

FACTOR V_a AND PLATELET CYTOSKELETONS. G.P. Tuszynski, P.N. Walsh, A. Koshy, J. Piperno, and B. White. Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA.

We have recently shown that the triton X-100 insoluble cytoskeletons from thrombin-activated platelets contain coagulation Factor V_a (Tuszynski, G.P. *et al.* J. Cell. Biol. 87, 219, 1980). Factor V_a activity was measured by two independent techniques, a one-stage clotting assay, and a two-stage chromogenic assay utilizing purified Factor X_a, prothrombin, and the chromogenic substrate S-2238. Further analysis of these cytoskeletons by SDS-PAGE and by immunodiffusion revealed the presence of fibrin. These findings suggest that a common mechanism may link alpha granular proteins to the platelet cytoskeleton. To investigate this mechanism, platelet cytoskeletons prepared from platelets treated under various conditions were assayed for Factor V_a activity. Both the rate of Factor V_a appearance and the final level of activity associated with the cytoskeleton were diminished 30-50% in platelets that were treated with aspirin (500µM) or with indomethacin (20µM). When secretion of Factor V, serotonin and nucleotides was inhibited greater than 90% by incubation of a platelet suspension with 2-deoxyglucose (30µM), gluconolactone (40µM) and antimycin-A (9µM), cytoskeletons prepared from this platelet suspension contained less than 10% Factor V_a activity of controls. The rate of appearance of cytoskeletal Factor V_a activity increased with increasing thrombin concentration while the final level remained constant. Calcium ionophore, A-23187 (1µM), released Factor V but less than 2% was associated with the cytoskeleton suggesting that Factor V_a, the activated form of Factor V, was required for binding. We conclude that prior secretion of Factor V and its further activation by thrombin are necessary for the association of coagulation Factor V_a with the cytoskeleton. These results are consistent with the hypothesis that released Factor V_a becomes associated with the cytoskeleton via cell surface components.

HUMAN PLASMA AND PLATELET FACTOR V LEVELS AS MEASURED BY RADIOIMMUNOASSAY. P.B. Tracy, J.M. Peterson, M.E. Nesheim, J.A. Katzmann, and K.G. Mann. Hematology Research, Mayo Clinic/Foundation, Rochester, Minnesota, 55901.

Highly purified human Factor V was used for the development of a competitive double antibody radioimmunoassay (RIA) using ¹²⁵I-human Factor V, burro anti-human Factor V antisera as the primary antibody and goat anti-burro antisera as the precipitating antibody. The standard curve allows the detection of as little as 20 ng of Factor V per ml of plasma. With this specific RIA for human Factor V, we have measured the level of Factor V in the plasma and platelets of normal individuals. The normal level of Factor V in plasma ranges from 4 to 14 µg per ml, with the average value equal to 7.0 ± 2.0 µg/ml (n = 64; 33 females; 31 males; 22 to 61 years of age). There appeared to be no correlation between antigen levels and age or sex. Factor V clotting assays were consistent with the RIA data for any given plasma preparation providing freshly drawn plasma was used in the bioassay. The bioassay data were quantitated based upon the specific activity of purified plasma Factor V; 1.7 units of Factor V equals 1 µg of protein. Plasma Factor V antigen levels were not affected by lyophilization of the plasma, prolonged storage of the plasma at -20°C or intentional conversion of the plasma to serum. The levels of Factor V present in washed human platelets were also determined using the RIA. Assay of washed platelets lysed in 0.2% Triton X-100 indicated that 0.6 to 0.85 µg of Factor V was present per 2.5 x 10⁸ platelets (4400 to 6200 molecules of Factor V per platelet). This result is in marked contrast to our observations for the bovine system, where we found that bovine platelets possess approximately 400 to 800 molecules of Factor V per platelet, and plasma Factor V levels range from 30 to 50 µg per ml. In the bovine system, the platelets possess approximately 1% of the total Factor V present, while in human blood, the platelets possess as much as 10 to 15% of the total Factor V present.

A NEW METHOD TO DETERMINE THE BINDING OF FACTOR X TO PHOSPHOLIPID BILAYERS. G. Tans, J. Rosing, G. v. Dieijen, J. v. Rijn, H.C. Hemker. Department of Biochemistry, University of Limburg, Maastricht, The Netherlands.

The binding parameters (dissociation constant and the number of binding sites) for factor X binding to phospholipid bilayers in the presence of Ca²⁺ ions can be determined using the factor X-activating protein present in Russell's Viper Venom (RVV-X). It is shown that RVV-X is only able to activate factor X molecules that are not bound to the phospholipid bilayer. Therefore, from the observed rates of factor X activation by RVV-X measured in the presence and absence of phospholipid the amount of factor X not bound to the phospholipid bilayer can be calculated. When the concentration of factor X added is known the bound factor X can be calculated and the binding parameters for factor X are obtained from a Scatchard plot. The technique presented here offers an advantage over other techniques published thus far. It is rapid and simple as compared to the Hummel and Dryer technique. There is also an advantage over techniques using light scattering. Since the technique presented here is not hampered by aggregation of vesicles it is possible to study the binding of factor X to vesicles containing a high mole fraction of PS and at high CaCl₂ concentrations. The binding parameters for factor X binding to vesicles of a mixture of phosphatidylserine (PS) and phosphatidylcholine (PC) are dependent on the amount of negatively charged phospholipid present. At increasing mole fractions of PS factor X binds more tightly to the vesicles and also the number of factor X binding sites increases. However, at high amounts of PS (60%) the number of sites present decreases dramatically which is likely due to aggregation of the vesicles. Binding to vesicles composed of a mixture of phosphatidylglycerol (PG) and PC is considerably weaker whereas the number of sites present is about the same. Prothrombin and factor X compete for the same binding site as was determined by competition experiments. Our data will be discussed with respect to the mode of interaction of vitamin-K-dependent clotting factors with negatively charged phospholipid interfaces.

IMPROVED PURIFICATION METHOD OF HUMAN PLASMA FACTOR V AND APPLICATIONS OF A RABBIT ANTI-HUMAN FACTOR V ANTIBODY. C. Breederveld, T. Bruin, A. Sturk, Th. Hakvoort, J.W. ten Cate. University Hospital "Wilhelmina Gasthuis", Amsterdam, The Netherlands.

Human factor V, purified from citrated plasma by a method recently described by us (Bolhuis *et al.* 1979) was found to be slightly contaminated with α₂-macroglobulin. A rabbit antiserum to this preparation, obtained by frequent subcutaneous immunization, showed factor V procoagulant neutralizing activity. Immune electrophoresis demonstrated precipitation lines with α₂-macroglobulin. This contamination was successfully removed from the factor V preparation by affinity chromatography with specific rabbit anti human α₂-macroglobulin bound to Sepharose 2B. The antiserum to the original, impure factor V preparation showed no more precipitation arcs after absorption with 3% normal plasma and ammonium sulphate precipitation of the complexes. Factor V procoagulant neutralizing activity titre was estimated 1: 8 (50% neutralization after 1 hour incubation at 37°C.) This antiserum was used for the following studies: 1. An inhibitor neutralization assay was developed for measuring antigenic activities of the factor V molecule. Antigen concentration in a range from 12.5-100% could be reliably measured. 2. Fab-fragments, obtained by pepsin digestion of the purified IgG (by protein A Sepharose chromatography) were labeled with ¹²⁵I by an immobilized lactoperoxidase method. These Fab-fragments fully retained their factor V procoagulant neutralizing activity. ¹²⁵I-Fab-fragments were used in specific binding studies to both non-activated and ADP and thrombin-activated human blood platelets.