Radioimmunoassay of Factor V in Human Plasma and Platelets

By Paula B. Tracy, Lisa L. Eide, E. J. W. Bowie, and Kenneth G. Mann

Homogeneous, single-chain human factor V was used to develop a double antibody competition radioimmunoassay to measure factor V concentrations in plasma and platelets. Standard curves were constructed that allow for the detection of as little as 20 ng factor V/ml of plasma. Normal factor V concentrations range from 4 to 14 μg/ml of plasma with an average value of 7.0 ± 2.0 μg/ml (n = 64). No correlation was observed between antigen levels and age or sex. The radioimmunoassay data are consistent with factor V clotting assays, providing freshly drawn plasma is used in the bioassay. Radioimmunoassay of washed platelets indicate that 0.63–1.93 μg of factor V is present per 2.5 × 10⁸ platelets (4612–14126 molecules of factor V per platelet). When normalized to individual hematocrits and platelet count, the data indicated that platelets contribute approximately 18%–25% of the factor V found in whole blood. In addition, two individuals with functionally deficient factor V were examined and found to be deficient in both antigen and activity.

Factor V is a single-chain, high molecular weight plasma protein that serves as a cofactor in the factor Xa-catalyzed conversion of prothrombin to thrombin. Even though this activity was first described in 1943 by Owren, the isolation of human factor V eluded investigators until very recently due to the protein’s susceptibility to proteolytic cleavage during manipulation. Consequently, the concentration of factor V in plasma has not been reported.

Factor V deficiencies, based on bioassay data, are well documented and have been shown to occur with liver dysfunction and with acquired factor V inhibitors. More recently, Griffin has postulated that some individuals, with a combined factor V and factor VIII deficiency, lack an inhibitor of activated protein C and suggested that these individuals may be devoid of factor V activity but not factor V antigen. Consequently, many reported factor V deficiencies may be the result of the inability of the factor V protein to function rather than a deficiency in the antigen itself.

Recently, our laboratory has described the purification of homogeneous, single-chain human factor V using a murine hybridoma antibody. This antigen has been used to develop a double antibody radioimmunoassay that has been applied to measurement of plasma and platelet concentrations of human factor V. Two individuals deficient in factor V activity have also been studied.

MATERIALS AND METHODS

Proteins

Homogeneous, single-chain human factor V was purified using a hybridoma antibody as described by Katzmann et al. Factor Va was prepared by incubation of factor V with catalytic amounts of thrombin as described previously. Factor V (Va) protein concentrations were determined based on a molecular weight of 330,000 with an ε₂₈₀ nm of 9.6. Kane and Majerus reported an ε₂₈₀ nm of purified human factor V of 8.9, which would result in only an 8% difference in our protein readings. Bovine thrombin was prepared as described previously for bovine thrombin as described previously. Factor V (Va) protein concentrations range from 4 to 14 μg/ml (described in this report). Human plasma ranges from 4 to 14 μg/ml (described in this report). When human plasma is used for the assay plasma standard, fully activated factor V has a specific activity 2–5 times higher than that observed with a bovine plasma standard, i.e., 3400–8500 U/ml of protein.

Antisera

Specific antiserum to human factor V was produced in a burro by 10 weekly subcutaneous injections of 0.1 mg of human factor V in a 1:1 emulsion with complete Freund’s adjuvant. Goat anti-burro IgG antiserum was prepared in an analogous procedure as described previously.

Radioimmunoassay

Plasma and platelet concentrations of factor V were determined using a double antibody competition radioimmunoassay similar to that described previously for bovine factor V. Burro anti-human...
factor V was the primary antibody and goat anti-burro antiserum was the precipitating antibody. Incubation mixtures in 1.0 x 6.0 cm plastic tubes contained the following: 0.2 ml of 125I-factor V (20 ng), 0.2 ml of either a working factor V standard solution (0.02–10.24 μg/ml) or appropriate plasma or platelet dilutions, and 0.2 ml of a 1:2500 dilution of burro anti-human factor V antiserum that had been diluted in 2.5% normal burro serum. Following a 1-hr incubation at 37°C, 0.2 ml of goat anti-burro antiserum was added and the tubes incubated for a minimum of 16 hr at 4°C. The tubes were centrifuged, washed with assay buffer, and then assayed for radioactivity. All reagents for this assay were diluted in assay buffer: 0.075 M Tris, 0.075 M NaCl, pH 7.0, containing 1% bovine serum albumin and 1% Triton X–100.

The working titer of the burro anti-human factor V antiserum was determined using an antibody dilution study in the above radioimmunoassay. An antiserum dilution of 1:2500 yielded an optimal initial binding (B/T), of 40%. Standard curves were prepared using known amounts of purified human factor V.

**Source of Samples**

Blood was drawn from normal volunteers by venipuncture into 2.85% Na2 citrate – 2 H2O (9:1, v/v). Platelet-free plasma was obtained by centrifugation of the citrated blood at 1500 g for 20 min. Platelets were obtained from citrated, whole blood to which 5 μM PGE1 was added immediately following venipuncture to prevent platelet activation. Platelets were collected and washed to remove plasma according to the method of Orloff and Michaeli17 in appropriate solutions containing 5 μM PGE1. All platelet counts were performed with a Coulter Counter. The platelet suspensions to be used in the radioimmunoassay were diluted with platelet wash medium to yield 2.0–5.0 x 10^8 platelets/ml. The platelets were completely lysed in 0.2% Triton X–100 as determined by light scattering.

**Factor V-Immunodeficient Plasma**

Seven parts blood was collected into one part CPD-A anticoagulant (0.16 M dextrose, 0.09 M trisodium citrate, 0.015 M citric acid, 0.016 M sodium biphosphate, 0.002 M adenine). The plasma obtained was rendered deficient of both factor V activity and antigen by passage through a column of a murine anti-human factor V hybridoma antibody coupled to Sepharose.1 This procedure results in less than 0.1% detectable factor V, by both bioassay and radioimmunoassay, remaining in the plasma.

**RESULTS**

**Factor V Radioimmunoassay Standard Curve**

Figure 1 shows a representative dose–response curve in which the bound 125I-human factor V is plotted as a log function of the addition of unlabeled, highly purified human factor V. Since this is a competition assay, the amount of radioactivity found in the precipitate is inversely proportional to the amount of unlabeled factor V added. Typically, the assay is linear from 0.016 to 1.024 μg factor V per assay tube, equivalent to 0.08–5.12 μg/ml plasma.

The accuracy and validity of the assay, as well as the specificity of the antiserum for factor V were examined. Line “c” in Fig. 2 illustrates a typical standard curve using factor V in assay buffer (slope = –3.10) in which B/B0 is plotted versus log concentration factor V. Line “b” represents a standard curve obtained when factor V is added back to factor V-immunodeficient plasma (slope = –3.32). The plasma dilution curve, covering a concentration range of 0.3 – 3.5 μg factor V/ml of plasma (line “a”) yielded a slope of –3.16. Both of the slope values generated in plasma are within 1 standard deviation of the mean obtained by averaging 6 assay standard curves (–3.19 ± 0.14). These results indicate that there appears to be no cross-reactivity of this antiserum with other plasma proteins since we can recover quantitatively, and measure accurately, the factor V present in plasma.

Our antiserum recognized both factor V and thrombin-activated factor V (factor Va). When unlabeled factor Va was used to generate a standard curve with this antiserum, the curve obtained was superimposable with that obtained with factor V. Plasma factor V antigen levels were not affected by lyophilization of the plasma, storage of the plasma at –70°C (6 mo), or intentional conversion of the plasma to serum.
The within-run precision of our assay (coefficient of variation), assessed by assaying 3 different plasma samples 10 times each within the same assay, was 4.1% (mean = 0.19 μg/ml), 8.0% (mean = 5.6 μg/ml), and 8.0% (mean = 10.2 μg/ml). In addition, when a single plasma sample was assayed 4 times over a 1-mo period, a value of 6.3 ± 0.58 μg (mean ± SD) factor V/ml of plasma was obtained.

**Factor V Plasma Concentration—Normal Range**

Based on radioimmunoassay results for 64 apparently healthy individuals, the normal concentration of factor V in plasma ranges from 4 to 14 μg/ml with a mean value of 7.0 ± 2.0 (Fig. 3). Thirty-three females (7.4 ± 2.2) and 31 males (6.5 ± 1.7) between 22 and 61 yr of age constituted the normal population used in this study. No correlation was observed between antigen levels and age or sex. Factor V clotting assays were performed on 22 of these plasma samples in order to determine total factor V concentrations based on bioassay. Bioassay data were quantitated based on the activity of highly purified human factor V, i.e., 1.7 U of factor V was equivalent to each microgram of protein. Factor V clotting assays were consistent with the radioimmunoassay data (Fig. 4), providing freshly drawn plasma was used in the bioassay. (Apparent concentrations of factor V in plasma decreased with time, even if stored at −20°C, when measured by bioassay, but remained constant when measured by radioimmunoassay.) Statistical analyses of these data indicate that the radioimmunoassay data gave a mean of 7.5 ± 1.7. These data are not significantly different as determined by a paired Student’s t test.

**Measurement of Platelet-Associated Factor V**

The distribution of factor V in whole blood was established by determining the amount of factor V present in the plasma and platelets of 8 different individuals. Platelets were collected and washed in the presence of 5 μM PGE₁ in order to prevent any platelet activation and possible secretion of platelet-associated factor V. Assay of the platelets lysed in 0.2% Triton X-100 (Table I) indicated that 0.63–1.93 μg of factor V was present per 2.5 × 10⁸ platelets (4612–14,128 molecules of factor V per platelet). For each individual,
Fig. 4. Factor V plasma concentrations determined by radioimmunoassay and bioassay. Twenty-three samples gave mean factor V concentrations of 6.8 ± 2.0 µg/ml by radioimmunoassay and 7.5 ± 1.7 µg/ml by clotting assay. These data are not significantly different as determined by a paired Student's t test.

The platelet data were normalized to his or her hematocrit and platelet count in order to determine the amount of factor V in whole blood contributed by the platelet. The amount of factor V contributed by the platelets of each individual ranged between 17.6% and 24.9%.

Assay of Factor V-Deficient Patients

We studied the plasma of two patients with functional deficiency of factor V (Table 2). Patient 1 is a factor V–factor VIII:C deficient individual; patient 2 is only deficient in factor V. We increased the sensitivity of our assay to detect as little as 20 ng factor V/ml of plasma (~0.3% of normal levels). We achieved this sensitivity by decreasing both the amount of radiolabeled factor V (5 ng) and the antibody (1:8000) used in the assay. The standard curve obtained in the assay was linear from 0.004 ng to 0.512 ng factor V per assay tube, equivalent to 0.02–2.56 µg/ml plasma.

Patient 1 has 9% functional factor V by bioassay and 0.63 µg factor V/ml of plasma (9% of normal values based on 7 µg/ml, normal mean), indicating that she is truly deficient in antigen as well as activity.

Patient 2 has no detectable factor V, established both by bioassay and radioimmunoassay, indicating he is truly devoid of antigen.

DISCUSSION

Our double-antibody radioimmunoassay indicates that the normal range of factor V concentration in human plasma is 4–14 µg/ml. The age or sex of an individual does not appear to influence the factor V concentration. Since our burro anti-human factor V antibody quantitates factor V levels identically in plasma and with intentionally activated factor V (factor Va), collection of plasma to preclude any factor V activation is unnecessary. Plasma lyophilization, prolonged storage, or intentional conversion of plasma to serum has no effect on factor V antigen levels.

The assay of well washed, Triton X–100-lysed platelets indicated that there are 0.63–1.93 µg of factor V present per 2.5 × 10⁸ platelets (4612–14,128 molecules of factor V per platelet). These results are in marked contrast to our observations for the bovine system. Bovine platelets possess approximately 400–800 molecules of factor V per platelet, and bovine plasma factor V concentrations range from 30 to 50 µg/ml. Therefore, in the bovine system, platelets possess approximately 2%–5% of the total factor V present in whole blood. In the human system, however, on an
individual basis, the hematocrit, platelet count, and plasma and platelet concentrations of factor V exist, such that the platelets contribute approximately 18%–25% of the factor V present in whole blood.

We increased the sensitivity of our assay in order to detect factor V concentrations as low as 20 ng/ml or 0.3% of normal concentrations. Two individuals with known factor V deficiencies were studied. One individual, totally devoid of factor V activity, but fully competent with regard to the other assayable coagulation factors, was found to be completely deficient in factor V antigen. Another individual, a factor V and factor VIII deficient patient, was found to have 9% factor V by both bioassay and radioimmunoassay.

Factor V deficiencies have been described that result from acquired inhibitors to factor V or from the lack of an inhibitor to activated protein C, a potent inhibitor of factor V (Va) activity. Since these types of deficiencies may result from factor V antigen which cannot express function, we intend to assay several factor V deficiencies to determine if they are truly devoid of both antigen and activity, or possibly possess a normal nonfunctional, or abnormal factor V molecule.

REFERENCES

2. Owren PA: Parahaemophilia, haemorrhagic diathesis due to absence of a previously unknown clotting factor. Lancet 1:446, 1947
Radioimmunoassay of factor V in human plasma and platelets

PB Tracy, LL Eide, EJ Bowie and KG Mann