Human Plasma Protein Z Antigen: Range in Normal Subjects and Effect of Warfarin Therapy

By Joseph P. Miletich and George J. Broze, Jr

In contrast to the other well-studied vitamin K-dependent proteins that circulate in plasma, protein Z antigen is much more variable. The concentration in plasmas collected in EDTA from 455 normal, healthy donors is normally distributed with a mean of 2.9 µg/mL (46 nmol/L) and a SD of 1.0 µg/mL (95% interval of 32% to 168% of the mean). No significant correlation to age or sex could be detected. In comparison, the concentration of protein Z antigen measured with the same type of assay on the same 455 samples has a log normal distribution with a mean of 4.0 µg/mL (65 nmol/L) and a 95% interval of 70% to 138% of the mean. Also in marked contrast to other plasma vitamin K-dependent proteins, the total protein Z antigen level is extremely low in patients on stable warfarin therapy (range 1% to 16% of normal). Moreover, even though >95% of the antigen in normal plasmas adsorbs to barium citrate (a crude reflection of the presence of γ-carboxyglutamic acid [Gla] residues), in the patients taking warfarin almost all of the small amount of the antigen failed to adsorb, suggesting that virtually no protein Z had its full complement of Gla residues. Total protein C antigen in the same 25 patients averaged 53% of normal (34% to 72%) and 54% (average) of the total remaining antigen still adsorbed to barium citrate. The concentration of protein Z antigen in the plasma of a normal individual given a loading dose of warfarin fell at an initial rate of ~20% a day, indicating a plasma half-life (t½) of 2 to 3 days.

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Materials. Affigel-10 was purchased from Bio-Rad Laboratories (Richmond, CA), and cyanogen bromide-activated Sepharose 4B, Sepharose S-300, and Sephadex G-25 were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Repligen (Cambridge, MA) was the source of recombinant Protein A. Bovine serum albumin (BSA), goat anti-mouse IgG alkaline phosphatase conjugate, Trizma base, fluorescein isothiocyanate (FITC), and latex beads (0.8 µm) were bought from Sigma Chemical (St Louis). Dulbecco's modified Eagle's medium l-glutamine, nonessential amino acids, penicillin/streptomycin, and bovine and horse sera were obtained from KC Biological (Lenexa, KS). Epicon assay plates, Epicon Performance Verification Particles, and Epicon Reference Particles (590/620 nm) were purchased from Pandex Laboratories (Mundelein, IL). All other chemicals were reagent grade or better and came from Sigma or from Fisher Scientific (Pittsburgh).

Plasmas. Plasma samples used to determine the distribution of protein Z antigen in the normal population were collected from participants in health fairs, in 5-mL Vacutainer tubes (Vacutainer Systems, Rutherford, NJ) that contain 48 µL of 15% K2 EDTA anticoagulant. Cells were removed by centrifugation at 3,000 g for 15 minutes and the plasmas were quick-frozen at -85°C. Serum was also obtained for routine chemistries. Subjects were considered normal if no more than three of the following laboratory tests were further than 2 SD from the normal mean and all results were within 3 SD of the mean: electrolytes (sodium, potassium, chloride, and total CO2); standard blood chemistry (blood urea nitrogen, creatinine, total bilirubin, uric acid, glucose, total calcium, serum phosphate, total protein, albumin, and cholesterol); enzymes (alkaline phosphatase, γ-glutamyl transpeptidase, aspartate aminotransferase, total lactate dehydrogenase, and creatine kinase); and routine hematology (erythrocyte count, hemoglobin, packed RBC volume, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, RBC distribution width, WBC count, lymphocyte count, monocyte count, granulocyte count, platelet count, and mean platelet volume). Subjects also answered a
questionnaire; there was no indication of concurrent antibiotic usage, liver disease, anticogulant therapy, or hemorrhagic or thrombotic disorders in any of the individuals included in this study. Participants were informed that their blood samples would be used in normal range studies; samples and histories were identified only by a coded number and not by name to insure confidentiality.

Plasma samples from blood donors randomly selected during a 2-week interval were generously provided by Dr. Laurence Sherman of the St. Louis Subchapter of the American Red Cross. After a unit of blood is collected from a donor, one sample for serum and one for cells and plasma (EDTA anticoagulant) are routinely taken (using Vacutainer tubes and a separate port of the same line) for testing at the Red Cross. The anticoagulated samples that were processed within 24 hours from the time of collection and that were of no further interest to personnel at the Red Cross, from donors with no evidence of infectious disease, were used to recover plasmas as described above. Samples were identified only by a bar-coded number and were not identified in any manner by donor name. Because donors must wait at least 8 weeks between donations, all samples were from different persons. The first day’s samples (n = 429) were pooled (1.0 mL from each) and aliquotted as a normal control standard; samples from subsequent days were individually analyzed.

Citrated plasma samples from 25 outpatients taking warfarin for various cardiac abnormalities were recovered from blood sent to the clinical hemostasis laboratory for routine monitoring of the prothrombin time. Each patient’s record was checked to insure that therapy had been continuous and uneventful for at least 6 months with no medical intervention for other illnesses.

Four different lots of pooled normal plasma (citrate anticoagulant, 20 donors) were obtained from George King Biomedical (Overland Park, KS).

Plasma containing <1% protein Z was prepared by immunoadsorption using either monoclonal antibody HZ-1 or HZ-7 attached to Affigel-10 at a concentration of ~10 mg/mL according to the manufacturer’s directions. Five-milliliter (1.1 x 5 cm) columns of the immunoadsorbant were equilibrated in normal saline buffered with 20 mmol/L of Tris at pH 7.4. Ten milliliters of unbuffered saline followed by 25 mL of pooled normal plasma (George King Biomedical) was passed through the columns at rates of 1 mL an hour for HZ-1 and 5 mL an hour for HZ-7. The saline and the first 5 mL of plasma eluted were discarded; the next 17 mL were pooled. Neither plasma had any detectable protein Z antigen as compared with a buffer control as measured with the standard two-site assay or the barium citrate adsorption assay (using either monoclonal antibody) as described below. In addition, both plasmas were free of protein Z antigen as assessed by Western blot analysis using a polyclonal antibody (raised in a goat) with a sensitivity of 0.1 ng (<1% of normal plasma given the 5 μL vol tested).

Estimates of affinity constants. Two methods were used. In the first (the free antigen method), antibody was held constant at a concentration known by trial to be close to its Kd. Purified protein Z (in Tris-buffered saline containing 100 μg/mL bovine serum albumin, BSA) was varied from roughly one-fifth Kd to 5 x Kd. After incubation for sufficient time to establish equilibrium at the lowest antigen concentration, latex particles coated with goat anti-mouse IgG were added in large excess (enough to adsorb at least 5 times the amount of mouse antibody present in 10 minutes). Monoclonal antibody specific for protein C was used to correct for nonspecific adsorption of protein Z. After removal of the particles by centrifugation at 12,000 g for 1 minute, the concentration of antigen in the supernatant samples was determined using the standard assay described below. Bound antigen was calculated and Kd was estimated from the intercept of a double-reciprocal plot. The second method (sequential saturation) was essentially the same except that incubations were set up (replicates of eight) in Epicon plates and the Screen Machine added the goat anti-mouse particles. Two minutes before collection of the particles, FITC-labeled protein Z was added to each well. With this method, bound fluorescence is proportional to the concentration of free antibody sites. Background fluorescence was determined from wells containing no monoclonal antibody, and the fluorescence corresponding to the total concentration of antibody sites was determined from wells containing no unlabeled protein Z. Calculations were performed on the IBM PC using software supplied by Pandex Laboratories (Mundelein, IL), but essentially involve Scatchard analysis of the bound and free concentrations, assuming that the labeled probe does not significantly alter the equilibrium protein Z and the antibody during the 2-minute pulse.

Monoclonal antibodies. Standard methods for immunizing Balb/C mice, fusing splenic WBCs to Sp 2/0-Agl4 mouse myeloma cells with polyethylene glycol (PEG), and isolating hybridomas with selective growth media were followed. Supernatant samples were tested with an enzyme-linked immunosorbent assay (ELISA) using goat anti-mouse IgG conjugated to alkaline phosphatase and wells coated with protein Z, protein C, protein S, factor IX, or prothrombin. Cell lines positive and specific for protein Z were subcloned on soft agar; positive sublines were subjected to at least one freeze/thaw cycle. Surviving hybridomas that still secreted specific antibody to protein Z after several weeks in culture were selected for further characterization.

Proteins. Protein Z and thrombin were isolated as previously described.1,2 Thrombin-treated protein Z was prepared by incubating 1.0 mg/mL of protein Z with 70 μg/mL of α-thrombin in 0.1 mol/L of NaCl, 20 mmol/L of Tris, pH 8.0, for 3 hours at 37°C. The thrombin-cleaved protein Z (Zc) was separated from both thrombin and uncleaved protein Z by chromatography using a Pharmacia fast protein liquid chromatography (FPLC) Mono Q column equilibrated in 0.1 mol/L of NaCl, 20 mmol/mL of MES, pH 6.5, and developed with a linear NaCl gradient.

Murine monoclonal antibodies were purified from ascites using protein A-Sepharose 4B prepared from recombinant protein A and cyanogen bromide activated Sepharose 4B, according to the manufacturer’s instructions (2.5 mg protein A per mL of packed gel). IgG was eluted with 0.1 mol/L of sodium citrate, pH 3.5, dialyzed against 0.1 mol/L of NaCl, 10 mmol/L of HEPES, pH 7.5, and stored at −85°C. Proteins to be fluorescein-labeled were dialyzed against 0.1 mol/L of sodium phosphate, pH 7.4, and adjusted to a concentration of 1 mg/mL. FITC was dissolved in freshly prepared 0.5 mol/L of sodium carbonate, pH 9.5, at a concentration of 0.8 mg/mL and added to the antibody at a ratio of 1:4 (vol:vol). After 2 hours of gentle mixing at room temperature, the labeled protein was separated from unbound fluorescein by chromatography through at least 5 mol of Sephadex G-25, equilibrated in 0.15 mol/L of NaCl, 20 mmol/L of Tris, pH 7.4. The molar extinction coefficient for FITC was taken to be 80,000 at 495 nm; the concentration of protein was calculated from the optical density at 280 nm after subtraction of 0.35 times the optical density at 495 nm to correct for the fluorescein, using an extinction coefficient of 14.5 for antibodies and 12.0 for protein Z. Between 5 and 12 mol of fluorescein were incorporated per mol of protein by this method.

Instrumentation. Plasma samples were handled with a Tecan model 505 Robotic Sample Processor (Tecan US, Chapel Hill, NC). The precision of pipetting 5.0 μL was >1% over a wide range of plasma viscosities; the drawn Teflon pipetting tip was washed inside and out with 2.0 mL of saline between samples, and no carryover could be detected. A 96-well Epicon assay plate can be loaded with standards, samples, and controls in <15 minutes; care was taken to guard against sample evaporation even within this short time.

Sample analysis was performed with a Screen Machine (Pan-
The well-to-medium coefficient of variation for all steps of particle and reagent addition, separation, washing, and quantitation as determined by using latex particles impregnated with fluorescent dyes is 4% to 8% for single-channel fluorescence and <2% when the ratio of fluorescence in two channels is calculated using reference particles. Raw data were transmitted to an IBM PC for storage and processing, using software supplied by Pandex. Linear regression coefficients were calculated using the least-squares method.

Assays. Standards for assays of protein Z antigen in plasma were prepared by combining purified protein Z (1.12 mg/mL) and the pooled plasma that had been depleted by immunoadsorption in appropriate volumes to give final concentrations of 0, 1, 2, 4, and 6 μg protein Z/mL; 100-μL aliquots were stored at −85°C and were discarded after a single day’s use.

For the standard assay of total protein Z, plain latex particles (0.5% wt/vol) mixed with reference particles (0.01% wt/vol) were coated with monoclonal antibody HZ-7 (100 μg/mL) in 0.1 mol/L of sodium phosphate, pH 5.5, for 2 hours with gentle agitation at room temperature, and then stored for up to 2 weeks at 4°C. Just prior to use, the required volume of coated particles was collected by centrifugation for 2 minutes at 12,000 g; the supernatant was replaced with twice the volume of TBSA. The coated particles were thoroughly dispersed in an ultrasonic bath. Five microliters of plasma sample diluted with 15 μL of TBSA were placed in the Epicon assay wells. The coated particles (20 μL) were added to each well by the Screen Machine, followed 10 minutes later by 20 μL of fluorescein-labeled monoclonal antibody HZ-1 at a concentration of 10 μg/mL in TBSA. After an additional 20-minute incubation, the particles were collected on the filters, washed twice with 50 μL of Tris-buffered saline and analyzed for bound fluorescence. All reactions were at room temperature. Separate experiments demonstrated that the reaction of both antibodies with protein Z was >90% complete in 10 minutes under the assay conditions. Further increase in signal was negligible at longer incubation times. The actual timing of addition of the reagents partially reflects a minimization of total assay time for the instrument, which can handle up to ten plates in the same batch.

For the assay of barium-adsorbable antigen, 20 μL of the appropriate fluorescein-labeled antibody (or antibodies) at a concentration of 10 μg/mL in buffer containing 75 mmol/L of sodium citrate, 40 mmol/L of NaCl, and 100 μg/mL BSA, pH 7.4, was added to samples diluted as above, followed in 20 minutes by 20 μL of 150 mmol/L of BaCl2. After 5 additional minutes, the barium citrate particles were collected, washed once with 50 μL of buffer (50 mmol/L of BaCl2, 100 mmol/L of NaCl, 20 mmol/L of Tris at pH 7.4) and analyzed for bound fluorescence. These conditions were found to “capture” >95% of the vitamin K-dependent antigens reproducibly whether the plasma was initially anticoagulated with citrate or EDTA. Reference particles could not be included as an internal standard, however, since they interfered in an unknown manner with the formation of barium citrate particles that were retainable by the filters.

RESULTS

Characterization of monoclonal antibodies. Six hybridomas from three separate fusions survived storage in liquid nitrogen and continued to secrete specific antibody in long-term culture. Each antibody was evaluated for cross-reactivity with the other antibodies, for affinity toward protein Z and apparent kinetics, and for its ability to retain reactivity when labeled with FITC or when bound to latex particles. One antibody (HZ-1) was clearly superior as an FITC-labeled probe for detecting bound protein Z; HZ-7 had the best characteristics for a “capture” antibody and was not cross-reactive with HZ-1. Both antibodies are IgG1κ. The estimates of Kd were 4.6 × 10−9 and 5.7 × 10−9 mol/L−1, and 5.2 × 10−9 and 2.7 × 10−9 mol/L−1 for HZ-1 and HZ-7, respectively, by the free antigen and the sequential saturation methods (described in the Materials and Methods section). Both antibodies are specific for protein Z by Western blot analysis of plasma (data not shown) and detect only protein Z in the protein fraction adsorbed from plasma with barium citrate (see below). By Western blot, these antibodies also recognize Zl, which is missing the NH2-terminal Gla-domain (described in Discussion section) and migrates slightly faster in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system.

Assays for human protein Z. The two antibodies were used as described in the Materials and Methods section in a two-site particle concentration immunoassay. The data shown in Fig I demonstrate that (a) fluorescence is directly and linearly proportional to the concentration of purified protein Z whether it is diluted in buffer or in the plasma immunodepleted of protein Z, and (b) that the protein Z in pooled normal plasma behaves in exactly the same fashion as purified protein Z, regardless of whether it is diluted in immunodepleted plasma or in buffer. No difference was detected between plasmas immunodepleted with either antibody. Protein Z missing the NH2-terminal Gla-domain (Zl) reacted normally in the assay, demonstrating that neither antibody is sensitive to changes in that region of the protein. The overall reproducibility of the assay was evaluated (using reference particles to correct for minor changes in well geometries) by assaying the normal pool (EDTA) in duplicate ten separate times on different days using the same reagents; the mean was 2.77 μg/mL with a SD of 0.04 μg/mL (calculated coefficient of variation of 1.3%). Even
when different preparations of reagents and standards were used, the coefficient of variation of the assay did not exceed 4%. The signal was linear with increasing protein Z up to a concentration of 10 μg/mL when samples were processed as described. The detection limit for the assay as described is 10 ng/mL (signal ≥ mean background + 1 SD), although this can be extended by simply using larger sample volumes.

More than 95% of the protein Z antigen in normal plasma can be adsorbed to barium citrate. Barium citrate particles are retained by 0.2-μm filters. Therefore, it seemed reasonable to use barium citrate to “capture” protein Z (as well as all the other vitamin K-dependent proteins) and quantitate it using either of the specific FITC-labeled monoclonal antibodies. Both antibodies gave very similar, linear standard curves and each detected the same amount of protein Z antigen (data not shown). The standards are made up in immunodepleted plasma that still had normal concentrations of the other barium-adsorbable proteins, thus attesting to the specificity of each antibody. When both FITC-antibodies were included, the fluorescence signal was additive, proving again that the antibodies recognize distinct epitopes and do not interfere with one another’s binding. Furthermore, neither antibody prevents protein Z from binding to barium citrate or vice versa. These assays are not as reproducible (coefficient of variation ~10%) as the two-site latex particle assay described above due to the added variability of barium adsorption and filtration and the lack of an internal reference standard, but they serve to reinforce the fact that each of the antibodies used detects the same antigen in normal plasma. When Z was added to buffer or to immunodepleted plasma, it failed to adsorb to the barium citrate (data not shown), as might be expected since it is missing the Gla-domain. Thus, although both antibodies bind to Z, neither could detect it in the barium adsorption assay since the protein and antibodies react but pass through the filter without binding to the barium citrate.

**Distribution of plasma protein Z antigen in the normal population.** The plasma concentration of protein Z antigen was determined in 455 normal, healthy adults (aged 19 to 80 years). Each sample was assayed in duplicate all on the same day to reduce assay variability to the absolute minimum. The mean level was 2.87 μg/mL with a SD of 1.00 μg/mL and a range of 0.63 to 5.67 μg/mL. Protein C antigen was determined in the same samples (using virtually identical methods and conditions) using two monoclonal antibodies specific for protein C (HC-2 and HC-4). In Fig 2, the data are plotted as frequency histograms with concentrations expressed as percentages of the means. The lines designate the predicted distribution frequency assuming a normal distribution for protein Z and a log normal distribution for protein C. Variability between individual plasma levels is at least two times greater for protein Z than for protein C, the latter being more typical of other known vitamin K-dependent plasma proteins. Furthermore, there was virtually no correlation between the concentrations of protein Z and protein C in these samples, when each was expressed as a percentage of the population mean (coefficient of correlation [COC] = 0.13). There was also no correlation of protein Z antigen concentration to age or gender (data not shown). Four different lots of citrated pooled normal plasma assayed in quadruplicate had mean antigen levels of 2.36 (GK 411C), 2.48 (GK 920C), 2.55 (GK 317), and 2.30 (GK 928) μg/mL; the overall mean, 2.42 μg/mL, is 84% of the mean for the 455 samples collected in EDTA anticoagulant, which is very close to the value of 83% calculated from plasma dilution by the citrate anticoagulant assuming a mean packed RBC volume of 44%. This also agrees well with the estimate of 2.2 μg/mL in citrated samples we previously reported using a rabbit antiserum and electrophoresis.

These normal range studies did not include infants, children, or adolescents, but 18 samples obtained from neonates (mean age 11 days, range 3 to 28 days) were checked. These newborns were hospitalized for various clinical conditions and cannot necessarily be taken as representative of normal healthy neonates. Nonetheless, protein Z antigen averaged 57% ± 23% (SD) of the normal adult level with a range of 29% to 107%. This was significantly higher and more variable than protein C antigen, which averaged 28% ± 8% (SD) of the normal adult level with a range of 21% to 48%. There was again a very limited correlation (COC = 0.29) between the two antigen concentrations.

**Decrease in plasma protein Z antigen with warfarin treatment.** Citrated plasma samples from 25 outpatients receiving stable warfarin therapy without incident for at least 6 months were analyzed for protein Z and protein C antigen (Table 1). Although the total amount of protein C antigen was decreased by only ~50%, total protein Z antigen was reduced nearly 13-fold. Making the samples 5 mmol/L in EDTA made no significant difference in the protein Z...
assay. When protein Z antigen was "captured" by adsorption to barium citrate and quantitated with either monoclonal antibody (HZ-1 shown), the mean concentration was only 0.02 μg/mL (1% of normal), indicating that most of the little remaining antigen is also deficient in Gla residues; 15 of the 25 samples had no detectable protein Z antigen in this assay. Similar assays using either of the monoclonal antibodies specific for protein C (HC-2 shown) demonstrated that roughly half the total amount of this antigen still had sufficient Gla residues to bind to barium citrate. Because both HZ-1 and HZ-7 react equally well (but at different epitopes) with Z, that has the Gla-domain completely removed, it is extremely unlikely that the binding of both is somehow adversely affected by deficiency of γ-carboxylation of Gla residues in that region. These data indicate that protein Z antigen in plasma is markedly more sensitive to chronic warfarin therapy than is protein C or the other vitamin K-dependent plasma proteins.6,4

Using the electroimmunodiffusion assay we previously reported that protein Z antigen becomes undetectable after 3 to 5 days of warfarin therapy, but we could not then absolutely distinguish a decrease in total antigen from a possible loss of reactivity with the antisera due to decreased carboxylation (although we recognized that a complete loss of reactivity would be unusual for a polyclonal antibody). The disappearance of antigen was investigated more closely with the assays described here, since neither of the monoclonal antibodies requires the presence of Gla residues for reactivity. Twenty months prior to the current experiments, one of us (J.P.M.) took warfarin during studies of human factor VII.7 Samples had been saved at -85°C and were assayed for protein Z antigen along with a freshly drawn, never-frozen sample (Fig 3). Several features are apparent. First, the plasma concentration of protein Z antigen in a healthy individual shows no significant variation throughout the day or across at least 20 months. Second, protein Z antigen is stable when frozen at -85°C for at least 20 months. Third, when plasma protein Z antigen falls in response to loading dosages of warfarin, the apparent plasma half-life (t1/2) is 2 to 3 days; the increase in the plasma level following administration of vitamin K is similar. Fourth, in this setting of short-term vitamin K antagonism, the concentration of antigen adsorbable to barium citrate parallels total antigen, showing that very little "Gla-less" protein Z appears in plasma. Therefore, the total contribution of newly-synthesized protein Z antigen in the plasma is negligible, which means that the true biological t1/2 of total antigen is in fact several days. Whether this is the true clearance rate from plasma or there exists a significant extravascular pool of protein Z antigen in equilibrium with plasma cannot be determined from these experiments. It should be noted that the protein Z antigen in this experiment never reached the same extremely low levels observed in patients on chronic, lower doses of warfarin because vitamin K was administered after 2 days when the factor VII level was <1%. Presumably, the protein Z antigen would have continued to decrease otherwise.

Verification of wide range of protein Z antigen in normal subjects. Twenty-five hundred blood samples (EDTA anticoagulant) collected during a 3-week interval were generously supplied by the American Red Cross (described in the Materials and Methods section). The distribution of antigen concentrations was the same as described above, i.e., approximately normal with nothing to suggest the presence of subpopulations. Nevertheless, 18 of the plasmas with very low and 18 with very high antigen concentrations were analyzed in more detail, as shown in Table 2. The measured level of protein Z was the same in every case regardless of whether the two monoclonal antibodies were used separately or together. Therefore, the wide range of concentrations found in normal subjects cannot be an assay artifact based on the presence of a polymorphism in the population unless it affects two independent epitopes to exactly the same extent. Within the limits of the assays, the protein Z antigen in every case was almost completely adsorbable to barium citrate as well, making it unlikely that protein Z can be synthesized independent of vitamin K in some individuals and therefore cause the wide range of values. Total protein C antigen measured in each group was entirely normal and showed the expected distribution, again verifying that there was no correlation between protein Z and protein C concentrations.

The possibility that some other substance(s) in plasma somehow affected the amount of antigen detectable by the antibodies (eg, by binding to protein Z) was also considered. Mixtures of two plasmas (with 31% and 179% antigen, 30% and 185% antigen, and 17% and 299% antigen) showed that the level of detectable antigen in plasma was not simply due to the percentage of antigen in the plasma.
Table 2. Protein Z and Protein C Antigen in Plasmas With Extremely High or Low Concentrations of Protein Z

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<th>Protein Z (Mean ± SD</th>
<th>Protein C (Mean ± SD)</th>
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<td></td>
<td>Barium Adsorbable</td>
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<td></td>
<td>Total</td>
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<td>High (n = 18)</td>
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<td>Mean</td>
<td>185 ± 21</td>
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<td>Range</td>
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<td>Low (n = 18)</td>
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<tr>
<td>Mean</td>
<td>27 ± 3</td>
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<td>Range</td>
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Plasmas (EDTA) with either high or low concentrations of protein Z were selected from 2,500 samples taken from blood donors. Duplicate determinations were averaged and expressed as a percentage of the mean for the 455 control samples used for the study of normal range.

respectively) were made, resulting in molar ratios from 0 to 1 in increments of 0.1. After a 2-hour incubation at 37°C, the mixtures were analyzed. The response was linear \(r^2 = .993\), indicating that if an interfering substance exists in one of the plasmas it is either incapable of interfering with protein Z from the other plasma or it is present in some limiting amount.

To address the latter possibility, the pooled control plasma (EDTA) was chromatographed on a column of Sephacryl S-300 equilibrated in the assay buffer. Only a single peak of antigen (eluting after IgG but slightly before protein C and albumin) was detected in the fractions. Thus, the pooled plasma did not contain a significant concentration of a substance of appreciable mol wt that could bind to protein Z in the assay buffer. Therefore, the wide range of protein Z antigen concentration observed in normal plasmas is not likely to be an artifact based on the presence of variable quantities of interfering substance(s) in some plasmas.

Finally, the possibility that protein Z was altered in some plasmas by the procedures for obtaining plasma was considered. Because protein Z is specifically cleaved by thrombin, which could conceivably be present at very low concentrations at the time of venipuncture, we tested Z1 in the standard assay buffer. Therefore, the wide range of protein Z antigen concentration observed in normal plasmas is not likely to be an artifact based on the presence of variable quantities of interfering substance(s) in some plasmas.

The concentration of protein Z antigen found in 95% of adult plasmas varies from 0.9 to 4.8 µg/mL (32% to 168% of the mean), with an actual observed range of 0.6 to 5.7 µg/mL. This distribution is considerably wider than has been found for the other vitamin K-dependent plasma proteins. No correlation of concentration to age or sex could be detected, although any minor dependence could be easily obscured by the nearly tenfold range in normals. Protein Z is also unique in its sensitivity to chronic warfarin therapy, which reduces the concentration of total plasma antigen to 8% of normal. Furthermore, even though nearly all of the antigen in normal plasmas will adsorb to barium citrate, suggesting that at least some of the Gla residues are present in every molecule, 60% of samples from patients taking warfarin chronically have no detectable adsorbable antigen. Whatever the function of protein Z may be, any given individual's plasma concentration clearly need not be maintained very near the population mean for any essential physiological activity. Whether this marked drop in antigen level in patients taking warfarin may be of clinical interest in some special settings (eg, monitoring compliance or refractoriness to warfarin therapy) has not yet been evaluated.

The possibility that protein Z is not a plasma protein in the sense that plasma is its site of action must be considered. If non-liver tissue (eg, bone, endothelium, etc) is the site of synthesis and of function for protein Z, and if plasma levels only reflect an average clearance, this could conceivably explain the wide range in normals, the greater sensitivity to warfarin, and the inability to find a function using plasma-based in vitro assay systems. A limited analogy for this concept might be the iron system. Ferritin can be measured in plasma even though it "functions" principally extravascularly, and it has a tenfold range of normal (25 to 240 and 12 to 130 ng/mL for adult men and women, respectively, in our laboratory). Transferrin, on the other hand, has a much tighter normal range (204 to 360 mg/dL) and acts principally intravascularly. When the role of protein Z is discovered, it will be essential to bear in mind the wide variation in plasma concentration in normal subjects before attempting to correlate any clinical problems with protein Z "deficiency" based on measurements in plasma.

The observation that thrombin-cleaved protein Z does not bind to barium citrate is interesting because cleavage of protein S by thrombin causes a similar change in that protein. The cleavage sites are not homologous, however. Dahlbäck and colleagues showed that bovine protein S has a region between residues 45 and 75 that is not homologous to the other vitamin K-sensitive proteins in plasma. Thrombin hydrolyzes two bonds (at arginines 52 and 70) releasing an 18 amino acid peptide; the NH2 terminus containing the Gla residues remains linked by a disulfide bond (involving cysteine 47) to the rest of the molecule. Human Z, on the other hand has only a single new NH2-terminus that is enzymatic activity. It may be a cofactor similar to protein S, which also lacks enzymatic activity. It may have nothing to do with hemostasis; the evidence linking protein Z to blood coagulation at this time is its structural similarity to six other vitamin K-dependent plasma proteins (factors II, VII, IX, and X, and proteins C and S).

The function of protein Z remains unknown. It seems unlikely that human protein Z or any portion of it has...
identical in 10 of 14 residues with the sequence of bovine protein Z beginning with residue 42 (G.J. Broze and J.P. Miletich, unpublished observations, May 1986). If a function for protein Z is discovered, cleavage by thrombin or other proteases may be a means of regulating interactions dependent on the presence of the Gla residues.

The two-site monoclonal antibody particle concentration fluorescence immunoassay described in this study has favorable characteristics when compared with other antigen assays. Sample requirement is low (5 μL), sensitivity is high (limit of detection <1% of normal), and reproducibility is great (coefficient of variation <2%). Data handling is simple since signal is directly and linearly proportional to concentration over the entire range, with very low background. Sample throughput is substantial; one person using the equipment described can assay ~80 samples an hour in duplicate. There are no toxic or radioactive substances, and the reagents have proved stable for 6 months, with no indication of deterioration as yet. Similar assays developed for factor VII and factor IX share these characteristics, indicating that many antigens of clinical interest can be measured rapidly and accurately in large numbers using these techniques.

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JP Miletich and GJ Jr Broze