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FACTOR VIII/VON WILLEBRAND FACTOR HAS POTENT LECTIN ACTIVITY

William J. Booth, Fiona H. Furby, Michael C. Berndt and Peter A. Castaldi

Department of Medicine, University of Sydney, Westmead Centre, Westmead, NSW 2145, Australia

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Summary. Highly-purified plasma and platelet Factor VIII/von Willebrand Factor had potent lectin activity when measured in a haemagglutination assay. This lectin activity was inhibited by monoclonal and heterologous antibodies to Factor VIII/von Willebrand Factor as well as by hexosamines, mannose and net-positively charged amino acids.

Plasma Factor VIII/von Willebrand Factor (FVIII/vWF) is a high molecular weight protein (1-20 x 10^6 daltons) which has two major roles in the haemostatic mechanism (1). It is the molecule responsible for the adhesion of platelets to component(s) of the subendothelium (2,3) and it is the carrier protein for Factor VIII procoagulant (FVIII:C) activity (4,5). The FVIII/vWF molecule contains specific antigenic determinants (FVIIIR:Ag) (6) and ristocetin cofactor activity (FVIII:RCo) (4). Platelets contain FVIII/vWF located in their α-granules (7,8) which is actively released upon stimulation by thrombin (9). The multimeric nature of the FVIII/vWF molecule (1,6) and its role in cell adhesion suggest that it is multivalent in character and, therefore, may have the properties of a lectin-like molecule. In this study, lectin activity was measured in a haemagglutination assay utilising trypsinized, fixed sheep erythrocytes. Potent lectin activity was demonstrated in fractions containing highly purified plasma FVIII/vWF and in FVIII/vWF-rich fractions derived from platelet α-granules. This lectin activity was inhibited by both monoclonal and heterologous anti-FVIII/vWF antibodies.

MATERIALS AND METHODS

Materials: Benzamidine was obtained from Aldrich Chemical Co., trasylol from Bayer Pharmaceutical Co., diisopropylfluorophosphate from Calbiochem, sugars and amino acids from Sigma, Triton X-100 from BDH, agarose from Seakem, and silver stain, acrylamide and sodium dodecyl sulphate from BioRad. All other chemicals were of analytical grade or the best grade available. α-Thrombin was the generous gift of Dr. J. W. Fenton II, Albany, New York.

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FVIII/vWF purification: Intermediate purity FVIII/vWF was prepared by a modification of the bench-scale fractionation procedure of Newman et al (10), from two litres of fresh human plasma containing 10 mM benzamidine and 10 U/ml of trasylol. FVIII/vWF in the ethanol cryoprecipitate was extracted with 0.02 M Tris solution, pH 7.0, containing 1 mM benzamidine. Treatment with $Al(OH)_3$ was omitted. The precipitate resulting from the 3-10% (w/v) PEG 6000 fractionation of citrated material at pH 6.1 was washed with ice-cold 8% ethanol-0.02 M Tris solution, pH 7.4, and solubilized in 10 ml of column buffer (0.05 M Tris, pH 7.4, 0.15 M NaCl, 0.02% (w/v) NaN_3) containing 0.5 mM diisopropylfluorophosphate (11). This was loaded onto a 75 x 2.5 cm column of Sepharose CL-4B (Pharmacia) and eluted at 25 ml/hr with column buffer. Fractions were stored at 4°C. Trace amounts of contaminating fibrin/fibrinogen in these fractions were removed by passage through a 10 x 1 cm column containing agarose coupled to affinity-purified antifibrinogen. Removal of fibrin/fibrinogen was confirmed by silver staining of electrophoretic gels. Alternatively, purified human plasma FVIII/vWF was obtained by Sepharose CL-4B gel filtration of glass pore-filtered FVIII/vWF concentrate (12) the generous gift of Dr. J. Margolis, Sydney, Australia. Purified human plasma FVIII/vWF was also kindly donated by Dr. M. A. Howard, Melbourne, Australia, and purified bovine FVIII/vWF was the generous gift of Dr. E. P. Kirby, Philadelphia, PA.

FVIII/vWF analysis: FVIII/vWF was quantitated by Laurell rocket analysis on gel bond film (Marine Colloids Products) following a modification of the method of Zimmerman et al (13). Suitably-diluted 15 µl samples were electrophoresed through 0.9% (w/v) ME agarose containing 0.5% (v/v) Triton X-100 and 1:1,000 rabbit antihuman FVIII/vWF antisera, at 15 mA per gel constant current for 16 hrs. Serially-diluted pooled plasma was used as a FVIII/vWF standard. Multimeric analysis was performed according to the method of Ruggeri and Zimmerman (14). FVIII:RCo assay was performed using formalin-fixed washed platelets and ristocetin as previously described (15). Fraction 19 from the Sepharose CL-4B elution profile (Figure 1) was used as standard (designated 100%).

Antibodies: The monoclonal antihuman FVIII/vWF antibodies, 3F8 and 2C9, were raised in mice by standard hybridoma technology (16) using purified FVIII/vWF as the antigenic source. Monoclonal antibodies were screened by the immunodot technique (17). Neither antibody caused an increase in the activated partial thromboplastin time of normal platelets. Antihuman fibrinogen antiserum was raised in rabbits and affinity-purified on a fibrinogen-coupled agarose column. Heterologous antihuman FVIII/vWF (affinity-purified) was the generous gift of Dr. J. Koutts, Sydney, Australia and antihuman FVIII:C immunoglobulin was the generous gift of Dr. I. Peake, Cardiff, Wales.

Storage: Aliquots of plasma FVIII/vWF were stored at -70°C for several months with minimal loss of lectin activity. However, repeated freeze thawing caused a marked loss of lectin activity. Plasma FVIII/vWF could be stored at 4°C for up to one month without significant loss of lectin activity which gradually deteriorated after this time.

Preparation of platelet fractions: Platelet proteins released by α-thrombin stimulation were fractionated by Sepharose 4B gel filtration as previously described (18).

Electrophoresis: Fraction aliquots (50 µl) were solubilized with 2% (w/v) sodium dodecyl sulphate, reduced with 5% (v/v) 2-mercaptoethanol and electrophoresed at 25V constant voltage for 16 hr following the procedure of Laemmli (19). Gels contained 3% (w/v) acrylamide in the stacking layer and either 7.5% or a 5-15% exponential gradient of acrylamide in the resolving layer. Gels were stained for protein with either Coomassie brilliant blue (20) or silver stain (21).

Haemagglutination assay: Haemagglutination of trypsinized, fixed sheep erythrocytes was assayed as previously described (22,23). Diluting buffer contained 0.01 M Tris, pH 7.4, 0.15 M NaCl and 0.02% (w/v) NaN_3. Platelet preparations from a normal control and a patient with severe von Willebrand's disease (FVIII/vWF undetectable in platelets and plasma) were tested for enhanced surface-associated and supernatant lectin activity, as previously described (22).

RESULTS AND DISCUSSION

Highly-purified human plasma FVIII/vWF (purified by three separate procedures) had potent haemagglutinin activity towards trypsinized, formaldehyde-fixed sheep erythrocytes (reciprocal titre 11,570/A280 in peak FVIII/vWF fraction, Figure 1). This activity was inhibited by hexosamines, mannose and net-positively-charged amino acids but not by other hexoses, N-acetylated hexosamines, other amino acids or EDTA (Table 1) and was not due to contaminating fibrin (24) (see materials and methods). Three lines of evidence suggest that the observed lectin activity is an intrinsic property of the FVIII/vWF molecule.

i) Lectin activity co-eluted throughout the FVIII/vWF peak on gel filtration concomitant with ristocetin cofactor activity which serves as an independent measure of FVIII/vWF (15) (Figure 1). The presence of FVIII/vWF in these

TABLE 1
PLASMA FVIII/vWF LECTIN ACTIVITY
INHIBITION OF HAEMAGGLUTINATION BY SUGARS AND AMINO ACIDS

TEST MATERIAL	RECIPROCAL TITRE
BUFFER	128
GLUCOSE	128
GALACTOSE	128
MANNOSE	8
GLUCOSAMINE	64
GALACTOSAMINE	0
MANNOSAMINE	0
N-ACETYL GLUCOSAMINE	128
N-ACETYL GALACTOSAMINE	64
N-ACETYL MANNOSAMINE	128
ARGININE	0
LYSINE	32
GLUTAMINE	64
GLYCINE	64
ASPARTIC ACID	128

The final concentration of FVIII/vWF in the first well of each row is 10 µg/ml; final concentration of sugar or amino acid is 50 mM in all tests. Buffer is Tris-buffered saline, pH 7.4.

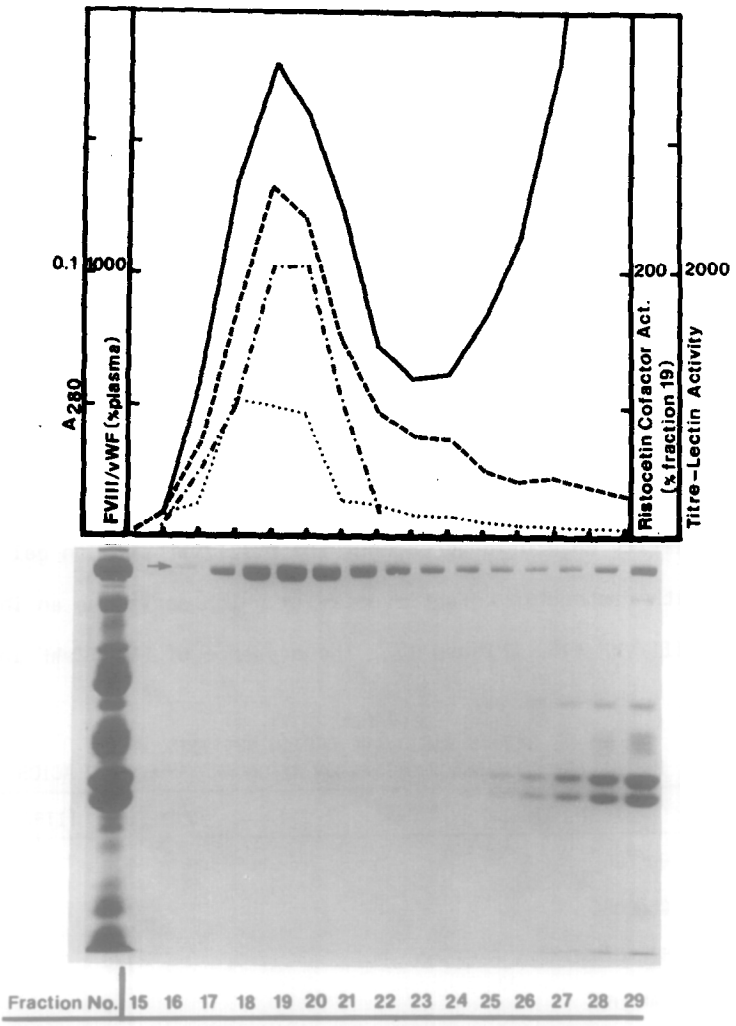

Figure 1. The FVIII/vWF elution profile after Sepharose CL-4B chromatography. Eluted fractions were measured for absorbance at 280 nm (———), for FVIII/vWF by Laurell analysis (– – – –), for ristocetin cofactor activity (·····), and for lectin activity (–·–·–·–) as described under materials and methods. The lower part of figure shows the corresponding electrophoretic profile of reduced proteins analysed on a 7.5% polyacrylamide slab gel. FVIII/vWF coincides electrophoretically with the M_r (reduced) = 230,000 band (arrowed).

fractions was established by electrophoretic, Laurell and multimeric analyses. High molecular weight multimeric forms of FVIII/vWF were prominent in fractions 16-22. Similarly, bovine FVIII/vWF had potent lectin activity in the haemagglutination assay.

ii) One of two monoclonal antibodies raised against FVIII/vWF, 3F8, markedly inhibited both lectin and ristocetin cofactor activities, while the other, 2C9, was without effect (Table 2). FVIII/vWF-associated lectin activity

TABLE 2
PLASMA FVIII/vWF LECTIN ACTIVITY
INHIBITION OF HAEMAGGLUTINATION BY SPECIFIC ANTIBODIES

TEST MATERIAL	RECIPROCAL TITRE
1. BUFFER	128
2. ANTI-FVIII/vWF MONOCLONE 3F8	16
3. ANTI-FVIII/vWF MONOCLONE 2C9	128
4. AFFINITY-PURIFIED ANTIFIBRINOGEN	128
5. AFFINITY-PURIFIED ANTI-FVIII/vWF	8
6. ANTI-FVIII:C IMMUNOGLOBULIN	128

The final concentration of FVIII/vWF in the first well of each row is 10 μg/ml; final concentration of antibody is 10 μg/ml for tests 2, 3 and 4, and 100 μg/ml for tests 5 and 6. Buffer is Tris-buffered saline, pH 7.4.

was also inhibited by heterologous anti-FVIII/vWF but not by anti-FVIII:C or antifibrinogen. The inhibition of both FVIII/vWF-associated lectin and ristocetin cofactor activities by the monoclonal antibody 3F8 and the lack of inhibition of either activity by the monoclonal antibody 2C9 suggest that the epitopes for these respective activities may be closely linked sterically. Both activities are associated with high molecular weight multimeric forms of FVIII/vWF.

iii) Platelet FVIII/vWF-rich fractions derived from platelet α-granules contained lectin activity (Figure 2) which was specifically inhibited by heterologous anti-FVIII/vWF and by the same sugars and amino acids which inhibited plasma FVIII/vWF-associated lectin activity. These platelet fractions contained high molecular weight multimeric forms of FVIII/vWF and were associated with ristocetin cofactor activity. This was confirmed with purified platelet FVIII/vWF (manuscript in preparation).

The specificity of FVIII/vWF-associated lectin activity is similar to that described for platelet-related lectin activities (23,25). Following stimulation by thrombin, platelets show enhanced lectin activity associated with the activated platelet surface (24,26) as well as a soluble supernatant lectin activity (23,24). In common with these platelet-related lectins, the FVIII/vWF-associated activity is inhibited by hexosamines and net-positively changed amino

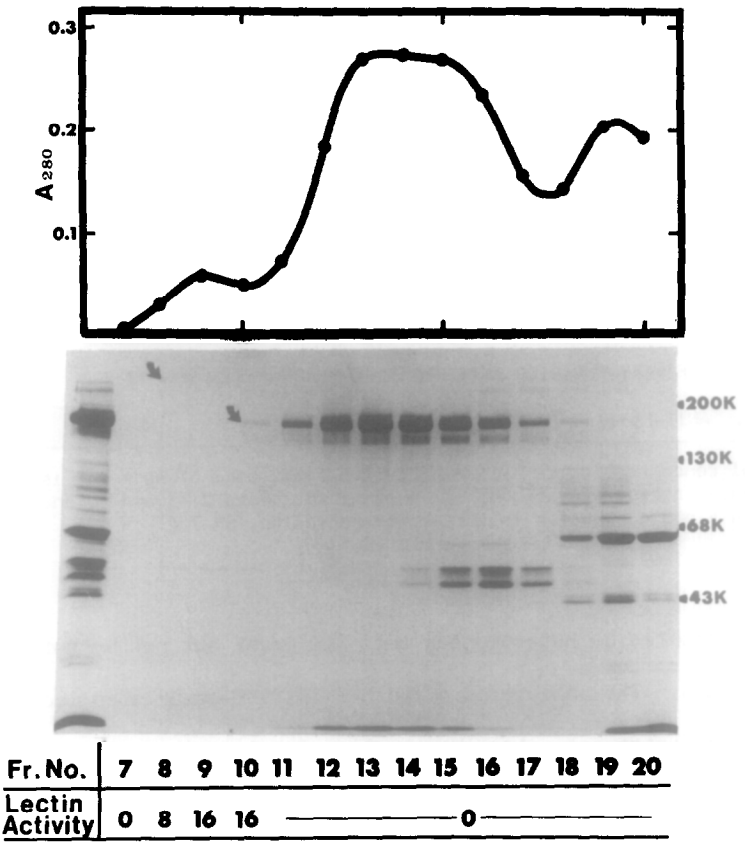

Figure 2. The elution profile of thrombin-released platelet proteins after Sepharose 4B chromatography. Eluted fractions were monitored by absorbance at 280 nm (upper figure) and analysed by 5-15% exponential gradient, sodium dodecylsulphate-polyacrylamide gel electrophoresis under reducing conditions (middle figure). The upper curved arrow indicates the M_r (reduced) = 230,000 subunit of FVIII/vWF, and the lower curved arrow the M_r (reduced) = 185,000 subunit of thrombospondin. Lectin activity (lower figure) was assayed as described under materials and methods.

acids (23,25) but differs with respect to its sensitivity to mannose (Table 1). Platelet FVIII/vWF does not account for the major part of either surface-bound or supernatant lectin activities, since neither activity was decreased in platelets prepared from a patient with severe von Willebrand's disease.

The finding that FVIII/vWF has lectin activity adds a new dimension to the characterization of this important molecule, particularly in relation to its binding functions. The interaction between FVIII/vWF and other proteins such as FVIII:C (4,5), the platelet membrane glycoproteins Ib (27) and IIb/IIIa (28) and the microfibrillar components of the subendothelium (3) remain the subject of intense investigation. The observed FVIII/vWF lectin activity may be involved in one or more of these binding interactions. The role of the lectin

binding site in platelet adhesion and FVIII/vWF-FVIII:C interaction is currently under investigation.

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