these treatments significantly altered either the functional protein S level or the free protein S antigen. Plasma samples from previously described patients with protein S deficiency (12) were evaluated with this assay and the results compared with those obtained with the previously described functional assay carried out in plasma. Protein S activity with the new assay averaged 30% (range 14–54%) as compared to 27% (range 10–45%) obtained with the previous assay.

Reproducibility of the assays. Within-day variations of the assays were determined with 11 replicate determinations of a normal plasma pool and on plasma from a warfarin-treated patient. Between-day variation was determined on 10 different days using aliquots of both plasmas. Coefficients of variation are shown in Table I.

Other coagulation assays. Protein C amidolytic and anticoagulant activities were determined as described (20). Factors VII and X activity were determined in one-stage clotting assays. Factor VII was assayed using activated rabbit brain thromboplastin (Dade Diagnostic, Inc., Aguardo, Puerto Rico) and factor VII-deficient plasma (George King Biomedical, Inc., Overland Park, KS). Factor X was assayed using Russell's viper venom in cephalin (Sigma Chemical Co.) and factor X- and VII-depleted plasma (Sigma Chemical Co.).

Patient population. Normal subjects and patients gave informed consent. Venous blood was collected in 0.13 M sodium citrate, pH 5.9. Platelet poor plasma was obtained by centrifuging blood at 4,800 g for 15 min at 10 C. Pooled normal plasma was obtained from 20 healthy donors. All plasma was stored at −70°C. The control population was composed of 39 normal individuals from 20 to 68 yr of age (mean age 43 yr). 20 patients on stabilized warfarin treatment were receiving the same dose of warfarin for at least 1 mo prior to testing. Patients starting oral anticoagulation because of venographically documented deep vein thrombosis with symptoms of < 5 d duration (n = 8) were treated with or without a loading dose of warfarin while on heparin therapy. Warfarin was started after 2–3 d of heparin administration. Baseline samples were obtained within 24 h of the admission of the patients, after phlebography was performed and while patients were on heparin treatment. Four patients received 60 mg of warfarin over the first 3 d of treatment. The others received only 30 mg of warfarin over the same time period. Subsequent dosages were adjusted according to the prothrombin time. 20 patients with severe chronic liver disease had laboratory data consistent with liver failure (albumin < 3 g/dl; gamma-globulin > 2.5 g/dl, elevated transaminases prolonged prothrombin time) with biopsy proven diagnosis of liver cirrhosis. 20 patients with laboratory evidence of DIC secondary to different clinical conditions (obstetrical, posttraumatic, infective) fulfilled diagnostic criteria indicated previously (fibrinogen degradation products > 20 μg/ml with markedly reduced platelet count and fibrinogen levels, and prolonged prothrombin and partial thromboplastin times).

Statistical analysis. Unless specified, geometric means and ranges of the values observed were calculated by log transformation of the single values. The significance of the difference between means was tested with one or two-way analysis of variance and Student's t test for unpaired or paired data. Linear regression and correlation coefficients were calculated with the least squares method.

Table I. Reproducibility of the Assays

	Within-day coefficient of variation		Between-day coefficient of variation	
	Normal	Warfarin	Normal	Warfarin
		%		
Protein S activity	8	18	12	22
Total S antigen	5	6	9	9
Free S antigen (HPS-2 eluate)	6	9	15	18
Complexed S antigen (HPS-2 supernatant)	4	6	11	13
C4b-binding protein antigen	7	8	9	9

Results

The mean functional protein S in the control group was 106% of normal pooled plasma, with a range of 72–158% (Fig. 3, *left*). Protein S activity was reduced to different extents in the three patient groups studied. The lowest mean level (29% of normal, $P < 0.001$) was observed in patients on stabilized warfarin treatment. Patients with liver disease and with disseminated intravascular coagulation had significant, but smaller, mean reductions of protein S activity (66% and 80% of normal, respectively, $P < 0.001$ (Fig. 3, *left*). Total protein S antigen levels were reduced in patients with liver disease (mean 80% of normal, $P < 0.001$), and in those on stabilized warfarin treatment (mean 68% of normal, $P < 0.001$), but were normal in patients with disseminated intravascular coagulation (mean 112% of normal, NS) (Fig. 3, *right*). These estimates of protein S antigen in disseminated intravascular coagulation are in agreement with the finding of Bertina et al. (11). Similar estimates in warfarin treated patients have been previously ob-

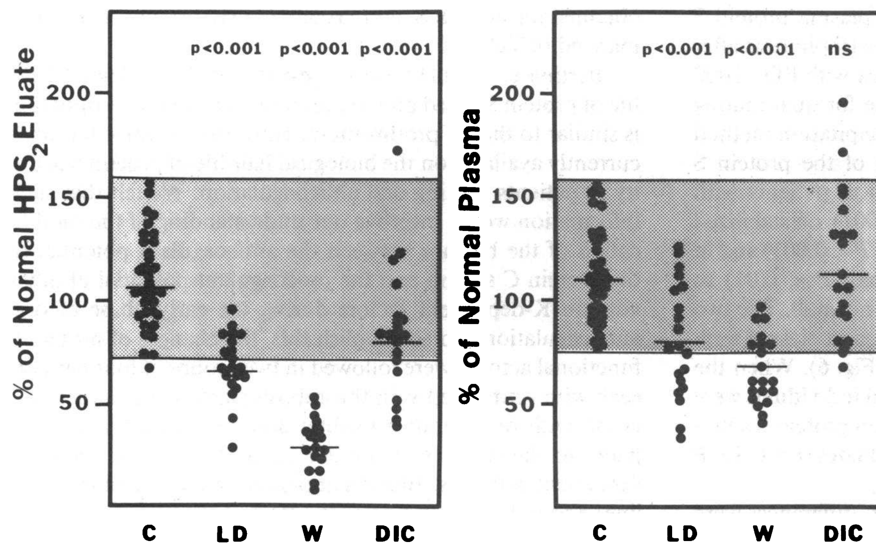

Figure 3. (*Left*) Protein S functional activity and (*right*) total protein S antigen in normal individuals, patients with liver disease, patients on warfarin therapy, and patients with DIC. 39 normal individuals (19 men and 20 women) were studied as were 20 patients with liver disease (*LD*), 20 patients on long-term warfarin therapy (*W*), and 20 patients with DIC (*DIC*).

served by others (11, 13). To investigate the relationship between total protein S antigen and protein S activity, the protein S distribution was evaluated in the different patient groups. Complexed protein S was measured in the HPS-2 adsorbed plasma, and free protein S was measured in the HPS-2 eluate. Complexed protein S antigen was significantly reduced in patients with liver disease (72% of normal complexed protein S antigen, $P < 0.001$) and in patients on stabilized warfarin treatment (82% of normal complex levels, $P < 0.001$) (Fig. 4, *left*). At variance, patients with disseminated intravascular coagulation had a higher mean level of complexed S antigen than did controls (126% vs. 108%, $P < 0.01$).

By dividing the absolute levels of complexed protein S antigen by the corresponding levels of total protein S antigen, it was possible to estimate the relative distribution of protein S in individual patient samples. The percentage of the patient's total protein S complexed with C4bBP varies with the different disease states. In liver disease, 43% of the protein S was in complex with C4bBP (Fig. 4, *center*). In patients on stabilized warfarin treatment, 60% was complexed and in DIC 64% was in complex. Control plasma had 52% of the protein S in complex. These findings received qualitative confirmation when the plasma samples from controls and patients were subjected to crossed-immunoelectrophoresis with EDTA in the first dimension. The plasma samples with high levels of complexed protein S antigen also had a larger area underlying the precipitation arc corresponding to the complexed protein S relative to the normal pooled plasma (data not shown).

Assuming a 1:1 stoichiometric complex between protein S and C4bBP, the estimates obtained of the relative concentrations of complexed protein S, together with the above reported total protein S antigen levels, would predict by the law of mass action reduced C4bBP in patients with liver disease, normal C4bBP in patients on stabilized warfarin treatment, and increased C4bBP in patients with DIC. The anticipated results were confirmed by direct measurement of C4bBP antigen in plasma. Mean C4bBP levels were 65% of normal in patients with liver disease ($P < 0.01$), 110% of normal in patients on stabilized warfarin treatment (NS), and 150% of normal in patients with DIC ($P < 0.01$) as compared to a mean level of 110% in controls (Fig. 4, *right*). Bertina et al. (11) using Laurell rocket electrophoresis found reduced C4bBP levels in liver disease, normal levels in warfarin treated patients, but reduced levels in DIC.

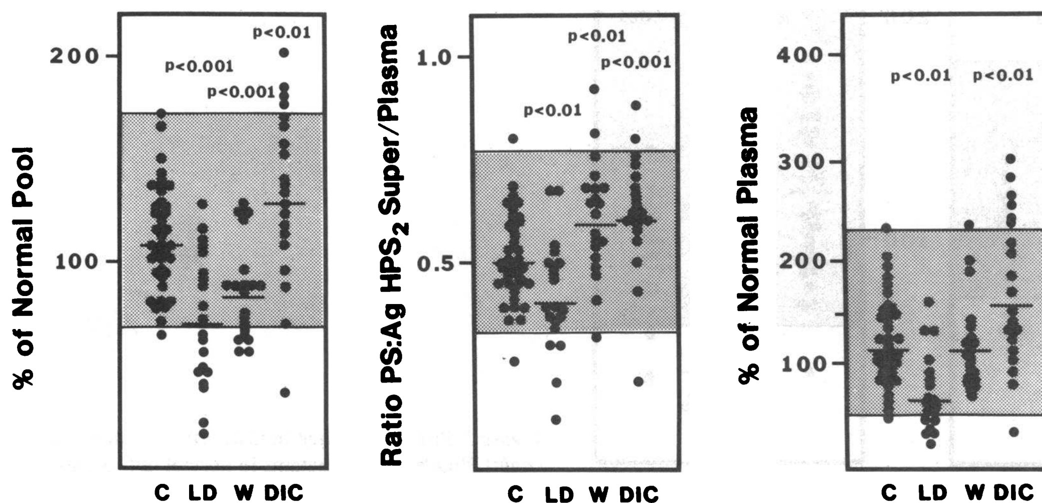

Figure 4. (*Left*) Protein S antigen in HPS-2 supernatant (adsorbed plasma), (*center*) ratio of complexed to total protein S antigen, and (*right*) C4bBP in normal individuals and liver disease, warfarin-treated, and DIC patients. The protein S remaining in plasma samples after adsorption was determined by radioimmunoassay as was the total protein S antigen in the initial plasma samples.

Previously it had been shown that free plasma protein S may also be quantitated by Laurell rocket electrophoresis after precipitation of the C4bBP-protein S complex with PEG 8000 (12). The new immunoadsorption technique for quantitating free protein S was tested against the PEG precipitation method (Fig. 5). Significantly reduced mean levels of the protein S antigen were observed using both methods in patients with liver disease (87% and 84% of normal, $P < 0.001$), on stabilized warfarin treatment (52% and 40% of normal, $P < 0.001$) and in patients with DIC (92% and 86% of normal, $P < 0.01$) as compared with controls (116% and 120% of normal). The two independent measures of the protein S antigen in plasma were positively correlated ($r = 0.87$, $P < 0.001$) (Fig. 6). When the data from all three patient groups and normal individuals were combined, there was good correlation between protein S activity and the antigen recovered in the HPS-2 eluates ($r = 0.80$, $P < 0.001$) (data not shown).

When examining the three patient groups, differences were found in the specific activity of the eluted protein S (Fig. 7, *left*). The mean ratio of protein S activity to protein S antigen eluted (specific activity) was 0.93 in the control group. Protein S specific activity was markedly reduced in patients on stabilized warfarin treatment (mean ratio 0.56, $P < 0.001$) and, to a lesser extent, in patients with liver disease (mean ratio 0.76, $P < 0.01$). Protein S specific activity was similar in controls and in patients with DIC (mean ratio 0.89, NS). To gain information about the possible mechanism(s) underlying reduced protein S specific activity during oral anticoagulation and in liver disease, crossed-immunoelectrophoretic analysis of the HPS-2 eluates from plasma of patients with markedly abnormal protein S specific activity was conducted in the presence of either EDTA or calcium ions in the first dimension (Fig. 7, *right*). Normal electrophoretic mobility of the eluted protein S was observed in presence of EDTA. However, when calcium ions were substituted for EDTA, the protein S isolated by immunoadsorption with HPS-2 from the plasma of patients on warfarin treatment showed a faster (anodic) mobility. The protein S isolated from one patient with liver disease and one patient with DIC both showing markedly reduced protein S specific activity (0.63 and 0.60) had also abnormal electrophoretic mobility in the presence of calcium ions (Fig. 7, *right*). The results obtained for protein S, and protein C measurements are summarized in Table II.

Bertina et al. (11) have suggested that the biological half-life of protein S based on total protein S antigen determination is similar to that of prothrombin. However, no information is currently available on the biological half-life of protein S activity in patients starting oral anticoagulation. We felt that such information would improve our understanding of the modification of the balance between the anticoagulant potential of the protein C system and the procoagulant potential of other vitamin K-dependent factors during the early phase of oral anticoagulation. To accomplish this, the changes of protein S functional activity were followed in two groups of four patients each with acute deep-vein thrombosis starting oral anticoagulation with or without a loading dose of warfarin and compared to the changes of protein C, factor VII, and factor X functional activities. Blood samples were taken before (baseline) and 4, 8, and 24 h after the start of therapy, after which additional samples were taken daily for 6 d. A last sample was taken after 15 d. Changes in the protein S levels following warfarin administration were normalized to the pretreatment level for each patient (Fig. 8, *left*). Since no difference was found in the rate of protein S decrease in patients treated with or without a loading dose of warfarin, the results obtained in the two groups were combined. No significant changes from the baseline values were observed for protein S and factor X 8 h after the start of treatment (data not shown). After 24 h of warfarin administration, protein S activity was decreased to 80%±4% of baseline values ($P < 0.01$). Corresponding levels of factor X activity were 79%±10% of baseline. In these samples, protein C and factor VII activities were reduced to 38%±6% and to 20%±6% of the baseline, respectively. The mean half-disappearance times of protein S activity, protein C anticoagulant activity, factor VII, and factor X calculated with a semilog regression method after checking for linearity were 42.4 h (range 24–58.6), 13.9 h (11.0–33.0), 8.1 h (6.2–36.3), and 38.5 h (22.0–3.1), respectively. The rate of decrease of protein S activity was compared to the rates of decrease of total and free protein S antigens (Fig. 8, *right*). Protein S activity (80%±4% of baseline) was significantly lower than total antigen (87%±3%, $P < 0.05$) and free protein S (89%±2%, $P < 0.05$) by

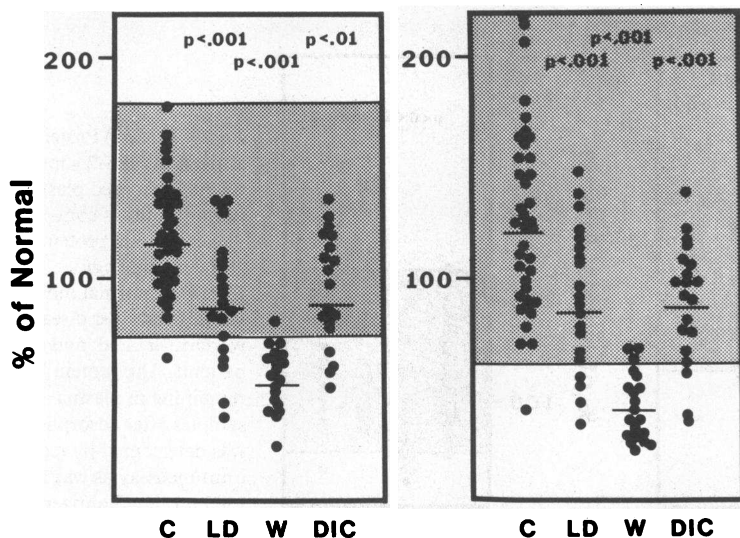

Figure 5. Protein S antigen in (*left*) HPS-2 eluates and (*right*) PEG-8000 supernatants in normal individuals, liver disease, warfarin therapy, and DIC.

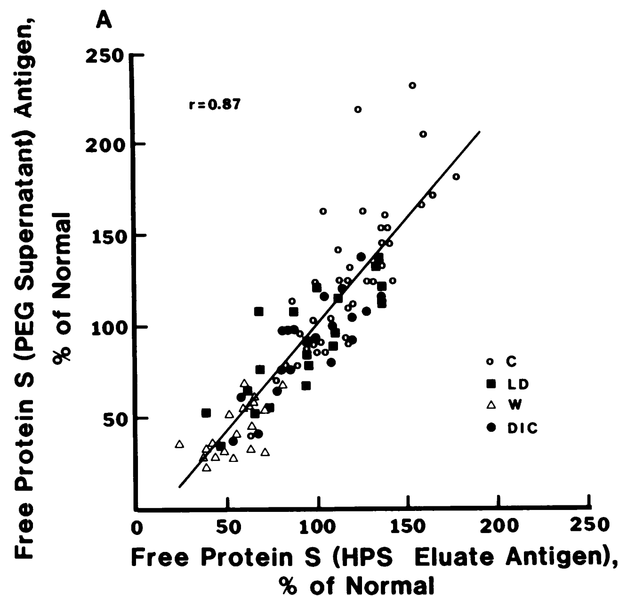

Figure 6. Correlation between protein S antigen in HPS-2 eluate and protein S antigen in PEG-8000 supernatant. Normal individuals and patients with liver disease, warfarin therapy, and DIC were examined. Correlation coefficient was 0.87 ($P < 0.001$).

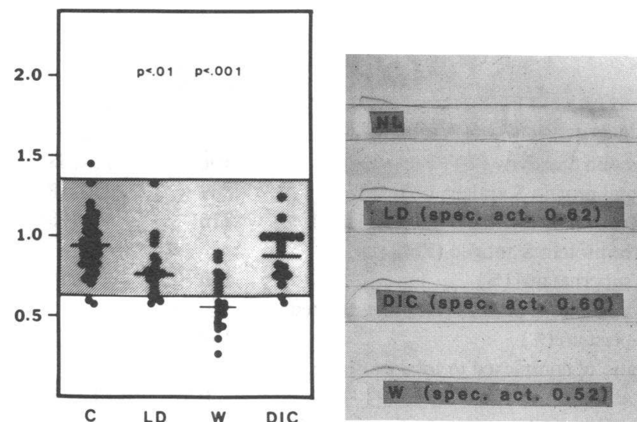

Figure 7. (*Left*) Specific activity of protein S in HPS-2 eluates in normal individuals (*C*), and patients with liver disease (*LD*), on warfarin therapy (*W*), and with DIC (*DIC*). (*Right*) Crossed immunoelectrophoresis patterns of a normal pooled plasma, a patient with liver disease, a patient with DIC, and a patient on long-term warfarin therapy. The protein S specific activities of the patient samples examined are indicated.

24 h. The extent of discrepancy increased over the following days reaching a maximum on day 5 of treatment. In no instance was a significant difference found between the rates of decrease of total protein S antigen and of free protein S antigen (HPS-2 eluate). This was surprising since, based on the information obtained from patients on stabilized warfarin treatment, one would expect the levels of C4bBP to be unaffected by oral anticoagulation and hence, by the law of mass action, a faster decrease of free protein S antigen relative to total protein S antigen would be anticipated. Table III shows the results of protein S and C4bBP determinations observed in the patients with acute deep-vein thrombosis while on heparin infusion prior to the start of oral anticoagulation. In the patients studied with acute deep-vein thrombosis, total protein S antigen levels were normal, but C4bBP levels were high. As a result, the relative concentration of complexed to total protein S was higher than normal resulting in reduced free protein S antigen and protein S activity. When the changes in the level of C4bBP antigen were followed after the start of oral anticoagulation, a normalization of the levels of C4bBP was observed within 6 d of warfarin treatment (Fig. 9). As a result of warfarin therapy, total protein S antigen levels decreased at a similar rate. As a result, there was no change in the observed ratio of complexed to total protein S antigen throughout the observation period (Fig. 9).

Discussion

Based on both clinical and basic studies, it is now clear that an understanding of the pathophysiology of the protein C anticoagulant pathway requires determination of both protein C and protein S activities in a variety of clinical conditions. Assays of protein S are particularly complex, since the protein exists both free and complexed with C4bBP. To gain insight into the response of protein S activity to common disease states and warfarin therapy, we examined protein S antigen and activity in patients with DIC, liver disease, and in those starting warfarin therapy after acute deep-vein thrombosis. In all cases, protein S functional levels were decreased, but the primary causes for the decrease were different.

In liver disease, although protein S levels are reduced, the extent of both antigen and functional decrease is much less than that observed (25) with other vitamin K-dependent factors. Interestingly, the level of C4bBP decreases more (to ~ 60% of normal) than protein S (~ 80% of normal). It is noteworthy that the specific activity of the protein S in patients with liver disease is significantly lower than that of controls. While the molecular basis of the reduced specific activity is uncertain, at least three potential mechanisms are possible: proteolytic inactivation, failure to adequately carboxylate the protein S, or two discrete populations of protein S molecules arising from different sites of synthesis. The latter possibility is suggested by two independent observations: (*a*) that relative to other vitamin K-dependent proteins, protein S is disproportionately high in these patients, and (*b*) the recent demonstration of endothelial cell synthesis of protein S (7, 8).

Protein S activity is reduced in patients with DIC. Unlike the patients with liver disease, total protein S antigen is not reduced. Instead, C4bBP is elevated, resulting in reduced free protein S. The elevation of C4bBP is in contrast to the findings of Bertina et al. (11) who reported C4bBP levels with a mean value of 81% of normal in DIC patients. Differences in assay methodology and patient selection may be responsible for this discrepancy. We find that the mean specific activity of the free protein S is not significantly reduced.

Protein S activity is markedly reduced in patients on stabilized warfarin therapy. Since total protein S antigen is reduced, there is an increase in the ratio of the C4bBP-associated protein S to free protein S. More important, the appearance of partially carboxylated forms of protein S in the circulation results in the reduction of protein S specific activity to ~ 50% of normal.

Knowledge of the half-life of protein S activity during the initiation of oral anticoagulant treatment has potential clinical

Table II. Summary of Results

	Controls (n = 39)		Liver disease (n = 20)		Warfarin (n = 20)		DIC (n = 20)	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Protein S activity (%)	106	72–158	66	29–95‡	29	9–53‡	80	40–173‡
Total protein S antigen (%)	109	75–158	80	30–126‡	68	42–97‡	112	68–196
Free protein S antigen (HPS-2 eluate) (%)	116	74–180	87	37–136‡	52	24–80‡	92	51–136*
Free protein S antigen (PEG supernatant) (%)	120	61–220	84	33–148‡	40	23–70‡	86	35–138‡
Complexed S antigen (HPS-2–adsorbed plasma) (%)	108	68–172	72	34–156‡	82	48–138‡	126	58–276*
Ratio of complexed to total protein S antigen	0.52	0.29–0.77	0.43	0.07–0.65*	0.64	0.14–0.82*	0.60	0.34–0.96*
Protein specific activity (ratio protein S activity: protein S antigen in HPS-2 eluate)	0.93	0.63–1.35	0.76	0.58–1.34*	0.56	0.27–0.89‡	0.88	0.60–1.25
C4bBP antigen (%)	110	50–240	65	20–168*	110	72–244	150	61–400
Protein C amidolytic activity (%)	93	64–136	30	9–78‡	54	33–84‡	41	8–80*
Protein C anticoagulant activity (%)	95	63–142	28	5–84‡	17	5–56‡	38	6–96‡

* $P < 0.01$. ‡ $P < 0.001$.

relevance since, by analogy to protein C deficiency, protein S deficiency might be implicated in the pathogenesis of warfarin-induced skin necrosis. One hypothesis for warfarin-induced skin necrosis in protein C-deficient patients is that protein C levels decrease rapidly after administration of warfarin, leading to a transient imbalance in the regulation of coagulation. Hence, if protein S functional levels also decrease rapidly, protein S deficient patients should also be at risk. We have investigated the rate of disappearance of protein S activity, relative to that of factor VII, protein C, and factor X in two groups of patients with active deep-vein thrombosis treated either with or without a loading dose of warfarin. The apparent half life of protein S activity was $t_{1/2}$ = 42.4 h. The half-life was not altered by the use of the loading dose. This is in contrast to results obtained previously with protein C (20). Based on this observed long half life for protein S, if protein S-deficient patients are found to develop warfarin-induced skin necrosis, as has recently been reported in one individual (26), then this cannot be attributed to a transient imbalance in the regulation of coagulation at the plasma level and alternative mechanisms would have to be proposed.

When protein S activity was monitored in patients being treated for active deep-vein thrombosis, an unexpected observation was that the rates of decrease of free and total protein S were similar. This finding was explained by the observation that C4bBP levels decreased with total protein S levels. Analysis of the pre-warfarin status of the patients with active deep-vein thrombosis revealed high levels of C4bBP with normal total S antigen levels, resulting in redistribution of protein S into the complexed form (Table III). Accordingly, both reduced free protein S antigen levels and reduced protein S activity were present before the start of oral anticoagulation. Elevated C4bBP levels in patients with active deep-vein thrombosis and patients with DIC suggest that C4bBP is an acute phase reactant. This acute phase response of C4bBP is consistent with the observation of Dahlback (27) and our observation in

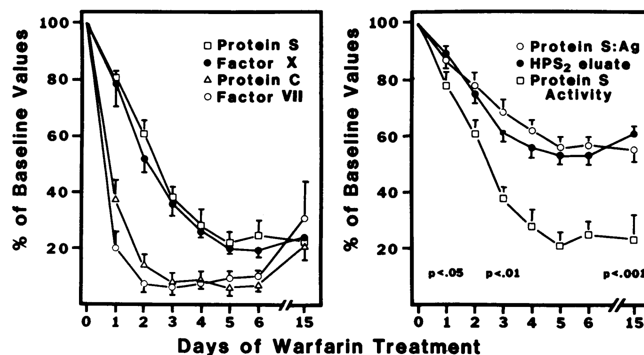

Figure 8. (*Left*) Effect of warfarin therapy on level of vitamin K dependent plasma proteins in eight patients with deep-vein thrombosis. (*Right*) Decrease in (●) total protein S antigen, (○) antigen in the HPS-2 eluate, and (■) protein S functional activity after initiation of warfarin therapy.

Table III. Protein S and C4bBP Determinations in Patients with Acute Deep-Vein Thrombosis

	Normal range	Patients with deep-vein thrombosis (n = 8)	
		Mean	Range
	% of normal plasma	%	
Total protein S antigen	75–158	117	84–192
C4bBP antigen	50–240	148	91–203*
Complexed/total protein S antigen	0.29–0.77	0.62	0.53–0.75*
Free protein S antigen (HPS-2 eluate)	74–180	85	51–112*
Protein S activity	72–158	83	45–109*

* $P < 0.05$.

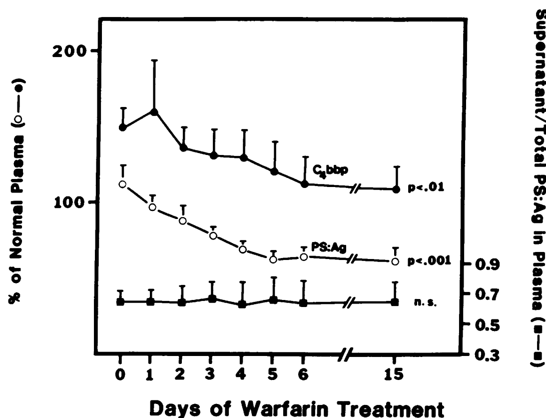

Figure 9. Levels of C4bBP and protein S antigen in patients with deep-vein thrombosis starting warfarin therapy. The antigen level (●) and the total protein S antigen level (○) are expressed as percentage of normal pooled plasma. The percentage of the total protein S complexed to C4bBP (■) is also indicated (mean±1 SD of eight subjects).

patients with inflammation (28). The normalization of C4bBP levels observed within the first week of warfarin treatment could then be related to the overall reduction of the inflammation secondary to the initiation of anticoagulation. Should the results obtained in this small series of patients with deep-vein thrombosis be confirmed in a larger number of patients with acute deep-vein thrombosis, a transient state of acquired protein S deficiency would also be established among the hemostatic abnormalities of acute deep-vein thrombosis. The major clinical implication of this is that diagnosis of congenital protein S deficiency may not be possible during the acute phase of the thrombotic process.

After this manuscript was submitted, Walker (29) described a protein S-binding protein in bovine plasma. This raised the possibility that this protein was depleted during immunoadsorption. The protein S-deficient plasma appears to have sufficient binding protein to function effectively, since the anticoagulant influence of activated protein C could be completely restored by adding purified protein S devoid of the protein S-binding protein. One of the characteristics of bovine plasma depleted of protein S-binding protein is that the full anticoagulant response is not restored by addition of protein S alone to plasma deficient in both protein S and the binding protein. Thus, we conclude that either the binding protein is not required in human plasma or, more likely, little or none is removed during immunoadsorption. The possibility that the binding protein prevents adsorption of the protein S by the immobilized HPS-2 is extremely unlikely since the depleted plasma is no longer effectively anticoagulated by activated protein C.

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