Assessment of the interrelationship of the biological activities detected by botrocetin and ristocetin on von Willebrand's factor from normals and patients with von Willebrand's disease.

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Master of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

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THESIS SUMMARY.

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Experiments have been undertaken to determine if the biological activities detected by ristocetin and botrocetin in plasma vWf are identical or separate. These cofactor activities have been shown to be distributed differently on vWf of different molecular size and that the multimers that interact with platelets in their plasma are also different. Although in the majority of vWd patients these cofactor activities are similar a small number of patients have been identified where this is not the case. Study of these patients has provided evidence that these biological sites on vWf are probably closely associated although distinct. It is postulated that the presence of these two distinct sites that interact with the glycoprotein Ib platelet site has a biological advantage, although the relative importance of each site separately in haemostasis is not clear and requires further study.

Key words: von Willebrand factor. von Willebrand's disease. Ristocetin. Botrocetin. Platelet Agglutination.

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1. HAEMOSTASIS.

HAEMOSTASIS.

Haemostasis is a rapid and balanced process designed to arrest blood loss from damaged vessels without compromising blood fluidity. The process involves a complex interaction of the blood vessel, platelets, specific proteinases, protein cofactors and the endothelium to form a stable fibrin clot that is subsequently removed by fibrinolysis. The localised rapid formation of the fibrin clot is achieved by complicated systems of activation and inhibition whereby unwanted thrombosis and excessive bleeding is minimised.

1.1. Role of the blood vessels and endothelial cells.

The intima of blood vessels consist of a single layer of endothelial cells which performs many important functions including; transfer of metabolic substances, formation of a barrier between the subendothelium and blood elements, synthesis and metabolism substances which regulate the interaction between vessel wall and blood components, maintenance of non-thrombogenic surface, vascular repair and cellular immunity.

Healthy intact endothelium has a non-thrombogenic surface which is maintained by passive and active mechanisms. The passive mechanisms involves the synthesis of glycoaminoglycans, predominantly heparan sulphate (80%),

which is found on the luminal side of the endothelial cell covalently bound to a protein backbone forming a proteoglycan (mucoprotein) (8,9). The heparan sulphate exerts its anticoagulant action by potentiating the inactivation of some plasma clotting proteins. Many of the latter are serine proteases and exist in the plasma in precursor form prior to conversion to the active state by an enzyme amplification coagulation process. The proteases are maintained in their inactive form by a physiological inhibitor of the cascade sequence Antithrombin III. Heparan sulphate promotes the retention of an active configuration to the Antithrombin III. This results in the progressive inhibition of thrombin and other clotting proteins. The heparan sulphate complex may also inhibit collagen-induced platelet aggregation by modifying or sheilding the collagen (8,9). Also, proteoglycans bind non-covalently to collagen and influence both conformational stability of collagen monomers and formation of collagen fibrils (8,9).

The active mechanism employed by endothelial cells to prevent thrombus formation includes: 1. The synthesis of prostaglandin PGI_2 which inhibits platelet-vessel wall interaction (10); 2. Synthesis and release of plasminogen activator which promotes fibrinolysis (11); 3. Degradation of ADP by membrane-associated apyrase (ADPase); 4. Uptake and degradation of vasoactive amines such as 5-

Hydroxytryptamine, prostaglandins F_2 , D_2 and E_2; 5. Uptake, inactivation and clearance of thrombin and the binding of heparin and alpha-macroglobulin (12,13). Finally, endothelial cells also secrete thrombomodulin, a cofactor in the activation of protein-C. This protein neutralises coagulation proteins factors V and VIII and thus inhibits the clotting process. Protein-C also promotes the release of plasminogen activator and thus enhances fibrinolysis and fibrin clot breakdown (14).

Endothelial cell synthesis of PGI_2 inhibits platelet adhesion by stimulating a platelet membrane adenylate cyclase (10). This elevates intracellular cyclic AMP with subsequent sequestration of calcium ions which reduces the release of platelet granular inclusions and inhibits phospholipases and cyclo-oxygenase activity (15). The fall in cytoplasmic calcium may be related to the ability of cyclic AMP dependent protein kinase to stimulate uptake of calcium and phosphorylation of certain proteins by a platelet membrane fraction (16). Consequently, thromboxane A_2, a product of the platelet release mechanism and a potent platelet aggregating agent is not produced. Thus the release of PGI_2 limits thrombus formation by inhibiting further platelet deposition.

The synthesis and release of plasminogen activator by endothelial cells plays an important role in the activation of the fibrinolytic mechanism. Fibrinolysis removes unwanted fibrin deposits to re-establish blood flow in the vessels occluded by thrombus formation. Normally this would prevent fibrin clot formation and promote destruction but thrombin once formed by the coagulation process inhibits the synthesis and release of plasminogen activator (17).

1.2. The role of platelets in haemostasis.

Platelets are the smallest of the formed elements of blood (3-4u diameter) and are very sensitive to a wide range of chemical, physical and environmental stimuli. In addition, platelets are capable of metabolic and synthetic activity, morphological change and in the non-stimulated state maintain their discoid shape by a cytoskeleton of microtubules. The surface membrane is a continuous open canalicular membrane system tunnelling throughout the cytoplasm. This sponge-like system of channels, the surface-connecting system, enlarges the surface area of the platelet membrane and serves as a conduit for the uptake of plasma substances and for the extrusion of granule-bound secretary products during the release reaction. A second membrane system exists known as the dense tubular system which may play an important role in stabilisation of the circumferential microtubules. Channels for both membrane systems form interwoven membrane complexes. This dual membrane system appears to constitute the calcium pump essential in maintaining a low calcium concentration in order to keep the microtubules polymerised and thus maintain the discoid shape of the platelet. Finally the exterior coat of platelets contain at least eight different glycoprotein receptors which mediate surface contact reactions of platelet stickiness and aggregation.

The functions of platelets include the maintenance of vascular integrity, the formation of platelet plugs to arrest bleeding, the provision of platelet phospholipid (PF3) to the coagulation system and the promotion of vascular healing through the release of platelet-derived growth factor (PDGF).

Platelet adhesion is the process by which platelets attach to cells, tissues, surfaces and particles, whereas platelet aggregation is the process by which platelets attach to each other. Platelet adhesion is the first event in the formation of a haemostatic plug and triggers a further series of reactions in the platelet. The contents of the alpha and dense granules are released; the ADP and serotonin from the dense granules synergistically cause platelet aggregation. Phospholipases are activated, freeing arachidonate, which is converted to products that cause platelet aggregation and may affect adhesion. The phospholipid of the platelet membrane is made available and allows binding of the coagulation factors to the platelets and accelerates the clotting process. Activation of the clotting mechanism at the site of injury and on the surface of the adherent platelet allows the local formation of thrombin. Thrombin causes further platelet aggregation, release of platelet granule content, formation of aggregating agents from arachidonate and causes changes in the platelet surface similar to those occurring

due to platelet adherence. Also, thrombin causes the formation of fibrin around and amongst the platelets to which platelets adhere to form a fibrin plug. Thrombus formation may be regulated by thrombin activating the natural inhibitor protein-C and stimulating PGI_2 formation from intact endothelium. Finally, binding sites on the platelet surface are exposed for proteins involved in platelet adhesion and aggregation. Thus the formation of the platelet plug occurs through the sequence: platelet adhesion, platelet aggregation and shape change, including secretion of granule content, contribution of PF3 and promotion of vascular healing.

1.2.1. Platelet adhesion to the subendothelium.

Normally, platelets do not adhere to healthy endothelium but to collagen exposed by damaged vascular endothelium. Collagen is the most platelet-reactive material in the the vessel wall. Types I, II and III are potent stimulators of platelet adhesion while type IV and A-B do not cause platelet aggregation. Platelets may also adhere to other constituents of the vessel wall including microfibrils, basement membrane and damaged smooth muscle cells. The precise nature of the interaction between platelets and collagen is not known but collagen must be in the form of fibrils to bind platelets and rigidly spaced polar groups of epsilon-amino groups of lysine are also necessary for collagen supported platelet adhesion.

The nature of the protein or glycoprotein receptors for collagen have not been determined. At low shear rates adhesion occurs independently of plasma proteins but at high shear rates (>1000/sec) platelet adhesion to collagen requires von Willebrand factor (vWf). The vWf acts as a link between a platelet membrane glycoprotein receptor (GPIb) and a possible receptor site on the subendothelial collagen. In the absence of vWf or some structural abnormality in it, platelet/collagen interaction is prevented. Likewise the absence of the platelet glycoprotein receptor (Bernard-Soulier syndrome) impairs adhesion.

1.2.2. Platelet adhesion and release of granule content.

Platelet adherence to collagen or other foreign surfaces causes the release platelet granules and their contents and the production of prostaglandin endoperoxides, PGG_2, PGH_2, and their product, thromboxane A_2. The extrusion of the platelet granule material occurs through a system of channels (surface connecting system) by a mechanism similar to exocytosis. Activation of the release mechanism causes the platelet granules to cluster to the centre of the platelet which are encircled by a band of microtubules. (1,2).

The dense (storage) granules of platelets contain ADP and 5-Hydroxytryptamine (5HT) which are released in response to a weak stimulus. The released ADP and 5HT induce secondary platelet aggregation on the adherent platelets, together with vasoconstriction induced by the released 5HT. The ADP binds to specific platelet receptors inducing shape change and a reduction in c-AMP activity. Furthermore, the fibrinogen binding sites (GPIIb/GPIIIa) are mobilised and phospholipase activity initiated resulting in the formation of thromboxane A_2. The platelet-platelet interaction occurs through calcium-dependent ligand formation between bound fibrinogen molecules. Thus the release of ADP leads to further platelet aggregation which causes further release of ADP and the rapid formation of a platelet plug at the site of injury.

In addition to the release of the dense granule contents, several proteins are released from the alpha-granules of the platelet. The granules, some of which are lysosomes, seem to release their contents more readily than the dense granules.

Low concentrations of thrombin release the contents of the alpha-granules; PF4 (anti-heparin protein), beta-thromboglobulin, thrombospondin (heparin-binding protein), fibrinogen, fibronectin, vWf, albumin, factor V, antiplasmin, alpha-antitrypsin, alpha-2 macroglbulin, PDGF, cationic

proteins, proteoglycans and other proteins whilst higher concentrations are needed to evoke release of materials (ADP, 5HT) from dense bodies. The secreted proteins vWf, and thrombospondin may be found bound to the platelet membrane. Although sufficient concentrations of these proteins already exist in the plasma, local high concentrations at the point of release from the platelet may be required to enhance platelet adhesion and aggregation.

1.2.3. Function of platelet granule constituents.

The lectin-material thrombospondin binds to the surface of the platelets that have released their granule contents and interacts with fibrinogen. Fibrinogen is required for platelet aggregation and binds to platelets on which the glycoproteins IIb and IIIa have formed a receptor as a result of exposure of the platelet to aggregating agents. The role of platelet fibrinogen is unclear but has been shown to be released following platelet stimulation by collagen, thrombin and plasmin.

Platelet factor-4 (antiheparin factor) interferes with the antithrombin III complex with heparin and thus potentiates thrombin; its function in the absence of heparin is unclear. The function of beta-thromboglobulin is not known but occupation of a receptor site on endothelial cells causes a decrease of PGI_2 production. PDGF induces

cell growth and has chemotactic activity which stimulates the migration and proliferation of smooth muscle cells.

Platelet factor V serves as a receptor for factor Xa and thus in the formation of the prothrombinase complex that catalyes the conversion of prothrombin to thrombin. Factor V is released following platelet aggregation induced by collagen but not by ADP. Fibronectin plays a significant role in the adherence of platelets to collagen and firm attachment to it. However, the precise role of platelet fibronectin is unclear. The proteolytic inhibitors alpha-macroglobulin, and alpha-antitrypsin inhibit the fibrinolytic mechanism. The released contents of the lysosomes may influence the interaction between endothelial cells and coagulation factors by the degradation of heparan sulphate and the destruction of the platelet aggregating ability of collagen.

1.2.4. Prostaglandin metabolism in platelets

Platelet adhesion to collagen or exposure to release-inducing agents causes activation of phospholipase and release of arachidonic acid from the platelet membrane phospholipid (20). Arachidonic acid is hydrolysed and released mainly from phosphatidylinositol and phosphatidyl-choline and undergoes oxidation mediated by cyclo-oxygenase to form thromboxane A_2 (20). Thromboxane A_2 is a potent platelet aggregator and vasoconstrictor (20).

Collagen activation of the phosphatidylinositol cycle is initiated by phospholipase C which causes the formation of inositol phosphates and 1,2-diacylglycerol from phosphatidylinositols (20). Diglyceride and monoglyceride lipases can free arachidonic acid from 1,2-diacylglycerol which may be important in thromboxane A_2 synthesis (19,21). Furthermore, 1,2-diacylglycerol may stimulate the calcium-activated, phospholipid-dependent protein kinase. This enzyme catalyses platelet membrane protein phosphorylation which results in the contractile reactions of stimulated platelets (22).

1.2.5. Role of platelet calcium.

The release of platelet granule content and the activation of the arachidonate pathway are closely linked and both require mobilisation of internal platelet calcium ions. Platelet shape change, aggregation and release of granule content require an increase in the concentration of calcium ions in the platelet cytosol. This occurs by mobilisation of calcium ions from sites of sequestration (23,25). These sites may include the dense tubular and open canalicular systems of the platelet (23). When phosphatidyl inositol turnover/hydrolysis is stimulated you will get inositol 3-phosphate (IP3) and 1,2-diacylglycerol (DAG). The former will mobilise calcium from the dense tubular system. There will then be calmodulin and protein kinase activation followed by P-20 (Mr 20,000) and P-40

(40,000) phosphorylation.(24). The phosphorylated protein (P-20) is thought to be the light chain of platelet myosin. Phosphorylation of this light chain is necessary for actin-induced activation of myosin ATPase activity (24). The phosphorylation and dephosphorylation of the myosin subunit is thought to regulate the contractile functions of platelets by actin-myosin interactions which are activated when platelets adhere to collagen or stimulated by other aggregating agents. A calmodulin-dependent protein kinase is thought to be responsible for this reaction. The effect of collagen on the phosphorylation of these proteins is mediated by an increase in cytosolic calcium ions.

1.2.6 Platelet coagulant activity

Platelets participate in blood coagulation by providing a protective and catalytic surface for the interaction of coagulation factors. The procoagulant activity, termed platelet factor 3 (PF3), is attributed to phospholipids or lipoproteins in platelet membranes and granules. PF3 is made available when platelets are aggregated with ADP or collagen. The mechanism is not fully understood but may occur by reorientation of the platelet membrane due to platelet rupture or injury. This increases the availability of both negatively charged and zwitterionic phospholipids of the inner surface of the platelet membrane. Calcium bridges link these negatively charged phosphate groups

with negatively charged carboxylate residues of the vitamin K-dependent clotting factors (II,VII,IX and X). Recent evidence has shown that the procoagulant activity of the platelet membrane involves the ability to bind the activated cofactor, factor Va, which in turn provides the binding site for factor Xa. Thus factor V plays a cofactor role in conjunction with factor Xa, calcium and platelet membrane surface to activate prothrombin to form thrombin, which in turn converts fibrinogen to fibrin (26). The interaction of the other cofactor of blood coagulation, factor VIII, is thought to be similar. A complex containing factor IXa, calcium and factor VIII is bound to the platelet membrane and activates factor X to factor Xa. Thus factor VIII with factor IXa catalyses the activation of a factor X molecule adjacent to a similarly bound factor Va molecule (26).

1.2.7. Platelet adhesion to fibrin

Platelets adhere to fibrin possibly by the fibrinogen receptors on the platelet surface. This fibrin is formed on the platelet surface by thrombin generated at the surface of the platelet via the platelet release reaction and the availability of PF3. The resulting acceleration of the intrinsic cascade pathway leads to local formation of thrombin. Platelets adhere to fibrin as it polymerizes and thus the formation of the haemostatic plug or thrombus. Haemostatic plugs and thrombi are stabilised by fibrin

formation on their surface and among the aggregated platelets at the periphery of the plug or thrombus.

2. BLOOD COAGULATION.

BLOOD COAGULATION.

The haemostatic process that follows vascular injury involves the interactions of the blood vessel wall, platelets and the plasma clotting proteins. The blood coagulation protein reactions become localised because of the interaction between platelets and components exposed or made available by endothelial cell injury at the surface of the vessel wall. Platelets adhere and aggregate in the region of damage and the coagulation pathway is activated as a consequence of proteins interacting with either the damaged vascular lining or the aggregated and adherent blood platelets themselves.

A minimum of 13 coagulation proteins are postulated to participate in a sequential and cascading manner to form a clot composed of polymerised fibrin. With the exception of fibrinogen and factor XIII, the coagulation proteins fall into two groups. Clotting proteins II, VII, IX, X, XI, XII and kallikrein are serine proteases and, except for factor VII, are zymogens which in a sequence of enzymatic reactions provide a mechanism whereby signals for initiation of coagulation are amplified. The second group of coagulation proteins are cofactors which localise and concentrate enzymes and substrates at the site of fibrin formation. In this second group of coagulation proteins are included high

molecular weight kininogen (HMWK),tissue thromboplastin and factors V and VIII.

Table 1. Blood Coagulation Proteins.

Factor	Synonyms	Molecular weight	Function
I	Fibrinogen	340 000	Polymer
II	Prothrombin	72 000	Protease
III	Tissue thromboplastin	50 000	Cofactor
IV	Calcium (*)		
V	Accelerator globulin	330 000	Cofactor
VII	Proconvertin	48 000	Protease
VIII	Antihaemophilic globulin	330 000	Cofactor
IX	Christmas factor	57 500	Protease
X	Stuart-Prower factor	66 000	Protease
XI	Thromboplastin antecedent	160 000	Protease
XII	Hageman factor	80 000	Protease
XIII	Fibrin stabilizing factor	320 000	Crosslinker
	Fletcher factor	90 000	Protease
	HMWK (Fitzgerald factor)	120 000	Cofactor

(*) Designation factor IV is given not to a protein but to the calcium ion Ca^{2+}

The plasma clotting proteins are summarised in Table 1 and are by convention labelled with Roman numerals. As yet some of the clotting proteins have not been numbered and the three original clotting proteins described by Schmidt and Morawitz, thromboplastin, prothrombin, and fibrinogen, are usually called by their names (27,28). The haemostatic process involves a series interactions between the clotting proteins the relationship of which is based on the classical cascade scheme proposed by MacFarlane and by Davie and Ratnoff (29,30). A better understanding of the interrelations, feedback mechanisms and the discovery of new clotting proteins has led to a modified and improved coagulation mechanism.

Clotting proteins II, VII,IX and X are vitamin K-dependent proteins and have several gamma-carboxylated glutamic residues on the heavy or light chain of the molecule. Glutamic acid is converted to gamma-carboxyglutamic acid in the presence of vitamin K during the last step in their synthesis in the liver (31). The residues occur at the amino-terminal portion of the clotting protein molecules and pairs of residues form a charge density that tightly chelates calcium ions (32). Without this modification the clotting proteins function poorly in the coagulation process.

The current concept of blood coagulation can be divided into three stages : the contact activation phase, extrinsic tissue factor (tissue thromboplastin) activation and generation of prothrombin conversion activity and subsequent formation of fibrin.

2. Intrinsic pathway.

Activation of the intrinsic pathway of coagulation occurs when human plasma is exposed to a highly negatively charged surface. The surfaces include glass, celite, collagen, kaolin, endotoxins, or phospholipid (platelet factor 3) and many other substances. Four clotting proteins, XII,XI, HMWK and prekallikrein, interact on the surface in a complex series of reactions (33,34).

a) Factor XII (Hageman Factor).

Factor XII is a glycoprotein with a single polypeptide chain of Mr 74-80,000 and is a serine protease zymogen that is activated by limited proteolysis by plasma kallikrein (35). Factor XII is activated by cleavage of a specific internal arginyl-valine peptide bond to give alpha-factor XIIa which by further proteolytic cleavage by plasma kallikrein generates beta-factor XIIa (36). The alpha-factor XIIa is composed of an amino terminal heavy chain (Mr 50,000) and a carboxy terminal light chain (Mr 28,000)

held together by disulphide bridges (36). The amino terminal heavy chain is responsible for the strong surface binding capacity while the carboxy terminal light chain contains the enzymatic sites. The beta-factor XIIa consists of two polypeptide chains of Mr 2,000 and 28,000 held together by a disulphide bond and although is still an active protease, it lacks the surface binding properties of alpha-factor XIIa.

b) Factor XI (Plasma Thromboplastin Antecedent).

Factor XI is a glycoprotein with two indentical polypeptide chains (Mr 80,000 each) held together by disulphide bonds; a unique finding among the coagulation zymogens (37). Each of the two monomeric chains is composed of 607 amino acids and the dimer circulates in the plasma as a bimolecular complex with HMWK (38,39). Factor XI is converted to factor XIa by limited proteolysis by alpha-factor XIIa at a single internal arginyl-isoleucine peptide bond in each chain. The factor XIa consists of two disulphide-linked heavy and light chains with Mr 48,000 and 35,000 respectively (38). Each factor XIa light chain has one active site with an amino acid sequence typical of serine protease whereas the heavy chain mediates the association with HMWK and is required for the calcium dependent activation of factor IX by XIa (38,39).

c) Plasma Prekallikrein.

Prekallikrein is a glycoprotein with a single polypeptide chain of Mr 80,000 and is composed of 619 amino acids. The glycoprotein is non-covalently complexed with HMWK in plasma and becomes associated with the activating surface during the contact activation reactions (41,42). Prekallikrein is a serine protease zymogen that is activated by factor XIIa by cleavage at a single peptide bond (Arg 371-Ile 372) resulting in the formation of two forms of kallikrein. The resulting alpha-kallikrein consists of an amino terminal heavy chain and a carboxy terminal light chain linked by disulphide bonds (42).The heavy chain region mediates the binding of prekallikrein-kallikrein to HMWK and the light chain region contains the enzymatic active site. A second product of activation by factor XIIa is beta-kallikrein, which consists of three peptide chains, which is also generated by beta-factor XIIa activation of prekallikrein and is a product of an autolytic process (43).

d) High Molecular Weight Kininogen

Plasma kininogens are large proteins containing potent vasoactive peptides termed kinins. High Molecular Weight Kininogens (HMWK) consist of one or possibly two single polypeptide chains (Mr 110,000) and contains 20% of the kinin content of plasma (44). A second low molecular weight kininogen exists (LMWK) which contains 80% of plasma kinin content and consists of a single polypeptide chain of Mr 60,000. LMWK has no procoagulant activity and no known interaction with other proteins of the contact activation system. HMWK is cleaved by plasma kallikrein at two internal peptide bonds (Lys-Arg and Arg-Ser) to liberate the vasoactive nonapeptide bradykinin. This gives rise to a two chain molecule consisting of an amino terminal heavy chain linked to a carboxy terminal light chain by a disulphide bond (45). The light chain of HMWK is the coagulant portion of the molecule and possesses a binding site for the surface as well as a binding site for the heavy chain of prekallikrein or factor XI (44,45).

e) Surfaces.

Activation of the contact system occurs when human plasma is exposed to a highly negatively charged surface. The best activators include glass, kaolin, celite, ellagic acid and dextran sulphate, all of which are non-physiological. The nature of the surface which could activate the cascade pathway under physiological circumstances is not properly established. Substances including endotoxins, phospholipids (platelet factor 3), heparin and lipopolysaccharides can also cause activation and may have pathological importance (46,47). The negative charge is required for binding of the contact factors but is not essential for the activating capacity of the surface as additional physical-chemical properties are thought to be required (46,47).

Recent work on the role of platelets in the initiation of the intrinsic coagulation pathway has suggested that stimulated platelets may contribute to factor XII and factor XI activation (48). Platelets stimulated by ADP or collagen promote the proteolytic activation of factor XII by kallikrein and HMWK, the resulting factor XIIa activating factor XI. Moreover, platelets treated with thrombin or collagen promoted factor XI activation in the presence of kallikrein and HMWK in the absence of added factor XII. Thus, as a negatively charged surface alone does not effect any

factor XI activation by kallikrein and HMWK in the absence of factor XII, activated platelets seem to provide a factor XII by-passing activity which is not fully understood. These findings partly explain the dilemma that factor XI, prekallikrein, and HMWK, but not factor XII, are required for in vivo haemostasis.

f) Contact activation reactions.

In vitro the initial event is the adsorption of factor XII to a negatively charged surface. The bound factor XII undergoes a conformational change to expose its active site and converts prekallikrein to kallikrein. HMWK acts as a non-enzymatic cofactor, its main function is to bind to negative charges and facilitate the association of prekallikrein and factor XI with the surface. The bound factor XII is highly susceptible to proteolytic activation by kallikrein to generate alpha-factor XIIa, which may eventually activate prekallikrein and factor XI. Additional alpha-factor XIIa and kallikrein are generated by reciprocal activation (49) (Fig 4).

2.2. Intrinsic pathway prothrombin conversion activity.

The middle phase of the intrinsic pathway of blood coagulation involves factor XIa, generated via the contact activation reactions, described above and its subsequent interaction with vitamin-k dependent

clotting proteins (IX,X and II) and platelet phospholipid. Firstly, Factor XIa activates factor IX in the presence of calcium ions, and then the activated factor IXa and X are bound to the negatively charged phospholipid surface of the platelet The gamma-carboxyglutamic acid residues in these proteins are linked via calcium bridges to negatively charged phospholipid on the platelet surface. Finally, factor VIII, modified by thrombin also binds non-ionically to the phospholipid surface and acts as a cofactor in factor Xa activation (50,51).

In the presence of calcium ions, factor VIII and phospholipid, factor IXa cleaves the Arg-Ile peptide bonds of factor X to generate factor Xa. The formation of thrombin from prothrombin occurs as the result of the action of Xa and three other components; factor Xa, calcium ions, phospholipid and factor Va. This collection of components is termed prothrombinase or prothrombin conversion activity (PCA). Factor Xa alone is physiologically inadequate in thrombin production and requires factor V as a cofactor, calcium ions and phospholipid (52). Thrombin activated factor V promotes the proteolytic activation of prothrombin by factor Xa. Factor Xa cleaves two bonds in the prothrombin molecule to form an intermediate which consists of a thrombin precursor. A second proteolytic cleavage converts the

thrombin precursor to thrombin. Once generated, thrombin is no longer covalently linked to the gamma-carboxyglutamic residue of the intermediate and enters the soluble phase to interact with fibrinogen.

2.3. Extrinsic tissue factor (tissue thromboplastin) activation pathway.

The extrinsic pathway is initiated by exposure of blood to injured tissue and the resulting interaction between factor VII and tissue factor. Tissue factor (also called thromboplastin) is a lipoprotein present in cell membranes of most tissues (hence extrinsic to plasma), which bypasses the earlier stages of the intrinsic pathway. Tissue factor is present in large amounts in brain, lung and placenta but absent in platelets and muscle.

Lipid comprises one third of the molecular weight of tissue factor and is essential for its activity. Tissue factor purified from bovine or human brain and placenta has been shown to consist of one species of protein called apoprotein III, which is complexed with a mixture of phospholipids (53). The protein moiety is devoid of enzymatic activity and has a molecular weight of 46,000. Purified apoprotein III does not possess any procoagulant activity, but if recombined with appropriate phospholipids (phosphatidyl choline,

phosphatidylethanolamine and phosphatidyl serine), regains its full potency (54).

Factor VII is a vitamin K-dependent single chain glycoprotein, molecular weight 40,000, which is synthesised in the liver. The protein consists of 406 amino acids and contains in the amino terminal region 10 -carboxyglutamic acid residues, which are involved in calcium-mediated phospholipid binding (55). The current concept for the interaction of tissue factor with factor VII is that factor VII activates the exposed tissue factor and in the presence of calcium ions, binds factor VII to form a complex which slowly activates factor X and IX. The resultant activated factors Xa and IXa then proteolytically activate factor VII to VIIa. Thus the function of the factor VII/tissue factor complex is to generate minimal amounts of factor Xa which rapidly back-activates factor VII to VIIa. Factor VIIa then rapidly activates both factors X and IX (56). The binding of tissue factor to factor VIIa induces a conformational change that enables factor VIIa to activate its natural substrate factor X. After binding a second conformational change in the factor VIIa occurs, resulting in a tighter binding to tissue factor (56).

Regulatory mechanisms exist which inhibit the tissue factor/factor VII-mediated activation of coagulation. A plasma component associated with the lipoprotein fraction (called extrinsic pathway inhibitor or EPI) binds to tissue factor/factor VIIa in the presence of calcium ions and also requires the presence of factor Xa for expression of its inhibitory activity (57). Other inhibitors which have been reported include plasma lipoproteins such as apolipoprotein AII which differ from EPI in their mechanisms of inhibition (162). The pathological importance of these inhibitors has yet to be determined.

2.4. Relationship between intrinsic and extrinsic pathways.

The two pathways converge at the factor X level into a common pathway (Fig 1). Factor X is activated by either tissue factor/factor VIIa or factors IXa-VIIIm (VIIIm:factor VIII modified by thrombin), Factor X is also a vitamin K-dependent clotting protein and circulates as a two-chained protease held together by disulphide bonds. Activation by either pathways generates factor Xa which in the presence of a thrombin-modified cofactor and factor V, facilitates the activation of prothrombin. In the conversion of prothrombin to thrombin, factor Xa cleaves two bonds (Arg-Thr and Arg-Ile) to form disulphide-bonded A and B

chains that represent the carboxy-terminal half of the precursor. Once generated thrombin enters the soluble phase to interact with fibrinogen. Thrombin not only modifies factors V and VIII, but is able to induce platelet aggregation. However, its best interactions are with fibrinogen and factor XIII.

2.5. Fibrin formation.

Fibrinogen is a large glycoprotein and is the most abundant clotting protein in plasma (2-5g/l). It is composed of three pairs of non-identical polypeptide chains interconnected by disulphide bonds. The molecular weight of the individual chains are alpha=63,000, beta=56,000 and gamma=47,000. The fibrinogen molecule contains three domains, the outer two are identical while the central domain is distinct. This central domain contains the amino terminal portions of the molecule which are held together by disulphide bonds. This central domain also contains fibrinopeptides A and B which are the amino termini of the and chains (163).The conversion of fibrinogen to fibrin, by thrombin, involves the thrombin-catalysed removal of the 16 residue fibrinopeptide A. The fibrin monomers that are formed undergo limited polymerization which promotes cleavage of fibrinopeptide B. This second proteolysis enhances polymerization by yielding further fibrin monomers

which then form a polymer. The fibrin polymer is then stabilised by the interaction with factor XIIIa (163).

Factor XIII is found in both platelets and plasma. Factor XIII is activated by thrombin and in the presence of calcium ions an essential cysteine residue at the active centre of the catalytic subunit is unmasked. The active enzyme (factor XIIIa) catalyses the formation of two isopeptide bonds between specific glutamine and lysine residues on adjacent gamma-chains and subsequently between alpha-chains in the fibrin polymer. These crosslinks protect the fibrin clot from premature dissolution by increasing its resistance towards plasmin digestion (163).

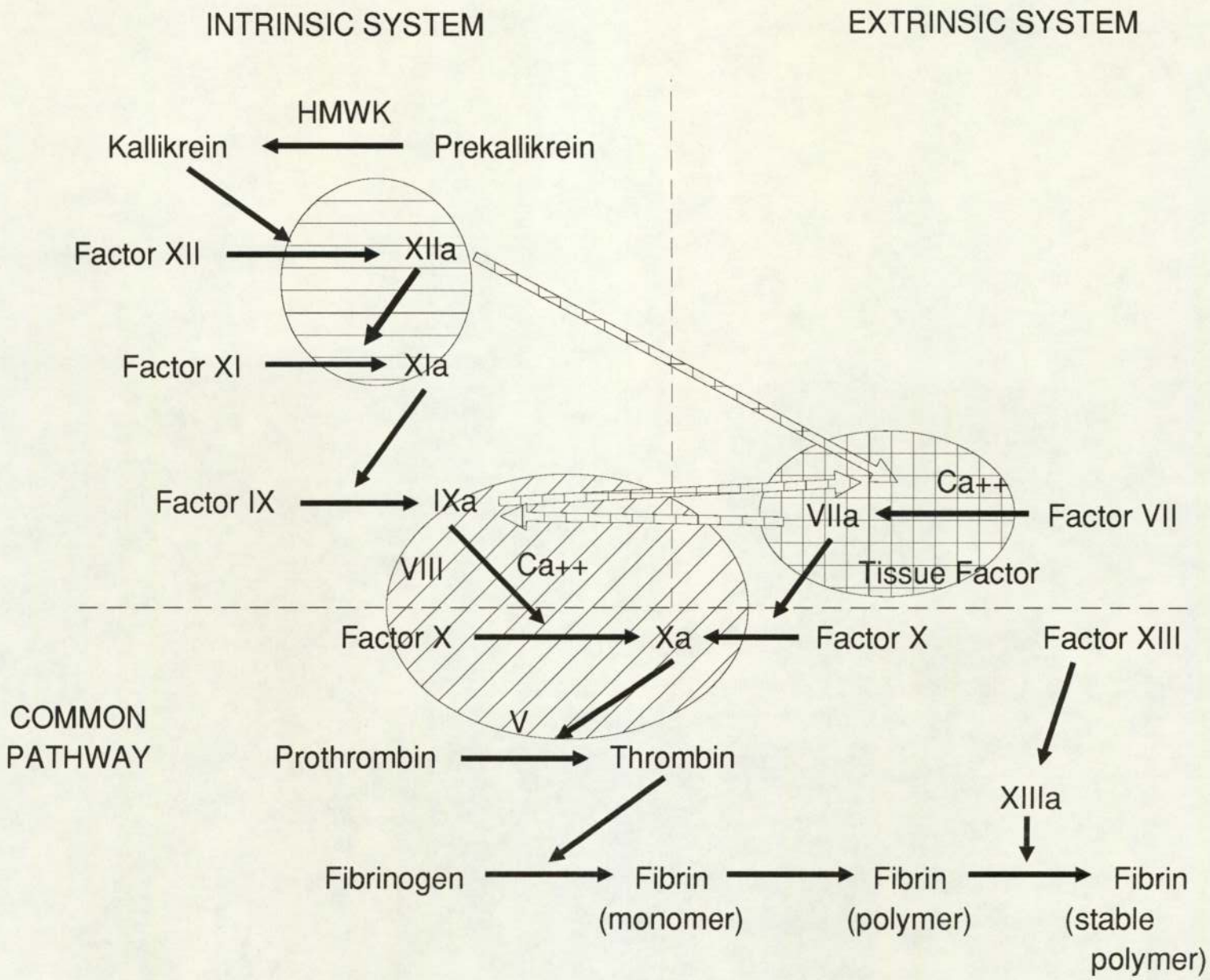

INTRINSIC SYSTEM
EXTRINSIC SYSTEM
HMWK
Kallikrein
Prekallikrein
Factor XII
XIIa
Factor XI
XIa
Factor IX
IXa
VIII
Ca++
Ca++
VIIa
Factor VII
Tissue Factor
Factor X
Xa
Factor X
Factor XIII
V
COMMON PATHWAY
Prothrombin
Thrombin
XIIIa
Fibrinogen
Fibrin (monomer)
Fibrin (polymer)
Fibrin (stable polymer)

Table 2. Nomenclature for Factor VIII and von Willebrand factor (202).

The nomenclature in this thesis has been taken from that advocated by the International Committee on Thrombosis on Thrombosis and Haemostasis (202).

Attribute	Abbreviation	
	Proposed	Outmoded
Factor VIII		
Protein	VIII	VIII:C
Antigen	VIII:Ag	VIIIC:Ag
Function	VIII:C	------
von Willebrand factor		
Protein	vWf	VIIIR:Ag, VIII/vWf
Antigen	vWf:Ag	VIIIR:Ag
Function	* Not abbreviated	VIIIR:RCo, VIIIR:vWf

* The abbreviation used to indicate ristocetin cofactor activity is vWfRiCoF and that for botrocetin cofactor activity is vWfBCof. Neither of these two tests nor any other in vitro test completely reflect vWf activity. The two proteins, VIII and vWf, form a bimolecular complex which is abbreviated VIII/vWf.

Table 2a. Selected properties of factor VIII and von Willebrand factor (202).

Assay	Factor VIII	von Willebrand factor
Functional	Partial Thromboplastin Time (PTT), Factor Xa formation	Bleeding time (BT) Platelet adhesion, Platelet agglutination with ristocetin (RIPA).
Immunologic	IRMA, ELISA, Immunoblot.	Quantitative: IRMA, ELISA Electroimmunoassay (Laurell) Qualitative: 2DCIE,* Multimer analysis, Immunoblot.

*The migration index (MI) is the distance of the arc's peak, as determined by 2DCIE, from the original point of application compared with a normal standard and can be used to express the results of this technique.

The PT (Prothrombin Time) involves adding thromboplastin and calcium to plasma and recording the clotting time. The principal use of the PT is to screen for clotting abnormalities of the extrinsic pathway of blood coagulaton.

3. VON WILLEBRAND DISEASE.

Von Willebrand Disease.

Von Willebrand disease (vWd) is a common inherited disease amongst bleeding disorders (12 per 100,000) (203), characterised by impaired platelet adhesion and thrombus formation at the site of vascular injury. The disorder was first described in 1926 by Erich von Willebrand who reported a family suffering from a bleeding diathesis in the Aland Islands (58). The condition was initially termed angiohaemophilia because of the possible role of vascular abnormalities, or pseudohaemophilia as some vWd patients had low levels of procoagulant activity (factor VIII:C), as in haemophilia A, which may contribute to the bleeding diathesis (59,60). However, over the last decade research has increased our understanding of the structure and function of the factor VIII/von Willebrand complex. Thus, we now know that the defective platelet function in vWd is due to quantitative and/or qualitative abnormalities of the von Willebrand factor (vWf). This haemostatically important glycoprotein is synthesised by vascular endothelial cells and megakaryocytes and stored in platelets. It is also present in the subendothelium and circulates in blood as a non-covalent complex with the procoagulant protein (factor VIII:C).

The heterogeneity of vWd is clinically expressed by a variety of conditions ranging from asymptomatic to severe conditions requiring treatment. Thus an appreciation of the role of vWf in haemostasis is essential to a understanding of the pathogenesis of vWd.

3.1. Historical background.

In 1957 a factor present in normal and haemophilic plasma was shown to correct the haemostatic defect in vWd (60). Furthermore, infusion of normal plasma into vWd patients stimulated the production of procoagulant activity (factor VIII:C) to a level much higher than could be explained by the factor VIII:C activity of the infused material. These observations were attributed to the effects of an unknown factor in the normal plasma (60).

A number of *in vitro* tests were subsequently developed to measure platelet function; the most widely used consisted of passing blood through columns containing tiny glass beads. When normal blood is passed through the column many of the platelets adhere to the glass beads, while a greater proportion of platelets escaped from the column when blood from patients with vWd was used (60,61). This abnormality was corrected when normal plasma or plasma fractions containing factor VIII were added to the column or to vWd whole blood.

By 1963 the diagnosis of vWd was based upon an autosomal mode of inheritance, prolongation of the skin bleeding time, decreased platelet retention in glass bead columns, low level of factor VIII:C activity and the unusual in vivo response to factor VIII:C infusions. The most important development was the demonstration and measurement of the plasma factor which corrected the abnormality in vWd. This protein was thought to be immunologically related to factor VIII, since the antisera used to detect deficiency in vWd by immunoelectrophoresis had been raised against protein fractions containing factor VIII, and was termed factor VIII-related antigen. These antibodies identified the factor VIII-related antigen now known to be the identifying antigen of vWf (ie vWf:Ag). The protein was found to be decreased or absent in vWd, whereas it was present in normal or increased amounts in patients with haemophilia A, despite their lack of factor VIII:C (62).

Further progress was made in 1971 when Howard and Firkin observed that the antibiotic ristocetin, which had been withdrawn because it caused thrombocytopenia, was able to induce platelet agglutination in normal platelet rich plasma (63). Platelet agglutination by ristocetin did not occur or was reduced in the platelet-rich plasma of patients with vWd unless normal plasma or even plasma from patients with haemophilia A was added. Lack of

ristocetin-induced agglutination of platelets in vWd in association with the decrease or absence of vWf has led to the development of a functional test to quantitate vWf activity and express it as ristocetin cofactor activity (vWfRiCof) (64). This test has proved to be useful in the diagnosis of most cases of vWd and is used in measuring the vWfRiCof activity of vWf using paraformaldehyde- or formalin-fixed platelets (65). In 1974 it was demonstrated that vWf was necessary for adhesion of platelets to the subendothelium of denuded rabbit aortas, and subsequent research has also demonstrated the presence of thrombin-induced receptors for vWf on platelets (67).

Considerable progress has been made in the characterisation of the factor VIII/von Willebrand factor complex over the last decade. In particular, immunological measurement of vWf by highly sensitive radiolabelled and enzyme immunoassays using polyclonal and monoclonal antibodies, isolation of highly purified human factor VIII and analysis of the multimeric structure of vWf in platelets and plasma from vWd patients have greatly improved our understanding of vWd and treatment of patients. (68,69).

Study of increasing numbers of patients have shown vWd to be a heterogeneous condition. Subtypes are defined by determining the vWf:Ag multimer pattern in these patients

and this has contributed further to the understanding of the structure of vWf.

3.2. Biosynthesis of vWf.

The vWf protein is synthesised in endothelial cells and megakaryocytes. The biosynthesis is complex and involves a primary translation protein of 2813 amino acids called pre-pro-vWf protein (158,159). The gene encoding for this protein is located on chromosome 12 and the corresponding mRNA is 8.5-9.0 kilobases in length (158,187). Of the 2813 amino acids 2050 are of the mature subunit, 741 correspond to von Willebrand antigen II and 22 to a signal peptide (Fig 2.). After removal of the signal peptide, pro-vWf protein undergoes a complex series of steps to form dimers which by means of inter-dimer disulphide bonds at the amino termini, form multimers. The homologous cysteine-rich D domains (D2a/D3 and D4) shared between the pro-vWf segment and mature vWf are thought to be involved in the intermolecular disulphide bonding found in vWf multimers (182). The homologous D domains (D1 and D2) within the pro-vWf segment may also be involved in directing the intermolecular alignment of free sulphhydryl groups that form disulphide links between the D2a/D3 regions of adjacent vWf protomers (182). The pro-vWf directed assembly of vWf multimers appears to be mediated through the formation of a transient disulphide-linked complex which may involve the sulphhydryl groups of the D domain

with the pro-vWf and the N-terminal region of the mature vWf subunit. (183). The mechanism by which pro-vWf binding leads to the formation of mature vWf subnuits at their N-terminal regions is not fully understood. However, pro-vwf may function as a catalyst for disulphide bond formation or direct vWf subunits to cellular components involved in the assembly of disulphide-linked proteins, or align the amino-terminal segments of vWf dimers to allow the formation of disulphide bonds (182). Furthermore, it appears that pro-vWf can direct the alignment of mature vWf protomers without it being a continious part of the pro-vWf primary structure (181). The vWf antigen II as a plasma and platelet protein is involved conformational changes in the pro-vWf protein which allows the formation of vWf dimers (188,189). Mature vWf is stored in endothelial cells in a organelle called the Weibel-Palade body and is released by a variety of stimulators (Fig.2) (160,161,162).

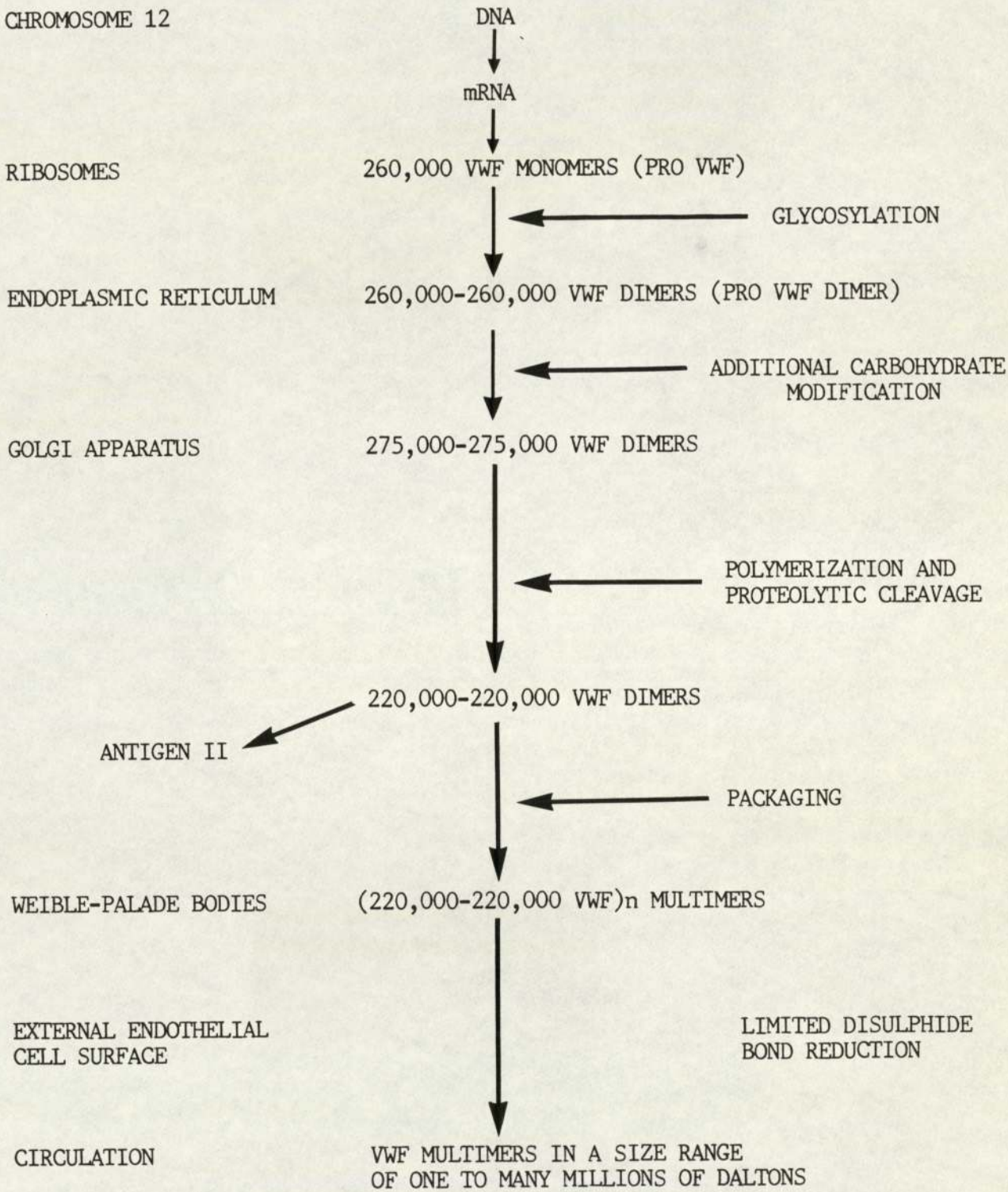
Four distinct types of domains have been identified on the vWf protein. The A domains contain 193 to 220 amino acids and are found in three tandem copies. The three B domains contain 25 to 35 residues and the two C domains contain 116 to 119 residues (191). Finally, four copies of the D domains exist and contain 351 to 376 residues. The first pair of D domains are tandemly arranged in the vWf:Ag II propeptide, whereas the remaining D domains are

separated by over 700 amino acids. The propeptide cleavage site lies between the second D domain and a D' fragment that occurs at the amino-terminal end of the third complete D domain (192). The glycoprotein Ib-binding domain of vWf resides in a 52/48-kilodalton (kD) fragment extending from Val_{499} to Lys_{728} (A_1 domain) (196). This same domain also contains binding domains for collagen (193) and heparin (194). The binding site for the platelet receptor GPIIb/IIIa appears to be in the C-terminal region in the tetrapeptide sequence Arg-Gly-Asp-Ser (residues 1744 through 1747), a sequence common to fibrinogen and fibronectin (197,198). A second binding domain for heparin (amino terminal region within residues 1 through 300) and collagen (residues 911 to 1114 (199,200). A factor VIII:C binding site exists at the amino terminal region of the vWf subunit (201).

In primary haemostasis, platelets covering injured endothelial cells involves bridging of vWf which binds to the components of the subendothelium, such as collagen and heparin-like glycosaminoglycans, and to the glycoprotein Ib on the platelet membrane. The binding of vWf to GPIb results in platelet activation and may promote recruitment of additonal platelets to the thrombus. The role of fibrinogen binding to GPIIb/IIIa is also important in platelet recruitment and may play a role in the

formation of the initial platelet monolayer. Finally, vWf localizes factor VIII at the site of a developing thrombus.

Fig. 2. Synthesis, secretion and processing of von Willebrand protein.



3.2.1 Von Willebrand Protein.

Von Willebrand factor is a high molecular weight glycoprotein which is lacking or structurally abnormal in vWd. Although vWf circulates in blood as a non-covalent complex with factor VIII the two proteins are distinct molecules (70,180,187). The physiological importance of the complex formation between vWf and factor VIII is not fully understood but vWf has a protective role in preventing the destruction and removal from the circulation of inactive factor VIII by proteases and activated protein C. vWf may also promote the localization of factor VIII at the site of thrombus formation. The most important functions of vWf are the promotion of adhesion of platelets to the subendothelium at high shear rates and as a carrier protein for factor VIII. Although direct evidence for the carrier function of vWf is lacking it is possible that factor VIII has only a short half-life in vitro without vWf. This function of vWf is suggested by the absence of circulating factor VIII in cases of vWd where vWf is absent. Furthermore, the stability of factor VIII in blood is dependent on calcium ions and the maintenance of the VIII/vWf complex is calcium dependent (71). Further evidence for the carrier function of vWf has been obtained from the observation that infusion of haemophilic plasma into vWd patients causes a rise in factor VIII:C (60). Factor VIII binds to vWf via the light chains of the VIII

molecule and is reversed by dissociating agents such as EDTA (71).

The plasma concentration of vWf is 7-10ug/ml whereas factor VIII is approximately 0.1ug/ml. Although there is a 100-fold excess of vWf over factor VIII it is not known whether some vWf multimer may be associated with more than one factor VIII molecule or whether there is only one factor VIII molecule for each multimer. Normally, vWf circulates as a complex non-covalently bound to factor VIII and represents over 95% of the mass of the complex (70). Historically, the two molecules could be separated by use of high ionic-strength buffers, showing that the two activities reside on separate molecules with no immunological relationship between them (72,73,74,75). It is now known that factor VIII and vWf are distinct molecules.

3.2.2. Multimeric structure of vWf.

vWf consists of a single subunit with a molecular weight of 195,000-270,000 which is linked at the carboxyl terminal end by disulphide bonds to another subunit to form a dimer (78,79,80). This dimer may be the protomer (smallest circulating multimer) of a range of multimeric forms with molecular weights varying from 1-20 millions (77). However, some studies have suggested that the protomer is a tetramer of four identical subunits linked by disulphide bonds (75,76). This difference in the

estimation of the molecular weight is due to the difficulty in assigning a correct molecular weight when working with a unreduced glycoprotein (77).

Endothelial cell culture experiments estimate the protomer molecular weight to be 440,000 and appears to be a homodimer (78). Approximately 15 to 20 multimers have been identified by SDS-gel electrophoresis, although larger multimeric forms could not be resolved into individual components (77). The repeating multimeric patterns visualised comprise of several bands, abnormalities of which have been described in certain subtypes of vWd (type IIC and type IID) (82,83). Thus the several bands seen in the multimeric pattern are an important feature in the structure of the vWf protein. Haemostatic efficacy of the vWf multimers is related to molecular size (184). The bleeding time is always prolonged in patients who lack the largest multimeric forms, regardless of the concentration of vWf protein in plasma (84,85).

3.2.3. Carbohydrate in vWf protein function.

The role of carbohydrate residues in the structure and function of the vWf protein is unclear. It has been suggested that modification of terminal sialic acid and mainly the penultimate galactose residues results in impaired platelet-vWf interaction. Early studies showed that enzymatic removal of sialic acid with neuraminidase

affected ristocetin-induced platelet aggregation (86). However, other investigators detected no reduction in ristocetin cofactor activity after treatment with neuraminidase (87). Some data has shown that the penultimate galactose residues are important in ristocetin cofactor activity; removal of or oxidation of galactose results in decreased activity (88). This was reversed by subsequent galactose reduction. The structural integrity of the multimer has also been shown to be dependent on such galactose residues (89). A loss of ristocetin cofactor activity in parallel with the disaggregation of the large multimers has been demonstrated to be proportional to the loss of galactose from the vWf molecule (89). This abnormality of the carbohydrate moiety has been suggested as a primary cause of the defect in type IIA vWd (89,90). Removal of sialic acid and galactose from the vWf protein render it susceptible to the degrading action of proteases, particularly plasmin. However, in the presence of protease inhibitors, approximately 80% of galactose can be removed without any significant loss of multimeric forms and ristocetin cofactor activity (91). These data suggest that the carbohydrate moiety plays a key role in protecting the vWf protein from proteolytic attack (91).

3.2.4. Platelet vWf.

Platelets contain approximately 20% of the total vWf but no factor VIII:C (92,93). Most of the platelet vWf is

of megakaryocytic origin and is stored in the alpha-granules.(94,95). Platelet alpha-granule vWf is released when platelets are stimulated with ADP, thrombin or collagen; a rapid increase in the amount of vWf is expressed on the cell surface after thrombin activation (96,97). The association of vWf with the platelet membrane is divalent-cation dependent and requires the glycoprotein IIb/IIIa complex (96). This is clearly demonstrated in patients with thrombasthenia, where normal release of vWf occurs via thrombin stimulation but no increase in membrane expression of vWf occurs due to the lack of glycoprotein IIb/IIIa complex (96). Platelet vWf may be haemostatically important by providing high concentrations of vWf protein locally after stimulation. The platelet vWf differs structurally from plasma vWf in that it contains larger molecular forms which may be significant since the larger multimers are known to be most haemostatically effective (97).

3.2.5. Interaction of vWf with the subendothelium.

The vWf protein binds to the vascular subendothelium although the components involved are not known. Only certain types of collagen (type III) bind vWf although types I, II and III are all capable of adsorbing vWf from plasma (98,99). The quaternary structure of the collagen is essential for binding to occur rather than the type of collagen (99). Optimal binding appears to require fibrillar

collagen and the presence of large vWf multimers (99). A specific domain of the vWf molecule, different from the one for the binding to platelets in the presence of ristocetin, is involved in the interaction with collagen type I and III (100). The exact mechanism for the interaction between collagen and vWf is not fully understood.

Although vWf is essential for the interaction of platelets with the injured vessel wall and for thrombus formation, it is not clear whether it is necessary for in vivo platelet aggregation (101). Perfusion studies have demonstrated that at high shear rates (>1300/s), vWf participates in platelet deposition on to exposed subendothelium. In these studies a significant platelet adhesion defect is observed in vWd patients which is corrected by the addition of normal plasma or plasma fractions (cryoprecipitate) containing vWf (102,103). At low shear rates (200/s), corresponding to large veins, no adhesion defect is measurable in vWd patients. Thus, vWf may act as a cofactor for platelet adhesion only when the residence time of the platelet at the subendothelial surface is extremely short.

It appears that the adhesion of platelets to the subendothelium and their subsequent activation is dependent on plasma and subendothelial vWf (104,105).

Endothelial cells deposit vWf in the subendothelial matrix but whose respective roles are not fully understood, and even less is known about the role of platelet vWf. The main biological function of the vWf is to enhance the attachment of platelets to the wall of damaged vessels and act as a carrier for factor VIII:C in order to achieve high concentrations of VIII:C at the site of injury (104,105).

4. METHODS OF VWF ANALYSIS.

Methods of vWf analysis.

The principles and usefulness of the more standard methods for examining vWf will be discussed, but further details of techniques used in this study are presented in chapter 6.

Deficient or abnormal vWf activity occurring in vWd can be ascertained by analytical and functional tests. Quantitation and multimeric pattern analysis of vWf protein can be determined by using polyclonal or monoclonal antibodies in electrophoretic and immunological techniques. However, such methods may not reflect functional/qualitative abnormalities of vWf activity in vWd. In contrast, the observation that ristocetin aggregated normal platelets, but not platelets from patients with severe vWd, provided a useful in vitro diagnostic test for functional vWf activity. Therefore methods for measuring vWf plasma activity, based on the the aggregation of platelets in the presence of ristocetin, were developed this activity and termed ristocetin cofactor activity (VIIIR:RCo). The quantitation of vWf activity may be performed by a variety of procedures including platelet aggregometry, platelet counting, macroscopic, radioisotope and immunoassay methods. The quantitation of plasma vWf, in conjunction with other tests used to evaluate various

properties of the factor VIII/vWf complex, has greatly improved the diagnosis and classification of vWd.

4.1. Skin bleeding time.

Although many methods of measuring vWf activity exist, the most direct method is correction of the skin bleeding time defect in vWd. This test is a valuable direct in vivo screening test for vWd, but it is not quantitative or specific for vWf as it is also prolonged in thrombocytopenia and platelet defects, both acquired and inherited. The skin bleeding time primarily determines platelet participation in small vessel haemostasis and identifies both qualitative and quantitative disorders in primary haemostasis. Thus the skin bleeding time will be prolonged (Ivy method >7 mins) (106,107,108) in most cases of vWd. However, numerous variables affect the performance of the test and many modifications of the original method of Duke and Ivy have been employed to standardise their effect; as a result a number of different techniques are currently used (106,107,108).

4.2. Platelet adhesion/retention method.

Glass bead retention methods have been extensively used in the past in the diagnosis of vWd, but have generally been abandoned because of the lack of specificity and reproducibility due to many technical factors (eg type of plastic tubing, size and quantity of glass beads, flow

rate). A more physiological test is that of vWf-mediated adhesion of platelets to the subendothelium of rabbit aorta, where platelet adhesion is reduced in vWd (109). This adhesion defect can be corrected by the addition of purified vWf to the blood. Again this technique does not specifically measure vWf activity.

4.3. Immunoassay of vWf Antigen.

Immunological quantitation of vWf antigen (vWf:Ag) aids in the diagnosis and classification of vWd. Although many immunological techniques are employed to assay vWf:Ag, those most frequently used are based on immunoprecipitation reactions which include the electroimmunoassay (EIA) and the two-dimensional crossed immunoelectrophoresis technique (2DCIE) (111,112).

The EIA technique suffers from the disadvantages of molecular size and charge influencing the result. This may give spurious results when measuring vWf:Ag in variant vWd patients (type II disease) who have have only low molecular weight vWf:Ag multimers present. These multimers have an increased anodal migration as demonstrated in 2DCIE techniques. Furthermore, the sensitivity of the EIA technique is approximately 5-10% which limits its use in measuring very low levels of vWf:Ag in severe forms of vWd. The principle of the EIA technique involves incorporation of a heterologous antibody against the

vWf:Ag into agarose gel poured on to a glass plate. Plasma containing vWf:Ag is applied to one end of the gel (cathode) and a constant electrical potential applied across the plate. The vWf:Ag moves out of the well as a concentrated peak and forms precipitin lines when the concentration of vWf:Ag and the anti-vWf:Ag reach equivalence. The height of the rocket formed is proportional to the concentration of vWf:Ag. The appearance of the peaks is determined by the quality and concentration of the antiserum used and there is a degree of subjectivity in measurement of the resulting peak heights. In addition, the pH of the gel buffer and the reproducibility of plate preparation are important. Careful control of the reservoir buffer pH, electrophoresis potential difference and temperature of the plate are necessary.

An alternative method for quantitation of vWf:Ag has been to apply the principles of enzyme-linked immunosorbent assay (ELISA) (113). This technique has the advantages of ease, speed and a sensitivity ten times greater than immunoelectrophoresis. The increased sensitivity makes the technique useful in the investigation of patients with severe vWd, and in monitoring replacement therapy in patients with vWd, where correction of vWf:Ag is important, the speed of this technique is also an advantage. Furthermore, the ELISA technique may be less

sensitive than EIA to differences in molecular size and charge of vWf:Ag. This has been demonstrated by comparing dose response curves in ELISA and EIA assays for type IIA vWd patients (114). Of the 15 type IIA vWd patients tested no parallelism was obtained by immunoelectrophoresis, whereas in 10 of these patients parallelism was demonstrated by the ELISA assay. However, no correlation between the multimer pattern of these patients and the degree of parallelism in the ELISA assay was observed (114).

In the ELISA assay the microtitre wells are coated with either monoclonal or polyclonal antibody (rabbit) against vWf:Ag. Test samples and standards containing the vWf:Ag are incubated with the sensitised microtitre plate. Non-reactive material is washed away from the surface and enzyme-conjugated specific antibody for vWf:Ag added and incubated with the microtitre plate followed by washing. Finally the enzyme substrate is added and the resulting colour changes is proportional to the amount of vWf:Ag in the test plasma.

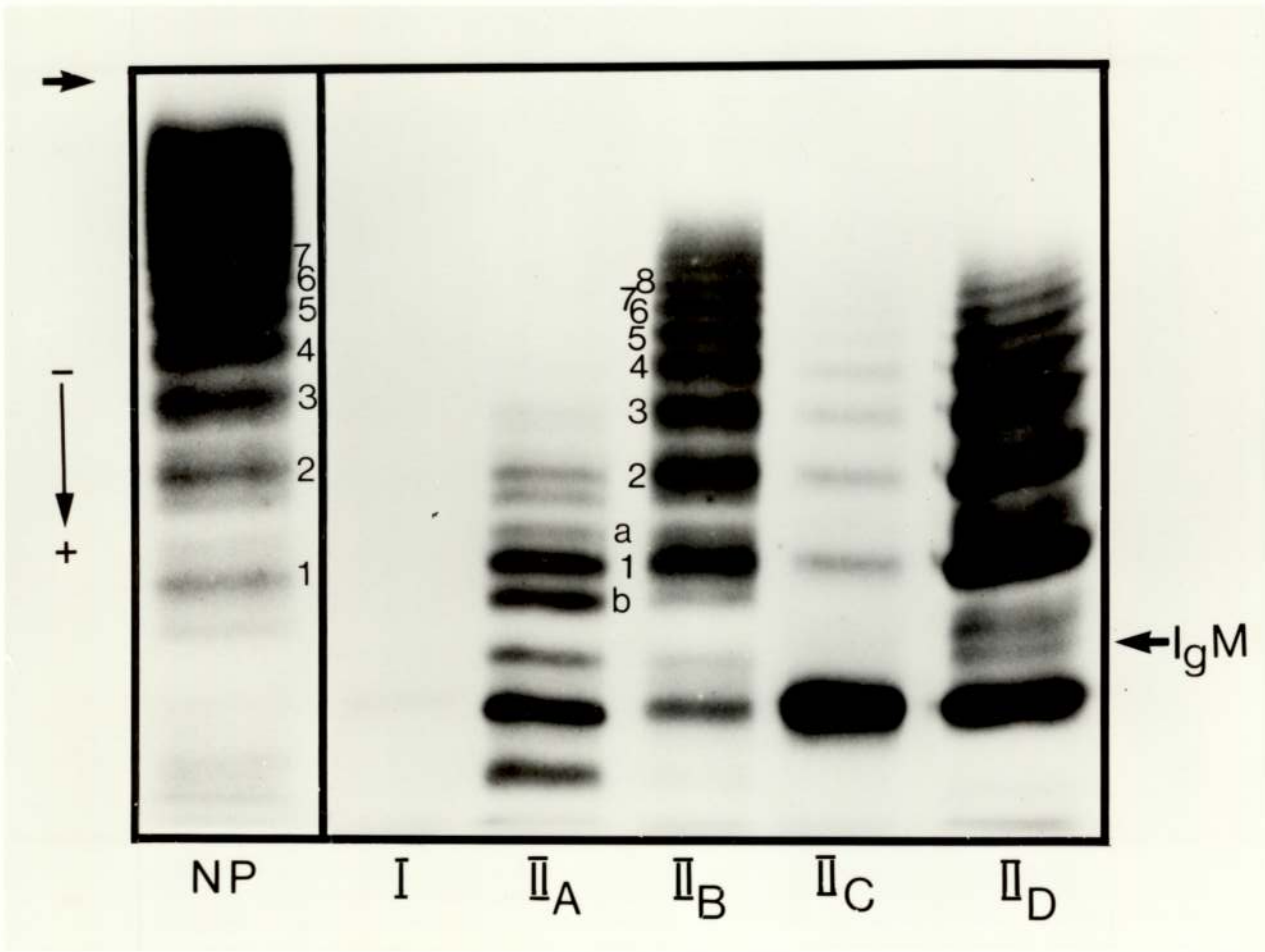
4.4. Qualitative evaluation of vWf:Ag.

Qualitative analysis of the vWf:Ag is most easily obtained by using two-dimensional crossed immunoelectrophoresis (2DCIE) as differences in electrophoretic mobility of patients vWf:Ag has been demonstrated in the type II or variant vWd patients (112).

In this test, vWf:Ag undergoes electrophoretic migration in one dimension in agarose containing no antibody against vWf:Ag, and then electrophoresised at right angle into agarose containing polyclonal anti-vWf:Ag. A precipitin arc is formed with length corresponding to the distance migrated by the vWf:Ag in the first dimension, and the curve of the arc corresponding to the relative concentration of the vWf:Ag of the components. Various molecular species may also be highlighted if their concentration is altered relative to normals, and such differences are reflected by a change in the shape of the precipitin arc.

Polyacrylamide gel electrophoresis (PAGE) has been used to study the structure of the vWf protein. The high resolution power of this technique has permitted analysis of the multimeric structure and the di-,tri- and tetrameric forms of vWf (Fig. 2). Electrophoretic separations are determined by the the physiochemical characteristics of

the proteins, steric network of the gel, composition of gel and electrode buffers and magnitude of electric field.


7
6
5
4
3
2
1
8
7
6
5
4
3
2
a
1
b
IgM
NP
I
II A
II B
II C
II D

Electrophoresis is performed on polyacrylamide gels containing agarose and sodium dodecylsulphate (SDS) which permits separation of protein by size rather than charge density. Polyacrylamide gels have a much smaller pore size than agarose gels allowing them to act as a molecular sieve, impeding the progress of high molecular weight proteins whilst permitting smaller proteins to migrate more rapidly (137). If a surfactant such as SDS is included in the gel and electrophoresis buffer, the hydrophilic characteristics of the protein are enhanced, masking its negative charge of variable density with a uniform negative charge. Polymeric proteins such as vWf may then be separated by size independently of charge density (137).

Polyacrylamide gel is formed from acrylamide and NN' Methylenebisacrylamide. The acrylamide chains cross link with each other to form a three-dimensional gel structure which has advantages over other gel materials in terms of chemical stability and pore size. In addition the gel are inert, insoluble, non-absorptive and do not display electro-osmotic effects.

Gels for multimeric analysis of vWf protein are prepared by dissolving agarose and SDS in alkaline buffer with heating. Cross linked acrylamide and a catalytic mixture is added to the molten agarose and the gel poured into a U-frame and allowed to solidify. Various catalysts can be employed and they are commonly used in combination

(eg 3-dimethylaminoproprionite and ammonium persulphate). The oxidative effects of ammonium persulphate can be avoided by incorporation of 2-mercaptoethanol in the polymerization mixture.

Once the polyacrylamide gel is prepared a second stacking gel containing agarose (large-pored) and SDS, is poured adjacent to it and the sample for vWf protein analysis is added to wells in the stacking gel. The vWf protein is concentrated in the stacking gel in the first phase of electrophoresis and then separated into its individual fractions in the smaller-pored separating acrylamide gel.

In the electrophoresis high ionic strength buffer solutions are required, so that the charge on the leading and following ions have the same sign. During electrophoresis the higher mobility of the leading ions in the gel cause them to move in advance of the vWf protein and the following ions in the vessel buffer, leaving a zone of lower conductivity and higher field strength. This accelerates the vWf proteins and the following ions so that they migrate at the same rate behind the leading ions and hence accumulate in the stacking gel. When the migrating vWf protein zone reaches the dividing line of the stacking and separating gel it comes into a buffer of such pH that the rate of the following ions almost attain

that of the leading ions and exceeds that of any of the vWf protein molecules; thus the following ions migrate behind the leading ions, but in front of the protein vWf (137). This second phase of electrophoresis separates the multimers by size, and the gel is then fixed and reacted with purified radiolabelled antibody to vWf:Ag protein and the vWf multimers visualised and identified by autoradiography (Fig.2).

Normal vWf protein shows a range of high, intermediate and low molecular weight multimeric bands. In high resolution gels the lower multimers appear to be composed of a triplet configuration with a dense staining central band and flanking pale bands above and below. The intensity of the bands may be assessed by computer aided laser densitometry. Multimeric analysis has made a major contribution to the study of the substructure of vWf and is also used as a way of classification of vWd.

4.5. Ristocetin-induced platelet aggregation.

The observation that platelet aggregation by ristocetin could distinguish between normal and vWd patients provided an important diagnostic test and allowed the development of quantitative assays to measure the biological activity associated with vWf protein. Normally, platelet rich plasma will aggregate at ristocetin concentrations between 0.7-1.5 mg/ml; this is due to individual and reagent batch to

batch variation (85). When platelet rich plasmas from vWd patients are tested with ristocetin a decreased or absent aggregation response is seen. The defective response is observed when the vWf protein, as measured in quantitative assays, is below 30% of normal plasma levels, or when a qualitative vWf protein defect is present.

Disease entities have been identified in which platelets are unusually hyperactive to ristocetin at low concentrations. These two conditions are type IIB vWd and pseudo-vWd (or platelet-type vWd). In type IIB vWd the defect is due to a structurally abnormal vWf protein, while in pseudo-vWd the defect is due to abnormal platelet vWf receptors (115,116,117). A distinct inherited platelet disorder, Bernard Soulier disease, is characterized by platelets that do not react with ristocetin because their platelet membrane lacks glycoprotein Ib, but can be distinguished from vWd because the addition of normal plasma to their platelet rich plasma does not correct the ristocetin agglutination defect. This contrasts with vWd when the addition of normal plasma always corrects defective ristocetin-induced platelet agglutination of platelet rich plasma.

The mechanism of action of ristocetin is believed to be an alteration of the surface charge of the platelet which permits vWf binding (118). Platelets carry a net negative

charge on their surface which if decreased in the presence of ristocetin facilitates formation of a vWf bridge between platelets. It also appears that exposure of vWf binding sites on the platelet surface due to the action of ristocetin seems important in ristocetin-induced platelet agglutination as neuraminidase-treated platelets, despite their surface charge being decreased by 50% of normal, show no demonstrable enhanced ristocetin-induced platelet agglutination (119). The platelet receptor, glycoprotein Ib, plays a significant role in this interaction and some evidence exists that it carries approximately 15% of the whole platelet negative charge. Thus ristocetin appears to have two principle actions on platelets which involve a non-specific decrease in the whole platelet negative charge and exposure of binding sites for vWf on the platelet surface. The interaction of the individual vWf multimers with the platelet binding sites is not fully understood, although some work has suggested that the large multimers bind with high affinity whereas oligomers of lower size bind less well (120). Also, a correlation of ristocetin-induced binding of vWf to platelets and to haemostatic function has not been demonstrated as situations have been reported where the skin bleeding time is prolonged despite normal plasma vWf activity (121,122,). The degree of aggregation of platelets needs to be considered together with other tests of haemostatic

function, bleeding time, vWf:Ag and vWf activity, to try to interpret the clinical bleeding problems of patients.

Modification of the ristocetin-induced platelet aggregation test (RIPA) for quantitation of vWf plasma activity was first described by Weiss in 1973, and termed ristocetin cofactor assay (VIIIR:RCo) (122). This assay is the single most discriminating test available for identifying affected individuals, although reduced or absent ristocetin-induced platelet aggregation in otherwise normal black Nigerians has been reported (123,124). Erroneous results may also be obtained due to conditions associated with raised plasma protein levels, for example in pregnancy, liver disease or paraproteinaemia, resulting in the inhibition of platelet aggregation. In addition false-negative results may arise in type IIB vWd, vWd in pregnancy and vWd patients transfused factor VIII concentrates rich in fibrinogen (125,126).

Quantitation of vWf activity (ristocetin cofactor) depends on the linear log-log relationship between the degree of ristocetin-induced aggregation of a standardised platelet suspension and the concentration of ristocetin cofactor (vWf) in the test system. In the assay procedure, fresh washed platelets may be used, but washing of platelets is time-consuming, and the need to perform assays regularly has prompted development of methods

using formalin- or paraformaldehyde-fixed platelets, which can be prepared in advance and stored until required. Thus, the use of a stable fixed platelet preparation reduces assay time, excludes variation in platelet response and improves reproducibility and quality control of the assay, without loss of sensitivity (127,128,129).

Platelet aggregometry is the method of choice for most laboratories and has been found to be a sensitive and reproducible method for the assay of ristocetin cofactor activity (VIIIR:RCo). Platelets, plasma and ristocetin are added to an aggregometer cuvette at 37°C and the change in turbidity of the platelet suspension due to platelet agglutination is traced on a chart recorder. The ristocetin-induced platelet aggregation response may be assessed by measuring the percentage aggregation in a fixed time interval, as indicated by the maximum percentage increase in light transmission (130). However, measurement of the maximum rate of aggregation, by measuring the slope of the steepest part of the aggregation trace, has been found to be more reliable in practice (131). The measurement of VIIIR:RCo is achieved by comparing the dose response curve obtained for patients' platelet poor plasma with a reference preparation.

4.6. Botrocetin-induced platelet aggregation.

Recently, the purified extract from the venom of the

snake Bothrops jaracaca, called botrocetin, has been shown to induce vWf-dependent platelet agglutination, and used in the measurement of vWf activity (botrocetin cofactor assay) without the disadvantages encountered with ristocetin of protein precipitation and species specificity (133). The venom contains both platelet aggregating material and a thrombin-like activity which are separated by ion-exchange chromatography. The discovery of botrocetin was the result of a search for new activators of plasma vWf when it was discovered that vWf in dogs was unreactive to ristocetin. Thus research using a canine model of vWd required a sensitive vWf activator which was found in several species of Bothrops snakes (133). Quantitation methods for the assay of vWf with botrocetin have been developed which are similar to the ristocetin method (VIIIR:RCo) (134). Although botrocetin and ristocetin are similar, several important differences exist which may help in our understanding of the structure-function relationship of the vWf protein. First, botrocetin reacts with low, intermediate and high molecular weight vWf multimers whereas ristocetin reacts with only the high molecular weight multimers (135). This is supported by the observation that the plasma of two patients with type IIA vWd, who lack the high molecular weight vWf multimers, support aggregation of platelets in the presence of botrocetin but not with ristocetin (136). Furthermore, factor VIII concentrates which also have relatively few

high molecular weight multimers show greater reactivity with botrocetin. Secondly, platelets from patients with Bernard-Soulier disease bind some vWf in the presence of botrocetin but not with ristocetin suggesting botrocetin may to some extent act differently in the binding of vWf to platelets lacking the glycoprotein Ib receptor (135). Finally, vancomycin inhibits ristocetin activity, but not botrocetin activity, the significance of which is not clear. Such differences between botrocetin and ristocetin may help in studying the pathophysiology of vWf-dependent platelet aggregation.

4.7. Measurement of factor VIII:C (Procoagulant activity).

Factor VIII:C (antihaemophilic factor) is a blood clotting protein defective or deficient in haemophilia A and is often decreased in vWd. Factor VIII:C is a cofactor in the middle phase of the intrinsic blood coagulation pathway and forms a complex with the activated clotting protein factor IX (IXa), calcium ions and phospholipid to promote the activation of factor X (Xa). Factor Xa, calcium ions, phospholipid and activated factor V (Va) form a complex called prothrombinase which converts prothrombin to thrombin. Thrombin interacts with fibrinogen to form a fibrin clot, which is the final coagulation reaction.

The most commonly used method for the measurement of factor VIII:C is the comparative bioassay. In this assay factor VIII:C is measured in terms of its biological activity which is related to the time taken for the formation of a fibrin clot. Two different bioassay methods are commonly used; the one-stage and two-stage assay. The one-stage assay is based on the ability of diluted samples containing factor VIII:C shortening the clotting times of a factor VIII deficient plasma. If all other clotting proteins are present in sufficient amounts in the assay system, the factor VIII:C added is rate-limiting and is a function of the clotting times if the correct dilutions are chosen. The correct dilutions will give clotting times, a measured by the Activated Partial Thromboplastin Time (APTT) method, which fall on the linear portion of the dose-response curve. The linear portion of the dose-response curve for the test preparation can be compared with that of a known standard and the concentration determined.

In the two-stage factor VIII:C assay, dilutions of factor VIII:C are incubated with excess concentrations of factors IXa, X, optimal concentrations of calcium ions and phospholipid. Over a given time period the amount of factor Xa generated will be proportional to the to the amount of factor VIII:C present. In the first phase of the incubation mixture, factor Xa phospholipid, calcium ions and factor V, which is also added, form a prothrombin

activating complex called prothrombinase. The amount of prothrombinase formed is measured by sub-sampling into a second tube containing calcium and a source of substrate fibrinogen. Alternatively, the substrate may be added to the initial incubation tubes and the clotting time recorded (138,139,140).

5. CLASSIFICATION OF VWD.

5. Classification of vWd.

Owing to the complexity of the vWf and the various abnormalities which may occur in vWd, the diagnosis and classification of the various subtypes reported is based upon a combination of clinical features, the mode of inheritance and analytical and functional tests. Frequently family studies are required to establish these features and may need repeating on several occasions due to a marked variability in some individuals and between different family members of the same kindred (141). The mode of inheritance of vWd is usually autosomal dominant but in some cases it appears to be autosomal recessive or due to the inheritance of two autosomal dominant genes. Thus both laboratory tests and the mode of inheritance are important considerations in the diagnosis and classification of vWd to enable appropriate genetic counselling. Finally, the group of laboratory tests usually employed in diagnosis may also be used to monitor both therapy and assess therapeutic materials (cryoprecipitate, factor VIII concentrate) used in the treatment of vWd.

5.1. Clincal features.

Von Willebrand's disease is characterized by mucous membrane bleeding (easy bruising, epistaxis, menorrhagia in women) and bleeding after operations or trauma. The

clinical severity can vary greatly from life threatening haemorrhage to mild symptoms that require no medical attention. The nature of the bleeding varies with different subtypes of the disease but unlike haemophilia, deep subcutaneous intramuscular haematomas are uncommon, while joint bleeding (haemarthroses) occurs only in the most severely affected patients without detectable vWf and very low levels of factor VIII:C.

The abnormal platelet function in vWd is caused by quantitative and/or qualitative abnormalities of the vWf protein. Variant forms of vWd have been described based on clinical, genetic and laboratory features.

5.2. Type I vWd.

Type I vWd is characterised by a quantitative reduction of vWf although all multimeric forms are present. This is the commonest type of vWd, approximately 70% of all cases, in which inheritance is autosomal dominant and often presents with large numbers of affected members in a family (142,143). Platelet vWf may be normal or decreased and endothelial vWf in the one case reported (moderately severe type I vWd) did have vWf in the endothelium, detected by immunofluorescence (144,145). The factor VIII, vWF:Ag and ristocetin cofactor are all reduced to the same degree and the vWf multimeric pattern appears to be normal (146). However, in 1983 the type I vWd group was

divided into two, on the basis of plasma multimeric patterns. In type IA the plasma vWf:Ag multimer pattern was the same as in normal plasma, whereas in type IB there was a relatively reduced amount of the larger multimers (147). Although platelet vWf multimer patterns in type I vWd are the same as normal platelets the vWf:Ag content can be normal or low (148). Von Willebrand antigen II is a protein normally present in plasma and platelets, which is synthesised and released by the endothelial cells in response to 1 deamino-[8-arginine]-vasopression (DDAVP). The function of this protein is unknown but is also reduced in content in the classical type I vWd (149,150,151).

5.3. Type III (Severe) vWd.

The vWf:Ag concentration is very low or even undetectable in some patients. There is some evidence for parental consanguinity and that severe vWd can occur in homozygotes for recessive or the inheritance of two dominant (compound heterozygotes) type I vWd genes. The genetic abnormalities have recently been investigated using cDNA probes. These studies demonstrated a complete or partial deletions of the translated regions of the vWf gene (185). Some patients are homozygous in that both chromosomes are affected, while asymptomatic parents are heterozygous for the deletion. Such homozygous individuals have very low or undetectable vWf:Ag levels in both

platelet and plasma, and no detectable endothelial vWf or vWf:AgII (149,145). Such patients can develope heteroantibodies after treatment with blood derivatives containing vWf. They also have a prolonged skin bleeding time, low or absent factor VIII:C and severe bleeding symptoms.

5.4. Type II vWd.

The variant types of vWd arise from a number of autosomal dominantly inherited separate qualitative abnormalities. Some of the more common vWf:Ag multimer characteristics are summarised in Table 3. and Fig 3. The type II vWd is characterised by a qualitative defect of the vWf protein which was first demonstrated in 1974 by 2DCIE (151). Subsequent subtypes have been described mainly based upon the multimeric pattern of vWf in plasma (see Fig 3 and Table 3) and platelets. Other features which have been reported are abnormal reactivity of platelets in the presence of ristocetin, a disproportionate low level of VIIIR:RCo relative to vWf protein level, and abnormal reactivity of the vWf:Ag in immunoradiometric assays. Data from a combination of analytical and functional tests has identified mainly four subtypes IIA,IIB,IIC and IID although rarer subtypes IIE,IIF,IIG and IIH have recently been described. Some of the more common plasma vWf:Ag multimer features are summarised in Table 3. and Fig 3.

5.4.1. Type IIA vWd.

Type IIA vWd is characterised by an autosomal mode of inheritance and an absence of large and intermediate plasma multimers. In addition the largest multimers are also absent in platelet and endothelial cells vWf (79,135). On SDS-agarose electrophoresis, only the five smallest oligomers are usually detected in plasma which appear as a triplet band. The triplet band is abnormal in type IIA vWd; the fastest migrating bands of each triplet is increased in quantity in comparison to the other bands (135).

Crossed immunoelectrophoresis shows an increased anodal mobility of the type IIA vWf which may partly explain the reduced vWf:Ag level when measured by IRMA, but not when measured by an electroimmunoassay technique. This is because the abnormal charge on the vWf protein may influence the electroimmunoassay but not the IRMA. The levels of VIIIR:RCo are reduced or absent while the factor VIII:C may be normal. The skin bleeding time is invariably prolonged but this disease has mild clinical bleeding unless the patients are traumatised or operated upon.

5.4.2.Type IIB vWd.

In type IIB vWd, there is absence of the large multimeric forms of vWf from plasma whereas a normal multimer pattern is found in platelet vWf in contrast to other type II vWd. There is an increased sensitivity of the

patient's platelet-rich plasma to low concentrations of ristocetin with agglutination of platelets induced by a lower concentrations of ristocetin than for normal platelet-rich plasma (115). Related to this is the observation that plasma vWf from type IIB vWd binds to normal platelets and washed autologous platelets at lower concentrations of ristocetin than normal. However, normal vWf binds to type IIB platelets at normal ristocetin concentrations; which is contrary to a possible platelet defect.

Infusion of the synthetic vasopressin analogue 1-deamino-8-arginine vasopressin (DDAVP) transiently corrects the abnormal multimeric structure of the plasma vWf, the larger multimers rapidly disappearing by comparison to normal or type I vWd patients. These endogenously released large multimers are removed from the circulation faster than normal vWf transfused into type IIB patients. This observation may indicate that type IIB vWf have an abnormally high affinity for tissue binding sites (153). Infusion of DDAVP into type IIB vWd patients has been reported to cause thrombocytopenia due to in vivo platelet aggregation, and is therefore considered contraindicated in treating these patients. Subsequent investigation found this aggregation related to adsorption of all multimers of vWf on to the platelets. The increased affinity of the type IIB vWf is thought to be associated with the platelet

membrane glycoprotein IIb-IIIa. In type IIB vWd the laboratory data shows a prolonged skin bleeding time, normal or slightly reduced factor VIII:C and normal or reduced VIIIR:RCo. The vWf:Ag as measured by EIA is either normal or slightly reduced but low by IRMA.

5.4.3. Type IIC vWd.

In type IIC vWd there is an absence of the largest multimers of vWf both in plasma and platelets, a disproportionate increase in the smallest multimers and a aberrant multimeric structure. The multimeric pattern shows a doublet band with a major and minor member (154). This characteristic multimeric pattern in type IIC vWd may result from the inheritance of two different genes for vWd. One of these genes affects the multimeric composition of vWf, heterozygotes having both aberrant fastest moving multimer and the normal high molecular weight multimers. This pattern is demonstrated as a double peak in crossed immunoelectrophoresis. The other gene may give rise to a quantitative reduction of vWf as seen in type I vWd. The affected individuals, type IIC vWd, are double heterozygotes giving rise to the characteristic multimeric pattern. Type IIC patients have prolonged skin bleeding times, low concentrations of vWf:Ag by IRMA (see Table 2a) and low VIIIR:RCo activity.

5.4.4. Type IID vWd.

Type IID vWd appears to have an autosomal dominant inheritance with patients possibly suffering with a severe bleeding disorder. Analysis on 3% SDS polyacrylamide gels demonstrates this variant vWd to be distinct from type IIA,IIB, and IIC, and shows absence of high molecular weight multimers with the smallest multimers lacking the normal triplet structure (155).

5.5. Pseudo- or Platelet-type vWd.

The basic defect in pseudo-vWd lies in the platelets and not in the plasma vWf. Normal vWf can bind to platelets in this disorder and cause platelet aggregation without ristocetin (116). Thus the plasma is depleted of the large multimers giving a multimeric pattern similar to type IIB vWd. The highest molecular weight multimers are present in the platelets and there is an increased sensitivity to ristocetin. Mild bleeding symptoms may occur associated with thrombocytopenia, and the disorder appears to be inherited as an autosomal dominant trait.

5.6. Acquired vWd.

Acquired vWd have been associated with immunological disorders such as systemic lupus erythematosus (SLE), autoimmune disease, multiple myeloma and malignancies such as Wilm's tumours (156,157). In some of these disorders antibodies bind to the factor VIII/vWf complex without inhibiting its biological activity in vitro, but may eliminate the vWf at an accelerated rate in vivo.

Designation	Features	Comments
IA	All vWf multimers are present in normal relative proportion.	No evidence of intrinsic functional abnormality of vWf.
IB	All vWf multimers are present in plasma but larger ones are relatively decreased.	vWF has less ristocetin cofactor activity than normal.
IC	All vWf multimers are present in plasma in normal proportion, but a structural abnormality of individual multimers is present.	vWF has less ristocetin cofactor activity than normal.
IIA	Large and intermediate vWf multimers are absent in plasma and platelets.	Increased proteolysis of vWF; some variability in the size of multimers present. Heterogeneous response to DDAVP; few cases show recessive inheritance.
IIB	Hyperresponsiveness to low doses of ristocetin; large vWF multimers are absent in plasma; all multimers present in platelets.	Increased proteolysis of vWf; thrombocytopenia following DDVAP; few cases show recessive inheritance.
IIC	Large vWf multimers absent; unique structural abnormality of individual multimers. Recessive inheritance (possible heterozygosity).	Decreased proteolysis of vWf; some heterogeneity in the multimeric pattern.
IID	Large vWf multimers are absent; unique structural abnormality of individual multimers.	Decreased proteolysis of vWf.

6. METHODS.

Methods.

6.1 Background to the study.

Von Willebrand's disease (vWd) is probably the commonest disorder of haemostasis. The observation that ristocetin agglutinates normal platelets but not those from patients with vWd provided the first useful *in vitro* diagnostic test for this disorder (63). Quantitative assays have been developed by using ristocetin-induced platelet aggregation in the presence of different plasma dilutions. Such assays reveal that this activity is absent or reduced in vWd but normal in haemophilic patients. The mechanism of platelet-vWf interaction has also been studied with ristocetin, but care has to be taken to use ristocetin concentrations that do not precipitate plasma proteins (eg fibrinogen) (126). Investigations of patients with different types of vWd have identified a subtype (IIB variant) in which the platelets are agglutinated at lower than normal ristocetin concentrations (115).

Recent studies have demonstrated that the purified component of snake venom from the species *Bothrops jararaca* will also aggregate platelets in the presence of vWf (4). As with ristocetin, this venom could be used to quantitate vWf levels. Plasma vWf levels determined by

ristocetin or botrocetin assays correlate well in normal subjects and a small number of patients with clinical vWd (type I) (5). Subsequently however an unusual variant of vWd was found to be devoid of vWf activity using the ristocetin assay whilst the botrocetin assay apparently showed normal levels (6). These observations suggest that ristocetin and botrocetin may be identifying two functional distinct biologically active sites on the vWf protein.

Because of these observations the investigations reported in this thesis have been undertaken to determine if there are two distinct biologically active sites (one detected by ristocetin, the other by botrocetin) on the von Willebrand protein. Furthermore, to determine if botrocetin-induced binding of vWf to platelets involves the whole spectrum of vWf molecular weight multimers and the glycoprotein Ib platelet receptor. A range of techniques have also been used to study vWf from normals and a variety of vWd patients, and compared with plasma botrocetin and ristocetin cofactor activities. Finally the importance of the carbohydrate side-chain of vWf and the effect of vWf proteolysis on the measurement of vWf activity and vWf:Ag by ELISA were investigated.

The following experiments have been conducted:

1. Evaluation of vWf activities as measured by botrocetin and ristocetin cofactor activities in patients with vWd to see if these activities are ever discrepant, and if this is associated with any particular structural abnormality of vWf.

2. Evaluation, in factor VIII concentrates, of the resistance or susceptibility to heating of the ristocetin-induced and botrocetin-associated vWf activities.

3. Evaluation of the venom-associated activities following endothelial cell release by 1-deamino-8-arginine vasopressin (DDAVP).

4. Evaluation of which vWf molecular forms interact with platelets in the presence of ristocetin and botrocetin, and to determine how botrocetin and ristocetin cofactor activities are related to vWf molecular forms separated by gel filtration.

5. Evaluation of botrocetin and ristocetin interaction with platelets from patients with vWd and inherited platelet defects.

6. Evaluation of monoclonal antibodies raised to vWf epitopes to determine if they selectively inhibit or enhance ristocetin and botrocetin mediated activities.

7. Evaluation of Con A binding of vWf:Ag in patients with vWd.

8. Evaluation of an ELISA for vWf and vWf:Ag of normal plasma collected in anticoagulants containing protease inhibitors.

In this section methods that are routinely used in this thesis are recorded, but additional methods used only in specific experiments are detailed in the relevant sections.

6.2. Blood collection.

Venous blood was taken into 0.11mol/L trisodium citrate (1 part to 9 parts blood). Platelet poor plasma (PPP) was prepared by centrifugation at 1500g for 15 minutes at 4°C. Separated plasma was used immediately for factor VIII:C assays, but stored at -20°C and thawed at 37°C prior to measurement of other factor VIII parameters.

6.3. Control plasmas.

The 10th, 11th and 12th British standard for blood coagulation, factor VIII human plasma, National Institute for Biological Standard and Control (NIBSC), was used as a standard for factor VIII:C, vWf activity and vWf:Ag measurement. A pool of 20 normal adult males was calibrated against a NIBSC standard and used as the standard for ristocetin and botrocetin cofactor assays.

6.4. Bleeding time (Ivy method).

The bleeding time is an indication of *in vivo* platelet and vascular involvement in haemostasis and identifies quantitative and qualitative disorders in primary haemostasis. The bleeding time is the simplest test in the

preliminary investigation of vWd, and in conjunction with other laboratory tests is used in the diagnosis and classification of vWd.

A sphygmomanometer cuff was placed round the upper arm just above the antecubital fossa and inflated to 40 mm of mercury. The outer aspect of the forearm was cleaned with an isopropanol swab and three pricks, 2mm deep, were made in rapid succession with a disposable lancet and a stopwatch started. Care was taken to avoid superficial veins and scar tissue. The bleeding points were gently blotted every 15 seconds with a filter paper disc until the bleeding stopped and the time noted. The average of the three individual times were reported to the nearest half minute.

6.5. Two-stage factor VIII assay (Diagnostic Reagents).

1. Preparation of absorbed plasma.

Factor VIII:C assays are employed for the diagnosis, classification and treatment of vWd and may in conjunction with other tests distinguish haemophilia A from vWd.

Labelled polypropylene torpedo tubes for standard and test samples were pre-warmed in a water bath at 37°C for 5 minutes. To each tube was added 200 ul of plasma and 20 ul of warmed aluminium hydroxide (1g of gel in 4 ml of distilled water). The tubes were capped, mixed gently and

incubated for 3 minutes at 37°C and then centrifuged for 2 minutes. The supernatant absorbed plasma was then diluted in citrate saline for assay.

2. Preparation of plasma dilutions.

Using plastic test tubes doubling dilutions of standard and test plasma were prepared in citrate saline (one part 3.8% tribasic sodium citrate is mixed with five parts of isotonic saline) ranging from 1 in 50 to 1 in 400.

3. Incubation phase.

To 400 ul of the factor VIII combined reagent (supplied with assay kit) was pipetted into each of 12 glass tubes. One glass tube was placed in a waterbath at 37°C, allowed to warm, then 100 ul of the first dilution of the factor VIII standard was added and the master clock started. At one minute intervals 100 ul of the remaining dilutions of standard and test were added to each of the prewarmed factor VIII reagent glass tubes.

4. Clotting phase.

At 12 minutes on the master clock 200 ul of substrate plasma (reconstituted with 5 ml of distilled water) was added to the first tube and the clotting time recorded. At one minute intervals, 200 ul of substrate plasma was added to the remaining tubes (including the blank) and the clotting times recorded.

5. Results.

The clotting times were plotted against plasma dilution on double log graph paper. The best straight line through the standard points and the best parallel straight line through the test points were drawn. The value for the test plasma was obtained by interpolation. A horizontal line was drawn from the point where the test sample intercepts the horizontal scale reading of 100 to the line of the standard. The intercept at this point on the horizontal scale gives the factor VIII value as a percentage of the standard.

6.6. Measurement of vWf:Ag by ELISA (167).

Immunological assays of vWf provide supplementary information to that gained from biological assay of functional activity. In order to fully evaluate the nature of any defect of vWf, it is necessary to quantify both biological and immunological activities.

One hundred microlitres of anti-human vWf:Ag diluted 1 in 500 in 0.05 mol/l carbonate buffer, pH 9.6 was added to each well of a M129B micro ELISA plate (Dynatech Laboratories), which was then incubated for 1 hour at room temperature in a moist chamber. The plate was washed three times with 0.5ml/l PBS-Tween for 2 minutes. Doubling dilutions of standard (NIBSC 10th British Standard for factor VIII) and test plasma from 1 in 10 to 1 in 80 were

made in 1ml/l PBS-Tween. One hundred microlitres of each dilution, and buffer blanks, were added to the wells and the plate incubated for 1 hour as above. One hundred microlitres of peroxidase conjugated anti-human vWf:Ag, diluted 1 in 500 in 1 ml/l PBS-Tween, was added to each well and the plate incubated for 1 hour as above. The plate was then washed twice in 0.5 ml/l PBS-Tween and once in 0.1 mol/l citrate phosphate buffer, pH 5.0. 100ul of substrate (80mg orthophenylalamine, 15mls citrate phosphate buffer, pH 5.0, 10ul hydrogen peroxide) was added to each well and the plate incubated at room temperature for approximately 15 minutes. The reaction was stopped by addition of 150ul of 1 mol/l sulphuric acid to each well. Optical density values were read at 492nm, using a plate reader, and the values obtained plotted against plasma dilutions on log-linear graph paper, vWf:Ag values being obtained by extrapolation from the standard curve (114).

6.7. Two-site monoclonal antibody based ELISA for vWf

An immuno-enzyme assay system (ELISA) has been developed using a monoclonal antibody which recognises the glycoprotein Ib binding site on the vWf molecule. The method closely reflects functional vWf activity measured by platelet agglutination methods (VIIIR:RCo) and is distinct from immunological measurement of vWf:Ag by polyclonal antisera. As results obtained reflect vWf activity, and not vWf:Ag, the assay provides a reliable,

specific and more reproducible alternative to platelet agglutination methods for functional vWf activity.

The monoclonal antibodies and method for this technique were kindly supplied by Dr A H Goodall, Academic Department of Immunology, Royal Free Hospital School of Medicine, London (165).

220ul of monoclonal antibody (RF-VIII:R/1 ascites) diluted 1 in 500 in carbonate buffer, pH9.6 (1.59g Na_2CO_3, 2.93g $NaHCO_3$, 0.2g NaN_3/ litre H_2O) was added to each well of a M129B micro ELISA plate and incubated at room temperature for one hour, followed by an overnight incubation at 4°C. The plate was washed three times with 0.05% Tween 20 phosphate buffered saline (PBS). Doubling dilutions of the standard and test plasmas from 1 in 10 to 1 in 640 were made in phosphate buffered saline containing 0.2% bovine serum albumin and 0.2% NaN_3, pH 7.2. 100ul of each dilution and buffer blanks were added to the wells and the plate incubated for 2 hours at room temperature. The plate was washed three times as before and 100ul of freshly diluted conjugate (RFF-VIII:R/2 monoclonal antibody conjugated to alkaline phosphatase) was added to each well and incubated for 2 hours at room temperature. The plate was washed three times as before and 100ul of substrate solution (1mg/ml p-nitrophenyl phosphate in 97ml of diethanolamine, 800 ml H_2O, 100mg $MgCl_3.6H2O$, 0.2g NaN_3 adjusted to pH 9.8 with 1M HCL and made up to 1 litre with distilled water)

was added to each well. After exactly 30 minutes 50ul of 3M NaOH was added to each row and the optical density values (OD) read at 405nm within 1 hour. The OD values were plotted against dilution on semi-log graph paper and the test results calculated by extrapolation from the standard curve.

6.8. Concanavalin A binding (Con A).

The measurement of binding of vWf:Ag to Con A may provide useful information about the carbohydrate side-chain of vWf:Ag. The method used is a modification of the method described by Peake and Bloom (171) in that an ELISA technique was used instead of an electroimmunoassay technique to measure the level of vWf:Ag.

Concanavalin A (acetate buffer, pH 6.3) was added to platelet poor plasma at a final concentration of 2mg/ml and incubated at 37°C for 2 hours. The plasma was centrifuged at 1500g for 10 minutes to remove the resulting precipitate and the vWf:Ag level determined on both the untreated and Con A treated test plasma by ELISA as previously described. The Con A treated sample vWf:Ag level was subtracted from the untreated plasma vWf:Ag level to give the amount of bound vWf:Ag. The details of how the optimal concentration of concanavalin A was determined are described in chapter 7.

6.9. Platelet based assays for ristocetin and botrocetin cofactor.

Although the measurement of the skin bleeding time and vWf:Ag measurement are key tests in the preliminary detection of vWd, the diversity of quantitative and qualitative defects in the syndrome makes the assay of plasma ristocetin and botrocetin activity essential. The property of ristocetin and botrocetin-induced platelet agglutination in the presence of vWf has been used to measure functional vWf activity by measuring the rate of platelet agglutination by optical detection in an aggregometer. Thus in conjunction with other tests used to evaluate the various properties of vWf, these assays improve the detection and classification of vWd.

In such assays either fresh washed platelets or a stable preparation of normal fixed platelets are used. The method used for preparation of washed and paraformaldehyde fixed platelets are described below.

6.9.1. Preparation of washed platelets (64).

Venous blood was collected by careful, clean venepuncture and dispensed into 4% w/v potassium EDTA (Ethylenediaminetetraacetic acid) (9:1 vols) in a tube. The blood was centrifuged for 15 minutes at 200g at room temperature and the platelet rich plasma (PRP) removed with a siliconised pasteur pipette. Approximately 0.7mls of

34% w/v bovine albumin was layered beneath the PRP and centrifuged at 650g for 15 minutes at room temperature. The platelets appeared as a layer at the interface of the bovine albumin and the plasma. The supernatant plasma was removed and discarded and the platelets harvested and resuspended in a volume of washing buffer, pH 7.4 (0.005M glucose, 0.006M potassium EDTA, 100mls 0.2M Tris-HCL, 1 litre 0.145M (0.89%) sodium chloride). A further 0.7mls of bovine albumin was layered beneath the resuspended platelets and centrifuged at 650g for 10 minutes at room temperature. The platelets are harvested and again resuspended in washing buffer and the washing process was repeated until the supernatant was crystal clear. After washing the platelets were again resuspended in a small volume of resuspending buffer (approximately 2mls), pH 7.4 (0.005M glucose, 50mls 0.2M Tris-HCL buffer, pH 7.4, 500mls 0.148M (0.89%) sodium chloride). A platelet count was performed and the concentration of platelets adjusted to 1200 x 10/9/l with Owren's buffer. pH 7.4.

6.9.2 Fixation of platelets with paraformaldehyde (65).

Venous blood was collected into 1/6th volume acid-citrate dextrose (ACD), pH 4.5 (0.084M trisodium citrate, 0.064M citric acid and 0.11M glucose). Whole blood was centrifuged for 8 minutes at 460g at room temperature and the resulting platelet rich plasma (PRP) removed. The PRP was then centrifuged at 1500g for 10 minutes at room

temperature to obtain a platelet pellet. The platelets were resuspended in approximately 20mls of citrate saline, pH 7.1 (0.15M NaCl, 0.006M trisodium citrate). This washing procedure was repeated twice more and the platelets resuspended in 40mls of 2% paraformaldehyde solution, pH 6.9 (9 parts 2% paraformaldehyde in 0.13M monosodium phosphate and 1 part ACD). The platelets were fixed for 48 hours at 4°C and then centrifuged at 1500g for 10 minutes at room temperature. The platelet pellet was resuspended in 0.15M phosphate buffer, pH 6.4 and stored at 4°C for a further 48 hours. This procedure was repeated and the platelets finally resuspended in 5mls of 0.15M phosphate buffer, pH 6.4 and stored at 4°C ready for use (65).

6.9.3. Plasma vWf activity using ristocetin (ristocetin cofactor assay).

Doubling dilutions of standard and test plasma from 1 in 2 to 1 in 16 were made in Owren's buffer, pH7.4. The platelet aggregometer, Biodata platelet profiler 4 (PAP-4), stir speed was set to 900 rpm and the zero position (blank) set with a clean siliconised glass tube containing 50ul of platelets, 150ul Tris buffered saline, pH 7.35 and a magnetic stir bar. One hundred microlitres of each dilution were added to 125ul of paraformaldehyde fixed or freshly washed normal platelets (400 x 10^9/l) and incubated for 2 minutes at 37°C in a platelet aggregometer. After warming, 25ul of ristocetin (10mg/ml)

was added and the rate of platelet agglutination and slope (automatically calculated by the aggregometer) of the resulting trace was plotted against dilution on log-log graph paper. Alternatively, the platelets and ristocetin were incubated together before the diluted plasma was added. This modification was adopted to reduced the problem of interference caused by increased turbidity which sometimes occurs with ristocetin. The vWf activity levels were calculated by comparing the test against a standard curve.

6.10. Preparation of botrocetin (venom coaglutinin) reagent (133).

Purification of commercially available snake venom was necessary to remove the fibrinogen clotting activity (FCA). This was done by ion-exchange chromatography using a DEAE-cellulose column; the FCA eluting off with the starting buffer and the platelet aggregating activity (PAA) eluting off with buffer containing 0.3M NaCl.

Preparation of DEAE column.

Approximately 30mls of degassed DEAE-cellulose gel (Pharmacia, Uppsala, Sweden) was equilibrated with the starting buffer (84mM imidazole/0.154M NaCl, pH 7.35) and a column of dimensions 1.6cm by 22cm prepared at room temperature. After packing, the column was extensively

washed with the same buffer before the snake venom powder was applied.

Preparation of sample to be chromatographed.

Approximately 60mg of snake venom powder (Sigma Chemical Ltd) was dissolved into 2ml of starting buffer and dialysed against 2 litres of starting buffer overnight at 4°C. Following dialysis, the particulate matter was removed by centrifugation at 100,000g for 10 minutes at room temperature. The supernatant was applied to the column and washed through with starting buffer until the absorbance reading at 280nm was zero. The bound PAA material was eluted off with 84mM imidazole/0.3M NaCl, pH 7.35 and collected in 3ml fractions using a fraction collector (LKB, Stockholm, Sweden), during a 5 hour period at room temperature. Each fraction was tested for FCA and PAA.

Test for FCA.

All collected fractions (18-111) were tested for fibrinogen clotting activity (FCA) by adding 100ul of the test fraction to 100ul of fibrinogen (3mg/ml) at 37°C and recording the clotting time. All fractions containing FCA were discarded.

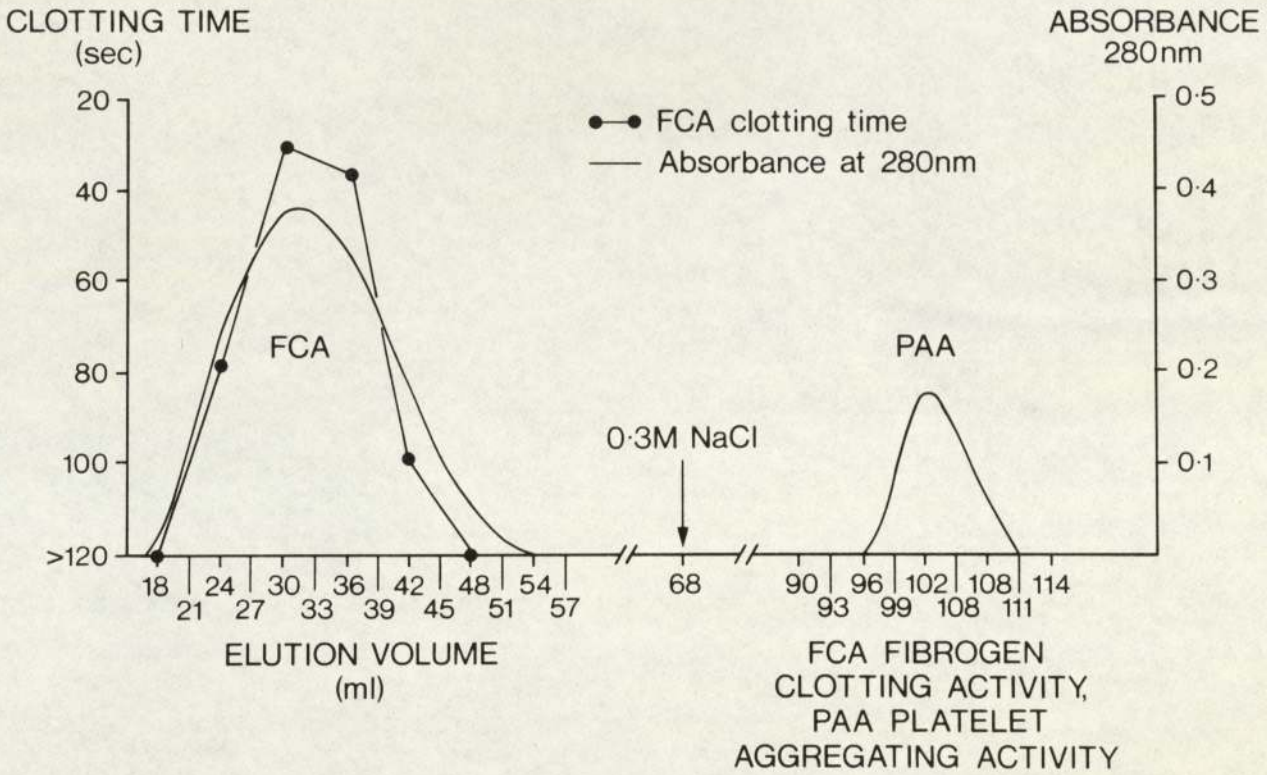
Test for PAA.

Only fractions with no FCA (96-111) were tested for

platelet aggregating activity (PAA). Into a clean siliconised glass tube was pipetted 300ul of 0.15M phosphate/saline buffer, pH 6.4 and 100ul of paraformaldehyde fixed platelets (800 x 10^9/l). To this mixture 50ul of normal pooled plasma and 50ul of test fraction was added. The platelet aggregating activity was monitored in an aggregometer at 37°C. No FCA was present in these fractions.

Pooled fractions containing only PAA were concentrated to 5ml final volume on an Amicon concentrator. Starting buffer was flushed through several times and the protein concentration determined by a Cobas Bio analyser. The purified botrocetin was then stored at 4°C ready for use.

PURIFICATION OF BOTROCETIN



6.11. Plasma vWf assay using botrocetin

(botrocetin cofactor assay).

In principle the botrocetin assay is the same as the ristocetin cofactor assay, having first found the standard botrocetin concentration, This assay has been suggested as an alternative the the ristocetin cofactor assay and has been evaluated in this study.

The details of how the optimal concentration of botrocetin to be used in this assay was determined are presented in chapter 7. The assay was performed in a platelet aggregometer at 37°C with a stirring speed of 900 rpm in the following manner. To 100ul of paraformaldehyde fixed platelets (800 x 10^9/l) was added 140ul of Owren's buffer, pH 7.4 followed by 25ul of test sample. The mixture was allowed to warm to 37°C before the addition of 20ul of botrocetin (107ug/ml). The degree of platelet agglutination was recorded graphically and the slope automatically calculated by the platelet aggregometer. The slope of the resulting trace was plotted against plasma dilution on log-log graph paper. Plasma botrocetin cofactor levels were determined by performing tests on doubling dilutions in Owren's buffer, ranging from neat to 1 in 8, of both test and pooled normal plasma (5).

6.11.1. Platelet aggregation studies.

Ristocetin-induced platelet aggregation (RIPA) is dependent on the presence of vWf in the plasma and

glycoprotein Ib on the platelet surface. Thus measurement of RIPA is relevant to the diagnosis of disorders due to abnormal platelets or vWf such as Bernard-Soulier syndrome and vWd. The use of a range of ristocetin concentrations allows the identification of vWd patients hypersensitive to low concentrations of ristocetin (type IIB), and may give an indication of the specific defect involved. Botrocetin-induced platelet aggregation (BIPA) is in principle the same once the optimum concentration of purified venom has been found.

Venous blood was collected into 0.11 mol/l trisodium citrate.$2H_2O$ (1 volume of citrate to 9 volumes of blood) and centrifuged for 8 minutes at 460g at room temperature. The resulting platelet rich plasma (PRP) was removed and the blood sample centrifuged again at 1500g for 10 minutes to obtain platelet poor plasma. The platelet rich plasma was then diluted with platelet poor plasma to achieve a platelet count of $250x10^9/l$. The platelet aggregometer was blanked on 200ul of platelet poor plasma with a stirring speed of 900 rpm. Into a clean siliconised glass tube, 200ul of test or control PRP was pipetted and allowed to warm to 37°C and then 20ul of ristocetin (final concentration 0.75, 1.00, 1.25 and 1.50mg/ml) was added and the resulting platelet aggregation trace recorded. Exactly the same procedure was followed for botrocetin-induced platelet aggregation studies apart from the final concentration of botrocetin

which was 16ug/ml. The details of how botrocetin-induced platelet aggregation were standardised are reported in chapter 7.

6.12. SDS Agarose-Acrylamide Gel Electrophoresis.

Multimeric analysis is a technique for the study of vWf involving electrophoresis and immuno-autoradiography. The development of multimeric analysis has yeilded the detailed substructure of vWf and shows the relative proportions of the different multimers. The intensity of the multimer bands may be assessed by computer-aided laser densitometry. Thus multimeric analysis is routinely used for the classification of the different subtypes of vWd (Fig 3).

The method used was that described by Enayat and Hill (172), which was a modification of the method of Zimmerman and Ruggeri (77). In these studies agarose (0.8%) and acrylamide (2.5% with 5% cross linking) gels were used.

6.12.1. Reagents.

a) Stock buffer.

1. 1.211g Tris
2. 0.372g disodium EDTA.
3. 100 mls distilled water.
4. Adjust pH with concentrated HCL to 8.0.

b) Sample buffer (10mM Tris-HCL, 1mM EDTA, pH8.0).

1. 10 mls of stock buffer (diluted 1 in 10).
2. 4.8g of Urea (= 8M Urea in final concentration).
3. 0.2g SDS.c)

c) Stacking gel buffer, pH 6.8 (0.125M Tris-HCL).

1. 1.54g Tris.
2. 100 mls distilled water.
3. Adjust pH to 6.8 with concentrated HCL.

d) Running gel buffer, pH 8.8 (0.5M Tris-HCL).

1. 12.11g Tris.
2. 200 mls distilled water.
3. Adjust pH to 8.8 with NaOH.

e) Electrophoresis buffer, pH 8.35 (0.0495M Tris, 0.384M glycine, 0.1% SDS).

1. 18g Tris.
2. 86.4g Glycine.
3. 3g SDS.
4. 3 litres of distilled water.
5. Adjust pH with NaOH.

f) Phosphate buffered saline (Veronal buffer, pH 7.2, 0.15M).

1. 8.0g NaCl.
2. 0.2g KCl.
3. 1.15g disodium hydrogen phosphate.
4. 0.2g potassium dihydrogen phosphate.
5. 1 litre of distilled water.

g) 20% Acrylamide (Stock solution).

1. 19g Acrylamide.

2. 1g bis-acrylamide

3. 100 mls distilled water.

4. Store at 4°C.h)

h) 2% Ammonium persulphate solution.

1. 2g Ammonium persulphate.

2. 100 mls distilled water.

3. Prepare fresh for use.

6.12.2. Running gel (0.8% agarose-2.5% acrylamide).

A solution of 1.07% agarose-0.135% SDS in 0.5M running gel buffer was prepared and transfered to a 56°C waterbath, and an acrylamide solution containing 3.31 mls of the stock acrylamide-bis-solution, 1.32 mls distilled water and 1.65 mls DMAPN solution was also simultaneously incubated at 56°C. After warming, 20 mls of the agarose-SDS solution was mixed with the acrylamide solution followed by 0.331 mls of 2% ammonium persulphate. The agarose-acrylamide mixture was then injected into a sandwich set (17x12x0.1cms). This was made up of a piece of gel bond film covering one glass plate and separated from a second glass plate by a 1mm thick U-shaped plastic spacer. The glass plates were held together with binder clips. After the running gel had set, the top glass was removed and a 3-5mm strip from the top of the gel is cut away and discarded. The top glass was reclamped into its

original position and 5mls of stacking gel (0.8% agarose-0.1% SDS) was poured above the running gel and left at 4°C to set. Thirteen rectangular sample wells (8 x 2mm) were cut in the stacking gel at 5mm intervals and 10mm from the interface with the running gel.

6.12.3. Sample preparation.

The supernatant plasma samples were diluted 1 in 20 in sample buffer containing 2% SDS and 8M urea. Approximately 1ul of a 1% solution of Bromophenol blue was added to 200ul of test sample, as a tracking dye, before incubating at 60°C for 30 minutes. For electrophoresis 20ul of each sample was applied to the wells.

6.12.4. Electrophoretic conditions.

Optimal electrophoresis was achieved with a constant current of 33mA per plate until the samples had migrated from the wells. The empty wells were filled with stacking gel and electrophoresis continued at 44mA until the tracking dye reached the end of the gel. After electrophoresis the gel was fixed in a solution of 25% isopropanol-10% acetic acid for one hour or overnight and then washed in several changes of distilled water for 2 hours before drying. In the thirteenth well a purified IgM was run as a molecular weight marker (900,000). This strip of dry gel was cut away and stained with Coomassie brilliant blue. The remainder of the gel was then

equilibrated in a 10mg/ml solution of bovine IgG in veronal buffer for 20 minutes and then in 1 in 10 diluted rabbit serum in the same buffer for a further 20 minutes. The gel was washed in distilled water and then autoradiographed.

6.12.5. Autoradiography

The dried gel was incubated overnight in a commercial rabbit anti-human vWf:Ag immunoglobulin (Dako), labelled with 125I by the chlorine gas method (173), at a specific activity of 10-20mCi/mg protein and diluted in 60 mls of veronal buffer to 1 x 10^6 cpm/ml. After incubation the gel was washed thoroughly in saline followed by distilled water and then dried. Autoradiography plates were produced using a Dupont Cronex film in a cassette fitted with Dupont Quanta 11 intensifying screen. The plates were kept at -70°C for 2-3 days to allow clear definition of the bands.

6.13. Two dimensional crossed immuno-electrophoresis (2DCIE).

2DCIE is a technique allowing qualitative evaluation of vWf. The main use of this technique is the identification of abnormalities in electrophoretic mobility and precipitin arc formation of vWf. Evidence from 2DCIE may also give some indication of presence/absence of the different molecular weight components of vWf which allows

classification of molecular variants of vWd.

Ten grams/litre agarose ME was dissolved in 0.05 mol/l barbitone buffer, pH 8.6 and kept at 100°C for 15 minutes. A 1mm thick gel was prepared on a glass plate (10 x 20 x 0.2cm) and cooled for 60 minutes in a humid chamber at 4°C. Twelve wells, 5mm in diameter, were punched out on the long side of the plate, the first six 1cm apart and the other six 2cm apart. To each well 10-20ul of test and control plasma were added, with crystals of bromophenol blue added as a marker. Electrophoresis, with cooling at a constant voltage of 20V/cm, was performed until the marker dye had migrated 3cm (usually 2 hours). Vertical strips of gel, six 1cm wide and six 2cm wide and all 3.3cm in length, containing the electrophoresed samples, were cut out and arranged with 2cm strips on the bottom of the glass plate and the 1cm strips above the mid point of the plate. The upper and lower troughs created were filled with 11.5 and 8.5 mls respectively of molten agarose in the same buffer containing 10mls/l rabbit anti-human vWf:Ag antiserum. The plates were electrophoresed for 16 hours with cooling at a constant voltage of 7V/cm. After electrophoresis, the plates were pressed, dried in hot air, washed in two changes of saline followed by two changes of distilled water, and then stained with 2g/l Coomassie Brilliant Blue R in methanol/acetic acid /distilled water (proportions 3.5:1:5.5), and then de-stained by two further changes. This

method described by Enayat and Hill (174),is a modification of other reported methods (55,151) which has the advantage of allowing the simultaneous evaluation of 12 samples including controls.

7. RESULTS.

RESULTS.

7.1. Introductory remarks.

In this chapter experiments have been done to compare the biological activities of ristocetin and botrocetin to determine if these two functional probes are identical, complementary or separate. The methods used in these experiments are presented in the appropriate sections of this chapter unless already described in Chapter 6.

Other workers have suggested ristocetin-induced platelet agglutination supposedly involves only the large molecular weight multimers of vWf (3). This finding has led to the concept that the platelet aggregating function resides in the high molecular weight multimers of vWf. However, botrocetin-induced platelet agglutination in the presence of vWf is said to involve the low and intermediate as well as the high molecular weight vWf multimers (136). If these findings are true they may form basis for selective testing of vWd patients in which different molecular weight fractions may be abnormal.

7.2. Gel filtration of normal plasma.

As gel filtration separates proteins on the basis of molecular size, normal plasma was eluted on sephacryl S400 columns to determine whether the different molecular vWf forms in sequential fractions showed differences in multimeric structure, vWf:Ag levels and vWf activity when assayed using ristocetin and botrocetin.

Plasma and plasma fractions.

To prepare plasma for subsequent gel filtration nine parts of blood were added to one part of anticoagulant 0.11 mol/l trisodium citrate. Platelet poor plasma (PPP) was then prepared by centrifuging the mixture at 1500g for 15 minutes at 4°C. The separated plasma was used immediately for factor VIII:C assays, but stored at -20°C and thawed immediately before assaying other factor VIII parameters.

Column chromatography.

A 27 x 1.5 cm column was packed with approximately 25 ml of degassed sephacryl S400 (Pharmacia, Uppsala, Sweden) using 0.15M NaCl/0.05M Imidazole buffer, pH 7.4. After packing, the column was extensively washed with the same buffer before applying the normal plasma. Then 2ml of pooled normal plasma (VIII:C = 1.30U/ml, vWf:Ag = 0.80U/ml, VIIIR:RCo = 1.00U/ml and VIIIR:BCo = 1.20U/ml) was applied to the top of a sephacryl S400 gel column and eluted with 0.15M NaCl/0.05M Imidazole buffer, pH 7.4 at room temperature with a flow rate of 28 mls/hour. Elution was monitored using UV absorbance at 280nm and fractions of 1ml were collected using a fraction collector (LKB, Stockholm, Sweden) and stored at -20°C. The void volume was determined using Dextran Blue 2000 (Pharmacia).

Fraction analysis.

Each of the 1ml fractions collected above were then assayed for botrocetin and ristocetin cofactor activity, vWf:Ag (ELISA) and the multimeric structure in each fraction determined using discontinuous SDS agarose-acrylamide gel electrophoresis. (See Chapter 6 for experimental details.)

7.2.1 Pattern of elution of vWf:Ag and botrocetin and ristocetin cofactor activities in gel filtered fractions of pooled normal plasma.

Immunoassay detectable vWf:Ag was found eluting from the column between 14-30 ml with a peak value at 17 ml. (Void volume Vo=14ml). However the ristocetin cofactor activity (VIIIR:RCo) was restricted to fractions 15 to 19 with a peak value in the 16 ml fraction. In contrast botrocetin activity (VIIIR:BCo) was found in all fractions from 15 to 24 ml with a peak value of 18 ml (Table 4 and Fig 5).

Thus fractions containing the higher molecular weight forms of vWf gave similar elution patterns for both VIIIR:RCo and VIIIR:BCo. A discrepancy appeared in fractions 17-24, containing the lower molecular weight forms of vWf, in that no platelet agglutination occured with low molecular weight vWf forms using ristocetin but highly significant agglutinating capacity still evident with botrocetin and these same low molecular weight vWf fractions (Table 4 and Fig 5). These elution patterns were constant in three separate experiments on three separate occasions. Similar elution patterns were also obtained on Sepharose 4B and 6B (single experiment), data not shown.

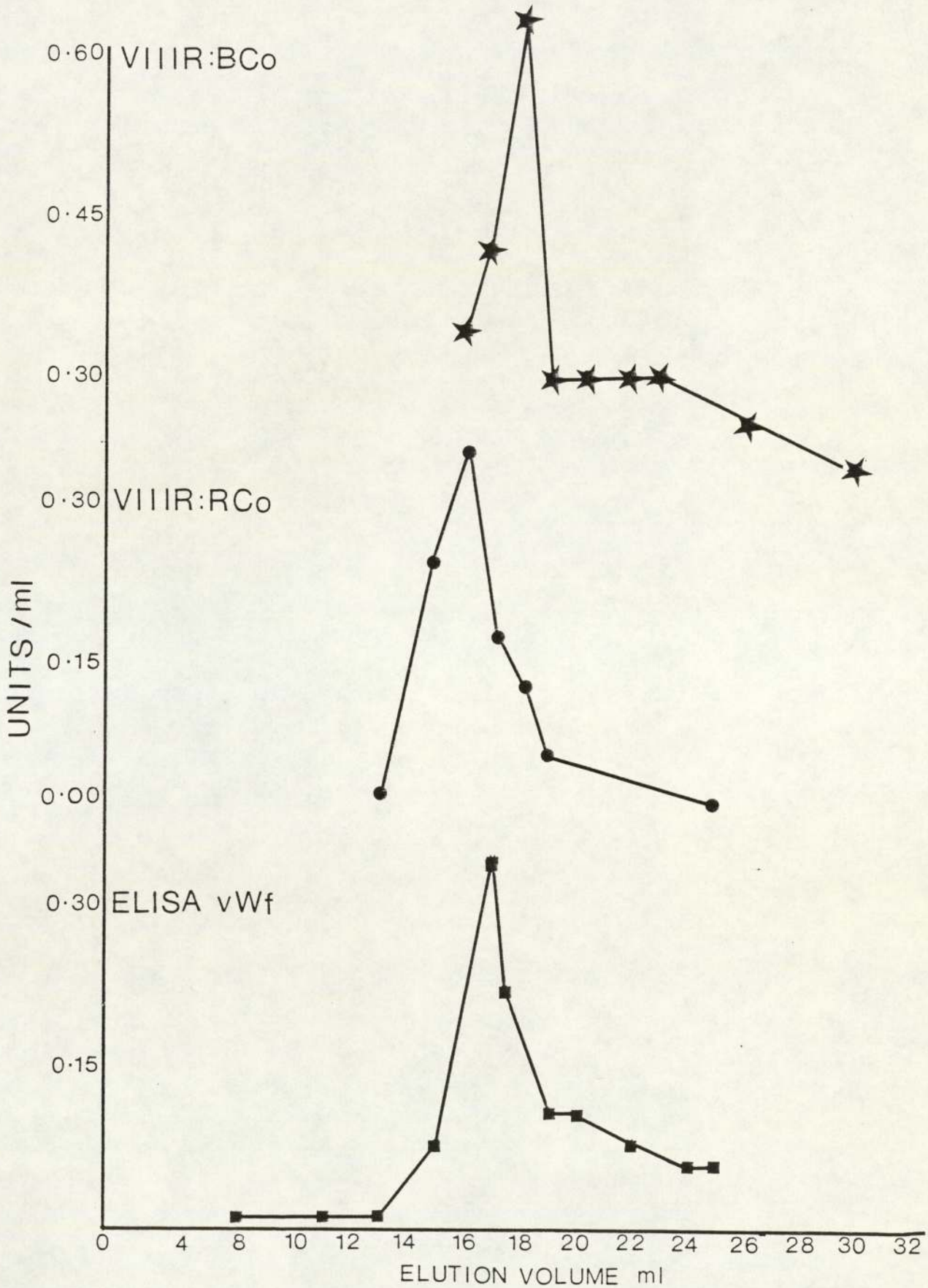
7.2.2. Evaluation of multimer structure of vWf:Ag in eluted fractions.

SDS agarose-acrylamide gel electrophoresis of the eluted fractions showed that fraction 14 contained only small amounts of high and intermediate multimers of vWf:Ag (Fig 6). The concentration of these multimers increased in the next two eluted fractions (15 and 16) and the low molecular weight multimers forms of vWf:Ag appeared in fraction 17. A full range of vWf:Ag multimers is then seen in fractions 17 to 21 although the concentrations of the high molecular weight multimers is decreasing in all subsequent fractions when the lower molecular weight multimers are increasing. By fraction 21 only a trace of the high molecular weight multimers is seen which disappeared by fraction 22. The appearance of high molecular weight forms of vWf:Ag corresponded with ristocetin activity (VIIIR:RCo). On the other hand, botrocetin activity (VIIIR:BCo) was detected in eluted fractions containing the whole spectrum of vWf forms (Fig 6) including those lacking the high molecular weight multimers.

In summary, botrocetin activity occurs in all fractions (15-24) and is associated with high, intermediate and low multimers, whereas ristocetin activity appears more restricted in its distribution, being detectable in fractions 15-19 which contain the highest concentration of high molecular weight multimers. Fig 5 shows the elution profile obtained for a typical fractionation of plasma vWf from a Sephacryl S-400 column (three similar experiments were performed). More botrocetin activity was present in both the earlier and later eluting fractions than ristocetin cofactor factor activity. This was a consistent finding with all gel filtration media used and is in agreement with the work of Howard et al who used semipurified vWf:Ag and Bio Gel A 15M (mesh 100-200) (195). A higher recovery from the column was obtained for botrocetin activity. This may be due to removal of a plasma blocker (inhibitor) to botrocetin activity on passage of the normal plasma through the column. Alternatively, separation of the different molecular forms of vWf may have exposed more vWf botrocetin binding site than are normally available in neat plasma.

Table 4. Comparison of vWf:Ag, ristocetin (VIIIR:RCo) and botrocetin (VIIIR:BCo) cofactor activities in fractions of normal plasma eluted from a Sephacryl S-400 column.

Elution Volume	Absorbance	VIIIR:RCo	VIIIR:BCo	vWf:Ag
ml	280 nm	U/ml	U/ml	U/ml
6	0.000	0.00	0.00	0.00
10	0.000	0.00	0.00	0.00
12	0.000	0.00	0.00	0.00
13	0.000	0.00	0.00	0.00
14	0.000	<0.05	<0.10	0.01
15	0.000	0.22	0.33	0.08
16	0.002	0.40	0.36	0.20
17	0.075	0.17	0.43	0.33
18	0.100	0.10	0.63	0.17
19	0.125	0.06	0.30	0.12
20	0.175	<0.05	0.30	0.12
21	0.200	<0.05	0.30	0.11
22	0.225	<0.05	0.30	0.10
24	0.295	<0.05	0.20	0.08
25	0.325	0.00	<0.10	0.08
26	0.375	0.00	<0.10	0.07
28	0.550	0.00	0.00	0.06
30	0.850	0.00	0.00	0.03
33	0.900	0.00	0.00	0.00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VIIIR:BCo
0·45
0·30
0·30
VIIIR:RCo
0·15
0·00
UNITS / ml
0·30
ELISA vWf
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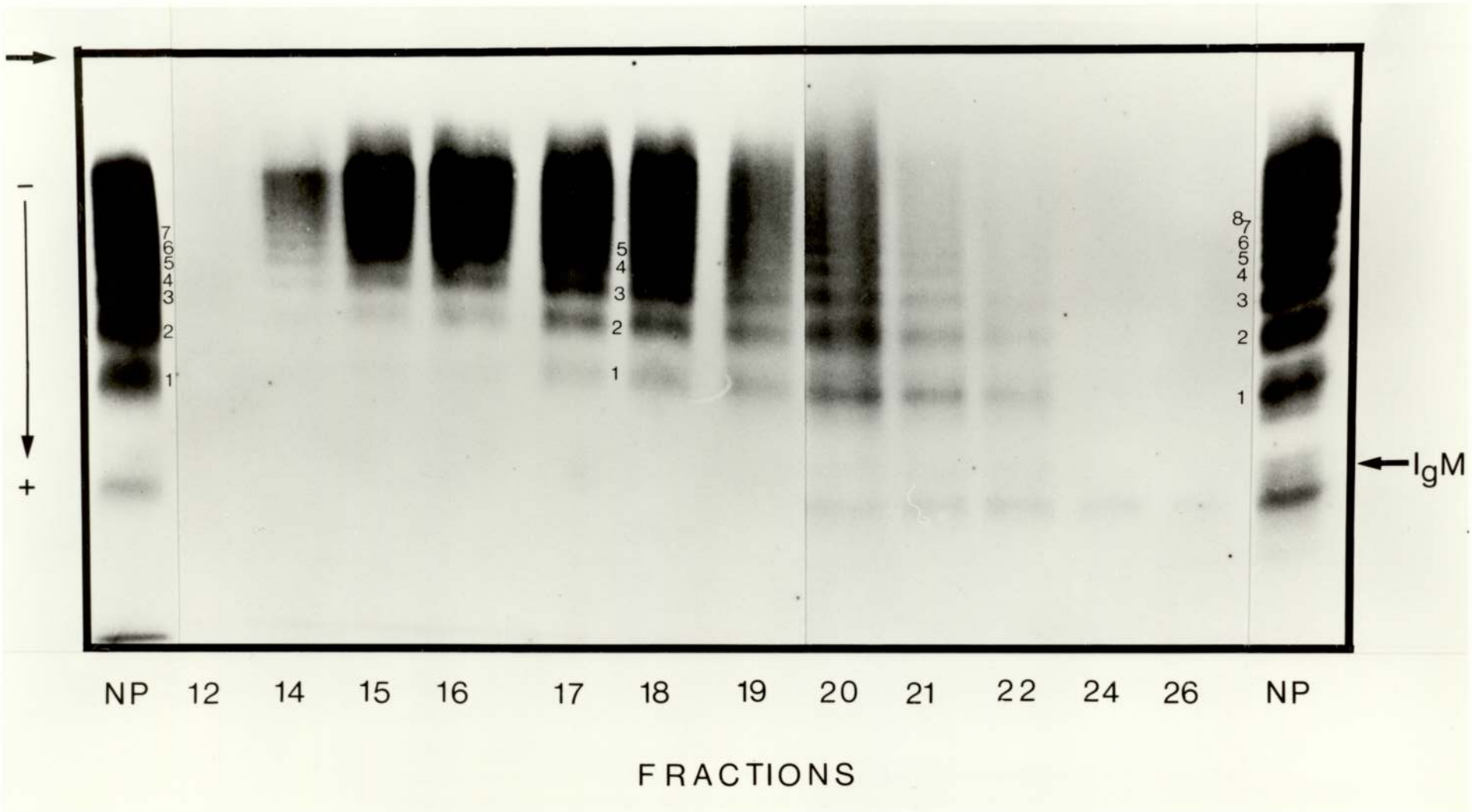
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FRACTIONS

7.3. Evaluation of platelet binding of vWf multimers in the presence of botrocetin and ristocetin.

Clearly vWf exists as protein multimers of varying size which exhibit some selectivity in binding with ristocetin and botrocetin. Since there may be selective deletions of vWf proteins of differing size in vWd it would be of considerable interest to establish which multimers are involved in platelet agglutination. Ristocetin and botrocetin therefore offer an opportunity to study such size-related platelet agglutination because of their selective binding.

Thus ristocetin or botrocetin at various concentrations with their associated vWf multimers of differing sizes were mixed with platelets inducing progressive ammounts of platelet agglutination. The latter therefore selectively precipitates and removes the platelet binding components and the remaining supernates can be examined to establish which components are involved.

7.3.1. Platelet binding experiment.

To 3 volumes of pooled normal plasma was added 1 volume of washed platelets (1200 x 10^9/l) and 0.1 volume of either ristocetin or botrocetin and incubated at 37°C for 30 minutes without stirring. The final concentration of ristocetin and botrocetin in the test system was 2mg/ml, 1.5mg/ml, 1.25mg/ml, 1.0mg/ml and 0.75mg/ml; and 10,8,6,4, and 2ug/ml respectively. The tubes were centrifuged at 1500g for 20 minutes at room temperature and the supernatant removed for analysis. A control of 3 volumes of pooled normal plasma and 1 volume of Owren's buffer was incorporated.

Analysis of supernatant.

The supernatant plasmas were assayed for botrocetin and ristocetin cofactor activity, vWf:Ag (ELISA) and the multimeric structure of vWf:Ag determined by SDS agarose-acrylamide gel electrophoresis followed by analysis using laser densitometric analysis. Residual ristocetin or botrocetin in the supernatants did not cause platelet agglutination when added to normal PRP. For methods see Chapter 6.

7.3.2. Results of platelet binding of vWf to platelets in the presence of botrocetin and ristocetin.

When all ability to bind to ristocetin, botrocetin or antibody against vWf:Ag has been removed via the platelet agglutination this is expressed as 100% binding. Thus in Table 3 and Fig 6 ristocetin at 1.50mg/ml shows 100% binding in the VIIIR:RCo assay, 76% in the VIIIR:BCo assay and 65% in the vWf:Ag assay.

If the multimeric studies of vWf:Ag are examined then it can be seen that the precipitin arc on crossed immunoelectrophoresis (Fig 8) changes with increasing loss of the most anodal component of the arc as the ristocetin concentration increases to 1.5 mg/ml final concentration. On SDS gel electrophoresis (Fig 9), it is apparent that in the presence of ristocetin concentrations of 1.0,1,25,1.5 and 2.0mg/ml there is increasing loss/removal of the highest molecular weight multimers of vWf:Ag. This is further exemplified in the densitometric scans of these gels (Fig 10) where with increasing ristocetin concentration there is progressive loss of the highest molecular weight multimers (ie large peaks on right hand side of trace). This data clearly confirms that the ristocetin cofactor activity is associated with highest molecular weight vWf:Ag multimers as anticipated. As these account for the largest proportion of vWf:Ag it follows that a significant fall in vWf:Ag levels will occur (as

shown in these experiments) when ristocetin induced vWf:Ag platelet binding occurs.

In contrast, in the experiments with botrocetin a steady state is observed at a concentration of 8ug/ml for the loss of botrocetin cofactor activity but at these concentrations a steady state has not been reached for either ristocetin cofactor activity or vWf:Ag (Fig 7 and Table 5). The fact that the residual vWf:Ag is lower with botrocetin than with ristocetin suggests that the botrocetin cofactor activity is associated with a smaller but different proportion of the vWf:Ag in the plasma.

Crossed immunoelectrophoresis shows that little or no change in the shape of the precipitin arc with increasing botrocetin concentration whereas SDS gel electrophoresis shows some loss of high, intermediate and lower multimers as evidence by the less intense bands on the autoradiographs after increasing botrocetin concentrations.

These experiments show that although botrocetin progressively removed material which can interact with ristocetin, botrocetin itself and vWf:Ag antibody the multimers which exhibit ristocetin binding are relatively unaffected. The multimeric SDS analysis and densitometric scans of these confirmed that different molecular species of vWf:Ag interact and bind with platelets in the presence of botrocetin. With a ristocetin concentration of 0.75mg/ml there is some loss of the highest molecular weight vWf multimers not detected by the ELISA vWf:Ag assay. This component of the vWf multimers has high ristocetin and botrocetin cofactor activity which binds to washed platelets but appears not to reduce the total vWf:Ag concentration to be detected by the ELISA vWf:Ag assay.

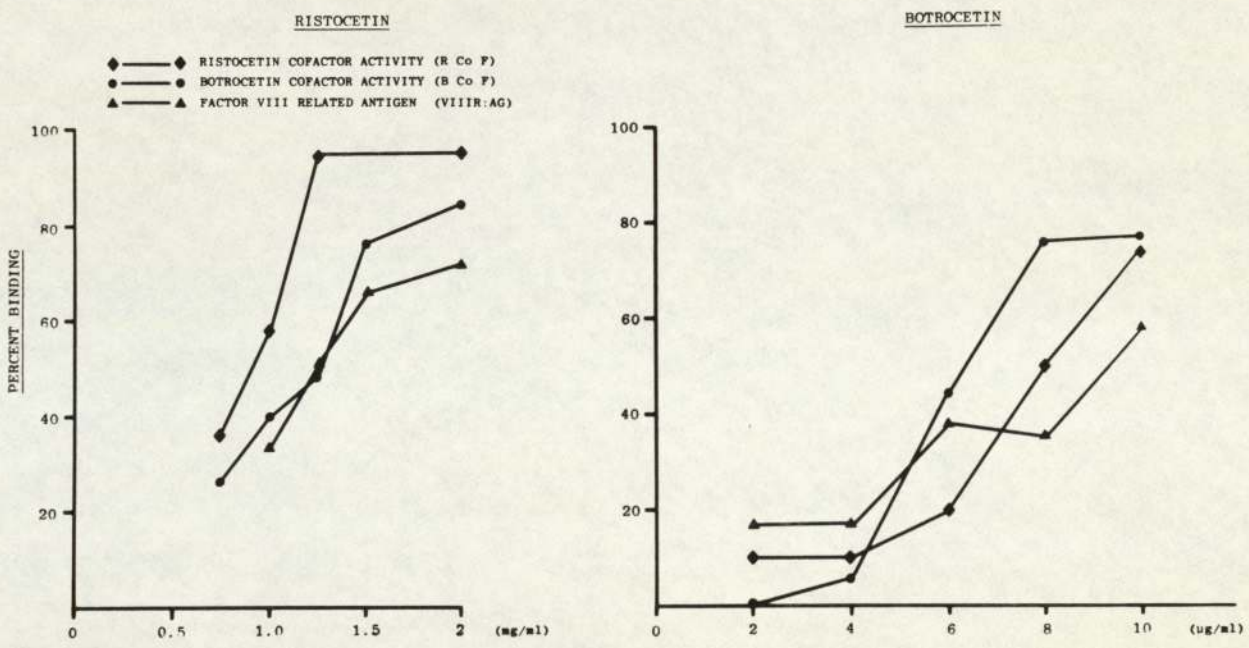
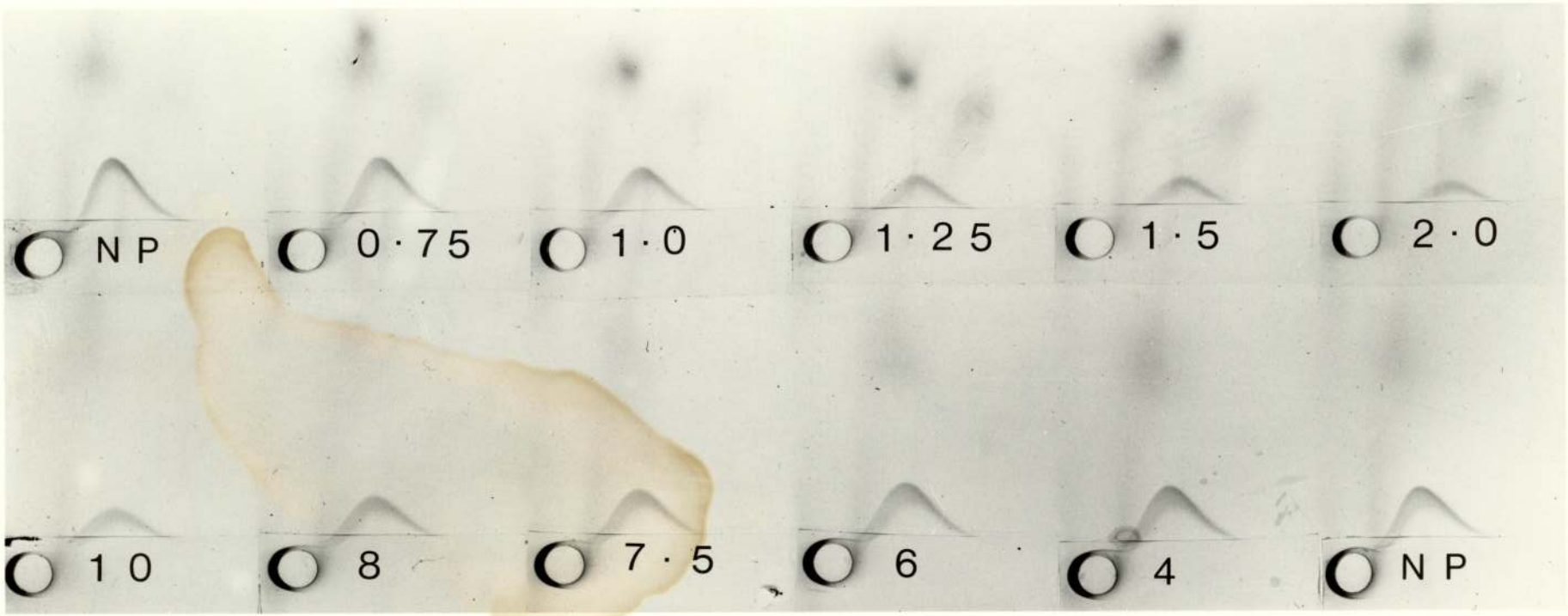
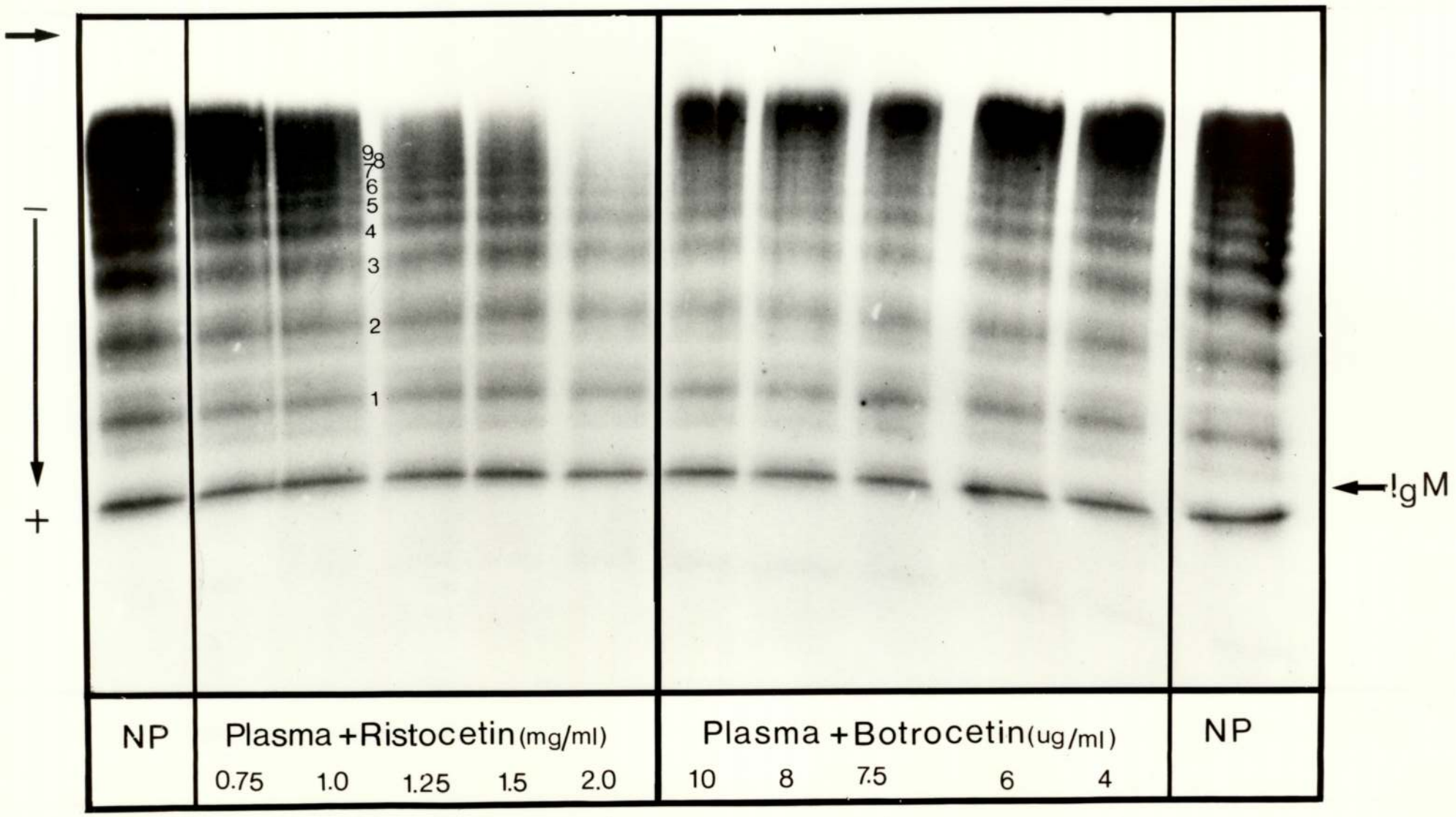

RISTOCETIN
RISTOCETIN COFACTOR ACTIVITY (R Co F)
BOTROCETIN COFACTOR ACTIVITY (B Co F)
FACTOR VIII RELATED ANTIGEN (VIIIR:AG)
PERCENT BINDING
100
80
60
40
20
0
0.5
1.0
1.5
2
(mg/ml)
BOTROCETIN
100
80
60
40
20
0
2
4
6
8
10
(ug/ml)

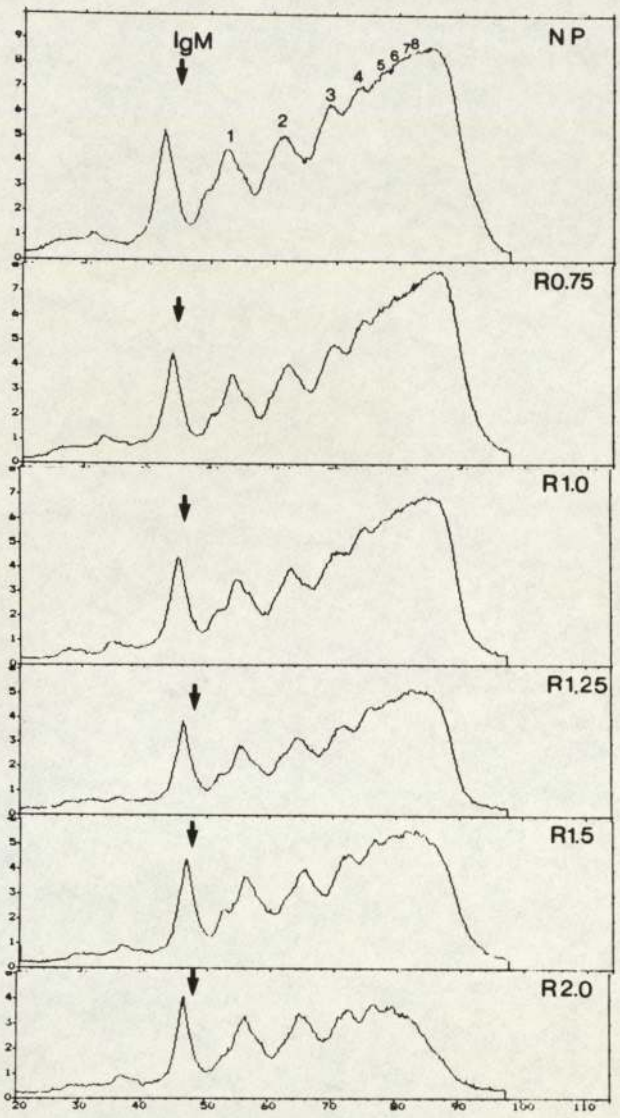
Table 5. Percentage binding of vWf:Ag, VIIIR:RCo and VIIIR:BCo after incubation of freshly washed platelets suspended in normal PPP in the presence of different ristocetin and botrocetin concentrations.

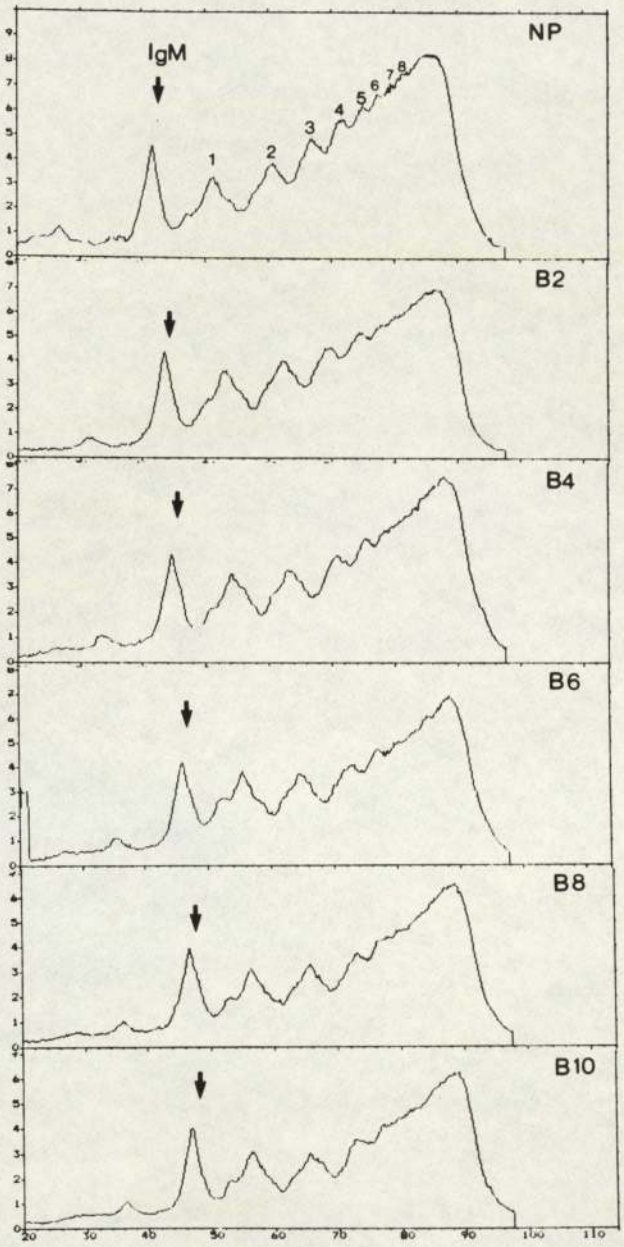
Agonist	Conc of agonist	vWf:Ag	VIIIR:RCo	VIIIR:BCo
		Percentage of starting activity.		
Ristocetin	2.00mg/ml	72 (12)	>90 (18)	84 (18)
	1.50mg/ml	65 (13)	>90 (18)	76 (18)
	1.25mg/ml	50 (18)	>90 (18)	49 (18)
	1.00mg/ml	33 (18)	58 (23)	40 (21)
	0.75mg/ml	0	36 (24)	27 (23)
Botrocetin	10ug/ml	58 (20)	75 (18)	76 (18)
	8ug/ml	35 (22)	50 (19)	76 (19)
	6ug/ml	38 (19)	20 (20)	44 (21)
	4ug/ml	17 (20)	10 (23)	5 (22)
	2ug/ml	17 (20)	10 (23)	0

Results are the means of five different experiments. The number in bracket ( ) = 2SD.


NP
0·75
1·0
1·25
1·5
2·0
10
8
7·5
6
4
NP


−
+
9
8
7
6
5
4
3
2
1
IgM
NP
Plasma + Ristocetin (mg/ml)
0.75
1.0
1.25
1.5
2.0
Plasma + Botrocetin (ug/ml)
10
8
7.5
6
4
NP


IgM
NP
1
2
3
4
5
6
7
8
R0.75
R1.0
R1.25
R1.5
R2.0


NP
IgM
1
2
3
4
5
6
7
8
B2
B4
B6
B8
B10

7.4. Comparison of botrocetin and ristocetin cofactor assays in normals and vWd patients.

In view of the plasma protein precipitation problems (fibrinogen precipitation at >2-3mg/ml ristocetin) encountered with ristocetin, botrocetin has been suggested as a possible replacement for the conventional ristocetin cofactor assay. To establish if this interchange is feasible, and our results suggest it may not be, the vWf activity was measured with botrocetin and ristocetin together with the bleeding time, factor VIII:C, vWf:Ag, and 2DCIE on 31 type I vWd, 26 type IIA vWd, 2 type IIC, 5 type IID vWd and 30 normal plasmas. The diagnosis of these patients has been established by Dr FGH Hill by considering their clinical history, family genetics, and haemostasis investigations (bleeding time, coagulation screening tests, factor VIII:C assays, vWf:Ag, vWf activity, RIPA, 2DCIE and multimeric analysis of vWf:Ag). See Appendix 2. Furthermore use of both ristocetin and botrocetin may well highlight whether in vWd subtypes there has been defective production of multimers of different sizes. Also ristocetin and botrocetin may be able to distinquish whether present and future therapeutic regimes such as DDAVP might restore certain multimers selctively.

Finally, botrocetin and ristocetin cofactor activity was compared with a monoclonal antibody-based (ELISA) for vWf in normal and vWd patients, using monoclonal antibodies

that inhibit the functional activity of vWf (165). Sample preparation and techniques were as previously described in Chapter 6.

7.4.1. Evaluation of botrocetin and ristocetin cofactor assays in normals and vWd patients.

Botrocetin, at a final concentration of 8-16ug/ml, caused platelet agglutination of fresh washed and paraformaldehyde fixed platelets in the presence of normal vWf, and PRP from normals but not from patients with severe vWd. The experimental details for determining the optimal concentration of botrocetin are described in section 7.5.

The measurement of vWf activity with botrocetin and fixed platelets was sensitive to 0.1U/ml of vWf activity. There was good correlation between botrocetin and ristocetin cofactor levels in both normals and vWd patients ($r=0.85$, $p<0.001$) (correlation coefficient, t-test) (Fig 11). In addition, the correlation between ELISA vWf assay and botrocetin activity was ($r=0.85$, $p=<0.001$), and between the ELISA vWf and ristocetin activity ($r=0.83$, $p=<0.001$). There was no direct correlation between the bleeding time and vWf:Ag, VIIIR:RCo and VIIIR:BCo. A comparison of ristocetin and botrocetin activities in vWd patients of various types,(for patient details see Appendix

2), is illustrated in Fig 12. In type I vWd the correlation appears better than in the other types of vWd (Tables 6,7 and 8). All the type IIA vWd patients had similar low VIIIR:RCo and VIIIR:BCo but vWf:Ag levels always significantly higher and often of normal value (Tables 7 and 8). In type IID vWd 2 of 5 had higher botrocetin cofactor activity than ELISA determined vWf activity and 1 of these 2 had normal botrocetin cofactor activity while the other had a very low level. Conformational changes in the vWf structure may have occurred on sample storage which may have altered the monoclonal antibody vWf epitope and consequently the ELISA vWf assay (204). The results for other vWd variants are shown in Table 8 but the numbers of each subtype are too small to comment on.

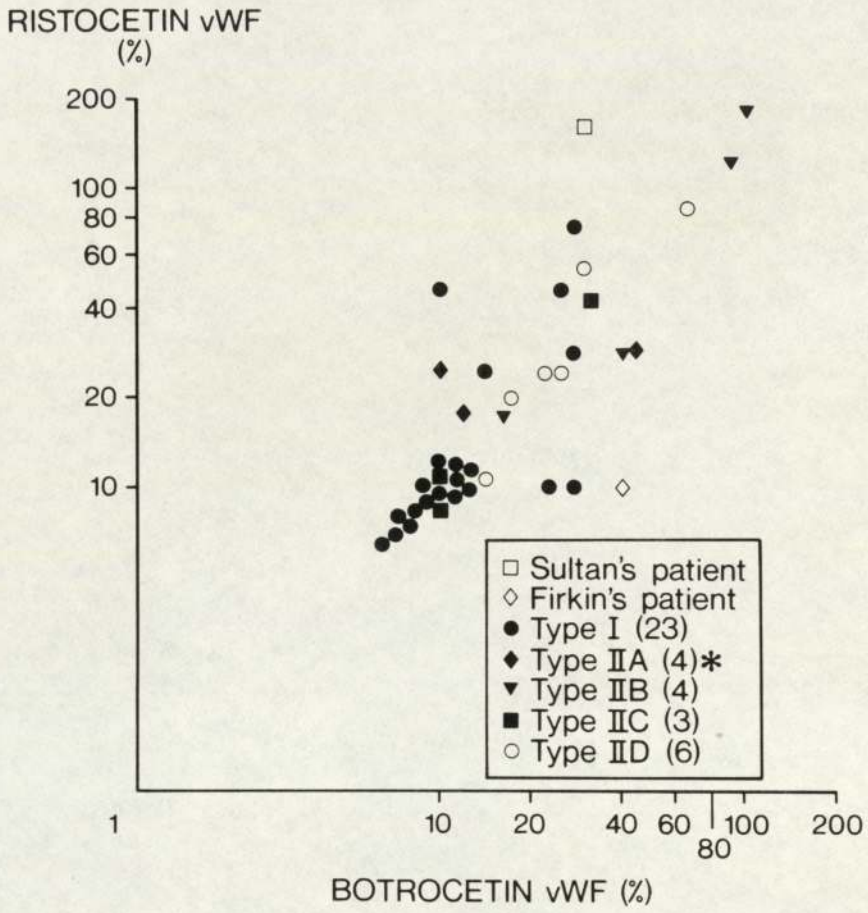
There is a good correlation between EIA and ELISA for vWf:Ag using the Royal Free monoclonal antibody in the type I vWd patients. However, in the type IIA vWd patients the vWf:Ag is higher by the EIA method (Table 7). This is probably due to the increased charge on the vWf molecule in type II vWd patients which is reflected by the high migration index (MI) values.

As a plasma sample was available from the unusual patient reported by Howard and Firkin (Table 9 and Fig 12) we were able to confirm the discrepancy between plasma VIIIR:RCo and VIIIR:BCo activity. Another vWd variant plasma

converse situation of a higher VIIIR:RCo than VIIIR:BCo. We also subsequently report a patient in this study (Section 7.11) who has some similarities but is not identical to the patient of Firkin and Howard.

Summary of results in vWd patients.

Botrocetin and ristocetin cofactor assays appear to correlate in patients with type I vWd and in the majority of type II patients. Furthermore, in these patients these assays correlate well with the ELISA assay for vwf using monoclonal antibodies that inhibit ristocetin activity. The finding of patients where the ristocetin and botrocetin cofactor activities are not in agreement suggest that the two activities can exist separately and therefore may be of different biological significance and that these two cofactor assays are not interchangeable.



* 12 Type IIA, vWF <10 by both assays (not shown)
Total vWD patients = 52

Table. 6. Factor VIII and vWf parameters and bleeding times in type I vWd.

No.	BT (min)	VIII:C U/ml	vWf:Ag(L) U/ml	vWf:Ag(E) U/ml	VIIIR:BCo U/ml	VIIIR:RCo U/ml
1	NT	0.14	<0.10	0.11	0.28	<0.10
2	>15	0.20	<0.10	0.16	<0.10	<0.10
3	10	0.25	<0.10	0.16	<0.10	<0.10
4	>15	0.23	<0.10	0.16	<0.10	<0.10
5	4	0.10	0.66	NT	<0.10	0.14
6	NT	0.18	<0.10	<0.10	0.11	0.11
7	7	0.48	0.30	0.44	0.11	<0.10
8	>12	0.12	<0.10	0.13	<0.10	<0.10
9	>12	0.26	0.22	0.27	<0.10	<0.10
10	>25	<0.10	<0.10	0.01	<0.10	<0.10
11	15	0.14	<0.10	0.21	0.27	0.28
12	>10	0.02	<0.10	0.02	0.12	<0.10
13	4	1.00	NT	0.40	0.26	0.47
14	5	1.00	NT	0.37	0.24	0.25
15	NT	0.04	NT	0.02	0.11	<0.10
16	NT	0.18	NT	0.05	0.11	0.11
17	NT	1.00	NT	0.50	0.27	0.46
18	NT	0.00	<0.10	0.02	<0.10	<0.10
19	NT	0.95	0.90	0.91	<0.10	0.48
20***				0.07	<0.10	<0.10
21***				0.28	<0.10	0.12
22***				0.09	0.11	<0.10
23***				0.09	0.23	<0.10

NT = Not tested. *** = Samples from ICTH multicentre trial.
E = ELISA. L = Laurell immunoelectrophoresis.

Table.7. Factor VIII andvWf parameters and bleeding times in patients with type IIA vWd.

Patients	BT (mins)	VIII:C U/ml	vWf:Ag U/ml EIA	vWf:Ag U/ml ELISA	2DCIE MI	VIIIR:BCo U/ml	VIIIR:RCo U/ml
1	>18	0.75	1.30	0.75	1.5	<0.10	<0.10
2	10	0.37	0.65	0.48	1.5	<0.10	<0.10
3	NT	0.61	0.59	0.75	1.7	<0.10	<0.10
4	NT	0.64	1.15	0.63	1.7	<0.10	0.26
5	NT	0.32	NT	0.28	NT	<0.10	<0.10
6	4	0.00	NT	0.78	1.5	<0.10	<0.10
7	3	0.72	NT	1.76	1.3	<0.10	<0.10
8	NT	0.48	1.55	0.80	1.6	0.12	0.18
9	NT	0.30	0.32	0.25	1.4	<0.10	<0.10
10	6	0.30	2.00	1.07	1.6	<0.10	<0.10
11	11	0.42	1.00	0.76	1.6	<0.10	<0.10
12	8	0.60	1.56	1.52	1.6	<0.10	<0.10
13	NT	NT	NT	**0.35	1.5	0.11	<0.10
14	NT	NT	NT	*0.09	NT	<0.10	0.12
15	NT	NT	NT	*0.22	NT	<0.10	<0.10
16	NT	NT	NT	*0.36	NT	0.40	0.29
17	NT	NT	NT	*0.12	NT	<0.10	0.10
Normal ranges	<7	0.5-1.5	0.5-1.5	0.5-1.5	0.88-1.1	0.5-1.5	0.5-1.5

** = Samples from ICTH Multicentre trial.
* = Sample kindly supplied by Dr Y Sultan

Table.8. Comparison of a monoclonal antibody-based immunoaasay (ELISA) for vWf and botrocetin and ristocetin cofactor assays in vWd patients.

VWD	ELISA vWf:Ag (U/ml)	RiCoF(U/ml)	ELISA vWf(U/ml)	BoCoF(U/ml)
I	0.16	<0.10	0.05	0.08
I	0.16	<0.10	0.04	<0.08
I	0.19	0.14	0.15	0.05
I	0.015	<0.10	0.01	0.11
Ia	0.07	<0.05	0.02	0.07
Ib	0.28	0.12	0.05	0.07
I	0.12	0.07	0.08	0.07
I	<0.05	<0.05	0.06	<0.05
IIA	0.35	<0.05	0.20	0.11
IIA	0.65	<0.10	0.08	<0.10
IIA	0.28	<0.05	0.06	<0.05
IIA	1.76	0.05	0.11	<0.05
IIA	0.80	0.16	0.16	0.12
IIA	0.75	0.05	0.07	<0.05
IIA	0.48	<0.05	0.10	<0.05
IIA	0.75	<0.05	0.08	<0.05
IIA	0.63	0.26	0.07	<0.05
IIC	0.80 (NP)	0.41	0.26	0.31
IIC	0.74	<0.05	0.04	0.07
IID	0.90 (NP)	0.10	0.21	0.31
IID	0.72	0.86	0.26	0.66
IID	0.90	0.16	0.09	0.50
IID	0.84	0.24	0.12	0.21
IID	0.84	0.24	0.21	0.21

NP = Non parallel lines.

Table. 9. vWf:Ag, VIIIR:BCo and VIIIR:RCo on plasma from normal subjects and two unusual patients with vWd.

	vWf:Ag (ELISA) (U/ml)	VIIIR:BCo (U/ml)	VIIIR:RCo (U/ml)
Normals (N=20)	0.64-1.30	0.50-1.50	0.50-1.50
vWd (type B)**	0.62	0.40	<0.10
vWd (type IIB)*	1.16	0.30	1.60

** Dr B Firkin's patient (6) * Dr Y Sultan's patient (see Appendix 2)

7.5. Comparison of platelet agglutination of platelet rich plasma induced by ristocetin (RIPA) and botrocetin (BIPA) in patients with von Willebrand's disease, Bernard-Soulier and Thrombasthenia.

Previous studies have shown that botrocetin, like ristocetin, induces vWf dependent platelet agglutination (133,135). Both agonists can utilize the high molecular weight multimers of vWf, although a discrepancy exists in some reports as to whether botrocetin can also utilize low and intermediate as well as the high molecular weight multimers of vWf (175).

When platelets bind to vWf in presence of ristocetin this involves glcoprotein Ib on platelet surface. This is supported by the inability of platelets from patients with Bernard-Soulier disease (platelets from these patients lack the glycoprotein Ib complex) to agglutinate in the presence of ristocetin. These platelets however seem to respond, albeit in a greatly reduced way, to a botrocetin vWf combination and thus would seem to imply that the site of binding of vWf induced by ristocetin and botrocetin are close but distinct (135).

Furthermore, differences in RIPA and BIPA have been reported in using plasma from some patients with type IIA vWd and one unusual case of vWd, called type B

vWd.(6,136). In type IIA large and intermediate multimers are absent and hence there can be no RIPA. In the very rare type B vWd an apparent normal multimer composition fails to give platelet aggregation in the presence of ristocetin but botrocetin-induced aggregation is seemingly absolutely normal (135). These reported differences suggest ristocetin and botrocetin not only can help to reveal differences in the multimeric pattern or composition of vWf but also may disclose more subtle differences in protein structure.

In order to investigate the reported difference between ristocetin and botrocetin induced platelet agglutination, platelet rich plasma (PRP) was prepared from:

1. Five patients with type IID vWd (large molecular weight vWf multimers absent). These patients were studied because of the observation that RIPA was normal although they had reduced VIIIR:RCo activity (<0.30U/ml). (See details of multimers in Appendix 2).
2. Two patients with Bernard-Soulier syndrome (platelets lacking membrane glycoprotein Ib).
3. Two patients with thrombasthenia (platelets lacking membrane glycoproteins IIb /IIIa).
4. Five normal controls with normal vWf multimers and platelet receptors.

Platelet-rich plasma (PRP) was prepared as previously described and 200ul pipetted into a clean siliconised glass tube at 37°C. To the PRP obtained from the type IID vWd patients, 20ul of either ristocetin or botrocetin were added to give a final concentrations for ristocetin of 0.75, 1.00, 1.25 and 1.50mg/ml and for botrocetin 16, 32, 65, and 130ug/ml. The degree of platelet aggregation was monitored on a platelet aggregometer, (Bio-data Platelet Aggregometer Profiler),for 3 minutes at 37 °C. The optimal concentration for botrocetin was determined by performing platelet agglutination studies on ten normals and selecting the lowest concentration which consistently gave maximum platelet agglutination (Fig 13). The PRP from the Bernard-Soulier and Thrombasthenia patients were only tested at the optimal concentration for botrocetin (8-16ug/ml), because of the limited supply of platelet-rich plasma.

7.5.1 Results of RIPA and BIPA.

For the normal controls the degree of RIPA and BIPA was expressed as percentage change in optical density and ranged from 65-90%. Table 10 shows the results of BIPA and RIPA on PRP from 5 type IID vWd. For RIPA, 3 out 5 type IID vWd patients showed a reduced percentage of platelet agglutination with ristocetin (4%, 9% and 7%) at the critical concentration of 1.25 mg/ml, (ie the concentration of ristocetin which in a normal patient would give maximum platelet aggregation). Of these three,

two patients showed normal RIPA (75% and 78%) at a higher ristocetin concentration of 1.5mg/ml. In the other two patients type IID vWd patients, RIPA at 1.25 mg/ml ristocetin was normal at 81 and 85% and a normal aggregation profile was seen for the complete ristocetin concentration range.

At 16ug/ml, BIPA for two of the five patients with type IID vWd was reduced, (28 and 40%) and both of these had reduced RIPA (4% and 9%) with ristocetin at a final concentration of 1.25 mg/ml. The PRP from the other three type IID vWd patients showed normal BIPA of 63%, 70% and 76%. The fact that these patients, who all have bleeding times in excess of 20 minutes and reduced cofactor assays on stored plasma, have RIPA and BIPA intact emphasises that other factors about their vWf protein are important in determining their abnormal haemostasis.

The PRP from two patients with Bernard-Soulier syndrome showed reduced RIPA (10% and 10%) and BIPA (13% and 20%). Also the PRP from two patients with Glanzmann's thrombasthenia agglutinated normally with ristocetin (60 and 65%) and borderline normal with botrocetin (50% and 50%) Tables 11 and 12.

These findings suggest that an intact glycoprotein Ib and normal vWf multimers are important for platelet-vWf

interaction in the presence of ristocetin and botrocetin. However, the aggregation results from two patients with Glanzmann's thrombasthenia would seem to exclude the platelet membrane glycoproteins IIb/IIIa.

BOTROCETIN - INDUCED PLATELET AGGREGATION

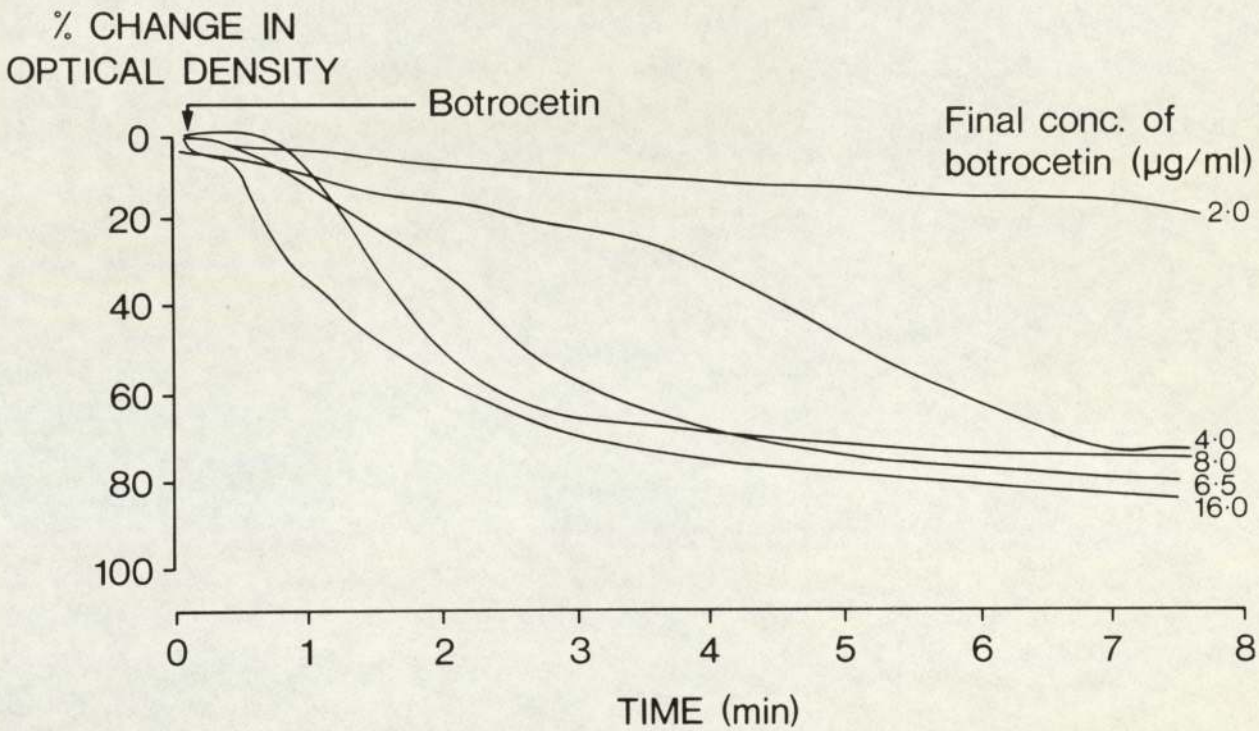


Table. 10. Comparison of percentage change in optical density for botrocetin (BIPA) and ristocetin-induced (RIPA) platelet aggregation in type IID vWd.

Sample	Concentration (ug/ml)	BIPA	Concentration (mg/ml)	RIPA
1	130	76%	1.50	69%
	65	70%	1.25	81%
	32	95%	1.00	36%
	16	76%	0.75	10%
2	130	81%	1.50	81%
	65	67%	1.25	85%
	32	74%	1.00	71%
	16	70%	0.75	11%
3	130	69%	1.50	75%
	65	59%	1.25	7%
	32	56%	1.00	3%
	16	63%	0.75	2%
4	130	67%	1.50	78%
	65	42%	1.25	9%
	32	47%	1.00	3%
	16	40%	0.75	4%
5	130	82%	1.50	10%
	65	67%	1.25	4%
	32	29%	1.00	7%
	16	28%	0.75	4%

The above results are the means of three determinations. Patient No1 is the uncle of No's 2,3 & 4. Patient No 5 is the daughter of patient No 4. Patients 3 & 4 are sisters.

Table. 11. Comparison of botrocetin and ristocetin-induced platelet aggregation in Bernard Soulier Syndrome.

Sample	Botrocetin	BIPA (%)	Ristocetin	RIPA (%)
1	10ug/ml	13	1.25mg/ml	10
2	10ug/ml	20	1.25mg/ml	10
Control		83		93

Table. 12. Comparison of botrocetin and ristocetin-induced platelet aggregation in PRP from two patients with Glanzmanns Thrombasthenia.

Sample	Concentration	BIPA	Concentration	RIPA
1	10ug/ml	50%	1.25mg/ml	60%
2	10ug/ml	50%	1.25mg/ml	65%
Control	10ug/ml	78%	1.25mg/ml	89%

7.6 The effect of DDAVP therapy on plasma VIIIR:RCo, VIIIR:BCo and vWf:Ag levels in normal individuals and patients with uraemia and vWd.

DDAVP (1-D-amino-8-D-arginine)-vasopressin can cause an increase in plasma levels of VIII:C, vWf:Ag and VIIIR:RCo in normals, milder forms of haemophilia A, patients with uraemia and some types of vWd (84). Although DDAVP has numerous advantages over plasma products including low cost, availability and freedom from viral contamination, it has been reported that DDAVP does not always correct haemostasis in uraemic patients and in some type IIA vWd, and is contraindicated in IIB vWd patients (84).

Previous studies have not examined the effect of DDAVP therapy on vWf activity using botrocetin nor have they compared this with the comparative ristocetin test. Therefore this study evaluated the response of plasma VIIIR:RCo, VIIIR:BCo and vWf:Ag levels following DDAVP therapy in 1 type I vWd, 2 type IIA vWd, 1 type IIB vWd and 2 patients with uraemia before and after two weeks of renal dialysis as samples were available from these patients to study.

DDAVP was administered in normal saline at a dose of 0.4 ug/Kg body weight over a 30 minute period. In the two patients with uraemia, DDAVP was administered and samples

taken 1 hour later. A further infusion of DDAVP was given after two weeks of renal dialysis. Blood samples were collected by clean venepuncture into 3.8% trisodium citrate (1 part citrate to 9 parts blood) immediately before infusion and at different times after the infusion. Blood samples were centrifuged at 3000g at 4°C and the platelet poor plasma stored at -50°C until tested. Plasma VIIIR:RCo, VIIIR:BCo and vWf:Ag levels were measured as previously described.

7.6.1. Plasma VIIIR:RCo, VIIIR:BCo and vWf:Ag levels following infusion of DDAVP.

In the normal patient, DDAVP produced a twofold increase in total vWf:Ag, VIIIR:RCo and VIIIR:BCo with the increase maximal at 1 hour (Table 11). At 3 hours the three parameters had returned to pre-DDAVP levels.

In the type I vWd patient DDAVP raised the vWF:Ag, VIIIR:RCo and VIIIR:BCo to normal plasma concentrations, reaching a maximum of 1.35, 0.75 and 0.65U/ml respectively. Once again values had returned to normal by 3 hours.

Infusion of DDAVP into two patients with type IIA vWd resulted in a threefold increase in vWf:Ag, VIIIR:RCo and VIIIR:BCo (Table 13). In both patients the vWf:Ag remained higher than the baseline value for at least 3 hours,

whereas the VIIIR:RCo and VIIIR:BCo levels declined faster returning close to pre-DDAVP levels by three hours.

In the type IIB vWd patient DDAVP infusion produced a similar rise in vWf:Ag, VIIIR:RCo and VIIIR:BCo to that seen in the type IIA vWd patients. However, the vWf:Ag and VIIIR:BCo reached a maximum at 3 hours whereas maximum VIIIR:RCo was at 2 hours.

In the two uraemic patients studied, the botrocetin and ristocetin cofactor levels were higher both before and after DDAVP. Only in one of the two uraemic patients (No 1.), did DDAVP produce a significant rise in VIIIR:RCo (3.80 U/ml) and VIIIR:BCo (>4.00 U/ml) at 1 hour; a similar response was seen when DDAVP was given after two weeks dialysis. By contrast in the other uraemic patient vWf:Ag rose after DDAVP but ristocetin and botrocetin cofactor activity were unchanged. Dialysis did not change this pattern of response.

Table. 13. Comparison of botrocetin and ristocetin cofactor activities in normal, uraemic and vWd patients after DDAVP therapy.

vWd	time(hrs)	vWf:Ag(U/ml)	VIIIR:BCo(U/ml)	VIIIR:RCo(U/ml)
I	0	0.35	0.40	0.20
	1	1.35	0.75	0.65
	3	0.95	0.50	0.39
IIA	0	0.15	0.10	0.17
	1	0.50	0.30	0.30
	3	0.34	0.20	0.22
IIA	0	0.16	<0.05	0.05
	1	0.44	0.14	0.14
	3	0.22	0.08	0.08
IIB	0	0.37	0.40	0.25
	1	0.51	0.60	0.32
	3	0.82	0.75	0.19
Normal	0	1.30	1.50	0.98
	1	2.88	2.60	2.20
	3	1.00	0.80	1.00
Uraemic (1)	0	0.98	2.25	2.20
	1	1.14	>4.00	3.80
Pre DDAVP	0*	1.09	>4.00	1.70
Post DDAVP	1	1.30	5.20	2.20
Uraemic (2)	0	1.10	1.80	1.00
	1	1.70	2.10	1.00
Pre DDAVP	0*	1.14	1.30	1.40
Post DDAVP	1	1.30	1.30	3.00

* = Second infusion of DDAVP after two weeks renal dialysis.

7.7. Botrocetin and ristocetin activity and vWf:Ag levels in factor VIII concentrates.

Since factor VIII concentrates are commonly used for the treatment of patients with haemophilia A and increasingly recommended if heat treated for patients with vWd, it is important to know the factor VIII related activities contained in the various concentrates. The potency and properties of factor VIII concentrates vary, with some preparations correcting the bleeding defect in vWd while others do not.

In order to study the effect of heat treatment on the vWf protein, five heated and unheated pairs of National Health Service factor VIII concentrate (8Y) from Elstree and five heated and unheated pairs of 8Y from the Plasma Fraction Laboratory (PFL), Oxford were examined. The products were supplied for study by Dr J Smith, PFL, Oxford. Also included were 2 heated and unheated pairs of 8 CRV, the old intermediate purity product. The 8Y samples were redissolved in 10 ml, and the 8CRV samples in 15 ml distilled water and assayed for vWf:Ag, VIIIR:RCo, VIIIR:BCo and subjected to multimeric analysis.

7.7.1. Experiments to see if botrocetin and ristocetin activities are heat sensitive or resistant in factor VIII concentrates before and after heating.

The values for vWf:Ag, VIIIR:RCo and VIIIR:BCo are given in Table 14. The concentrates CRV 8 (intermediate purity) when made up to recommended therapeutic concentrations generally had lower concentrations of vWf:Ag (37-40U/ml), VIIIR:BCo (7-9U/ml) and VIIIR:RCo (18-47U/ml) than the other samples. In all these latter concentrates vWf:Ag levels were generally higher than VIIIR:RCo activity but VIIIR:BCo activity was higher than both the vWF:Ag and VIIIR:RCo.

In only two of the concentrates was the vWf:Ag level lower after heat treatment in nearly all the batches of factor VIII concentrates and VIIIR:RCo activity was lower after heating in two batches of concentrates (Elstree 1 and 2). The Elstree 8Y concentrates had higher VIIIR:BCo than the others and this fell significantly with heating. Three of the PFL concentrates and all of the CRV8 concentrates had low VIIIR:BCo levels before and after heating. Thus heat treatment rarely affected vWf:Ag but the functional assay using botrocetin was severely impaired in almost all cases. In contrast heating did not affect the ristocetin-induced platelet agglutination.

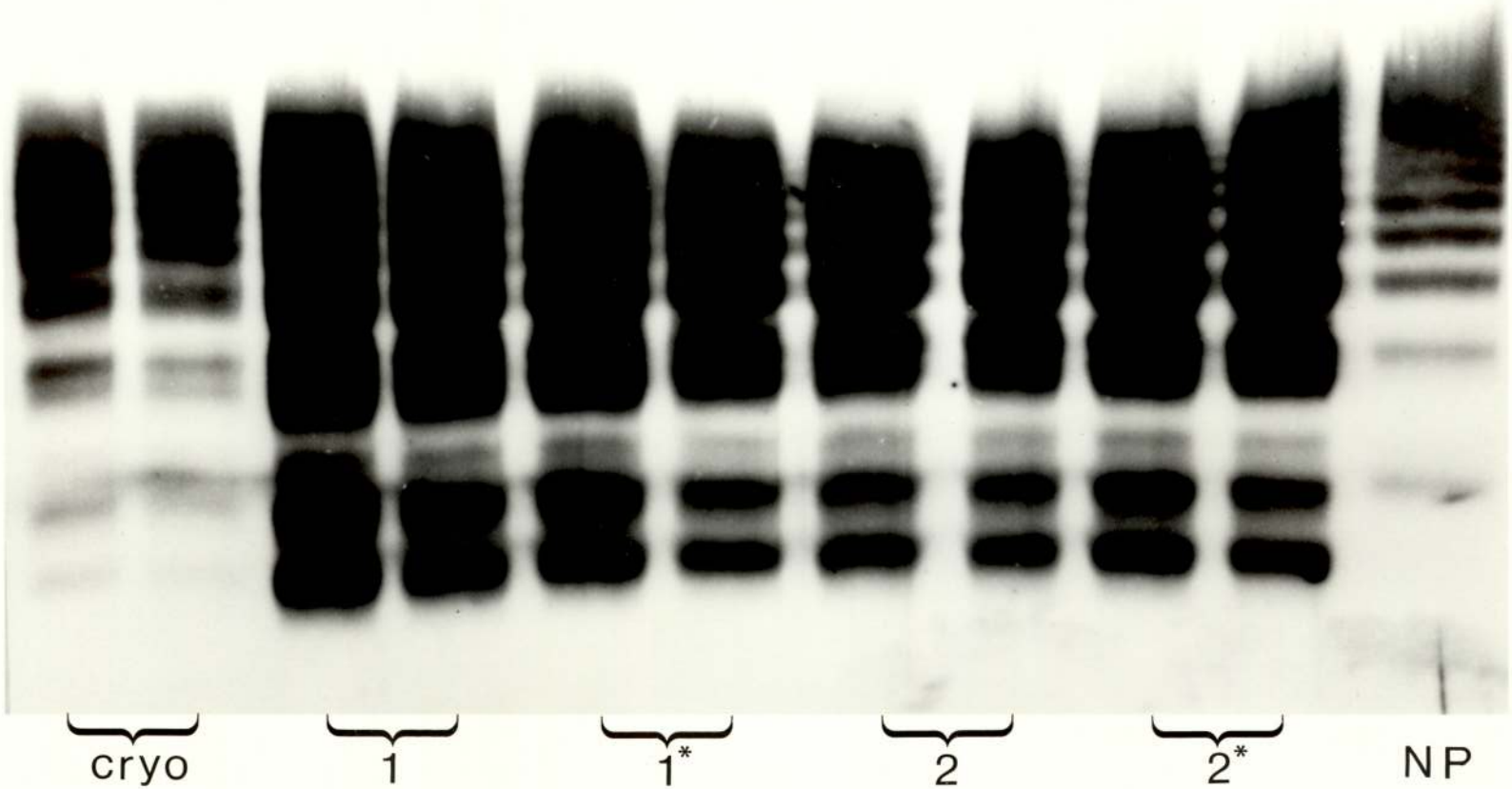
There was no statistical difference between results obtained on the heated and non heated concentrates as determined by a paired t-test. The statistical results were as follows: E1 to E5 vWf:Ag ($t = 2.041$, $p = 0.110$), VIIIB:Cof ($t = 1.545$, $p = 0.196$) and VIIIR:Cof ($t = 1.391$, $p = 0.2363$). For PFL6 to PFL10 vWf:Ag ($t = 1.448$, $p = 0.221$), VIIIB:Cof ($t = 2.981$, $p = 0.0412$) and VIIIR:Cof ($t = 0.084$, $p = 0.935$). Insufficient data was obtained for statistical analysis on CRV8.

SDS agarose-acrylamide gel electrophoresis of all the VIII concentrates confirmed the presence of the whole spectrum of multimer sizes of vWf with no apparent change occurring as a result of heating of the concentrate (Fig 14). This emphasises the need to measure functional activities in addition to looking at the multimeric profile as heating of these concentrates resulted in loss of functional activity although the multimer pattern remained normal. This would suggest that multimerisation is not dependent on functional activity and further evidence of this is postulated in section 7.11 in which an unusual variant is described.

Table.14. Botrocetin, ristocetin and von Willebrand antigen (vWf:Ag) activities in factor VIII concentrates.

Concentrates		vWf:Ag (U/ml)	VIIIB:CoF (U/ml)	VIIIR:CoF (U/ml)	Fig 14 Number
E1		96	320	100	1
E1	(heated)	88	200	40	1*
E2		147	170	47	2
E2	(heated)	90	140	24	2*
E3		113	170	48	3
E3	(heated)	88	140	48	3*
E4		80	200	48	4
E4	(heated)	74	140	55	4*
E5		79	140	55	5
E5	(heated)	74	180	48	5*
PFL6		65	250	32	6
PFL6	(heated)	55	210	40	6*
PFL7		69	83	55	7
PFL7	(heated)	69	35	55	7*
PFL8		60	83	40	8
PFL8	(heated)	44	35	40	8*
PFL9		50	19	47	9
PFL9	(heated)	56	7	40	9*
PFL10		73	7	47	10
PFL10	(heated)	65	7	47	10*
CRV8-11		40	7	47	11
CRV8-11	(heated)	40	7	24	11*
CRV-12		37	7	24	12
CRV8-12	(heated)	35	9	18	12*

E = Elstree Concentrates.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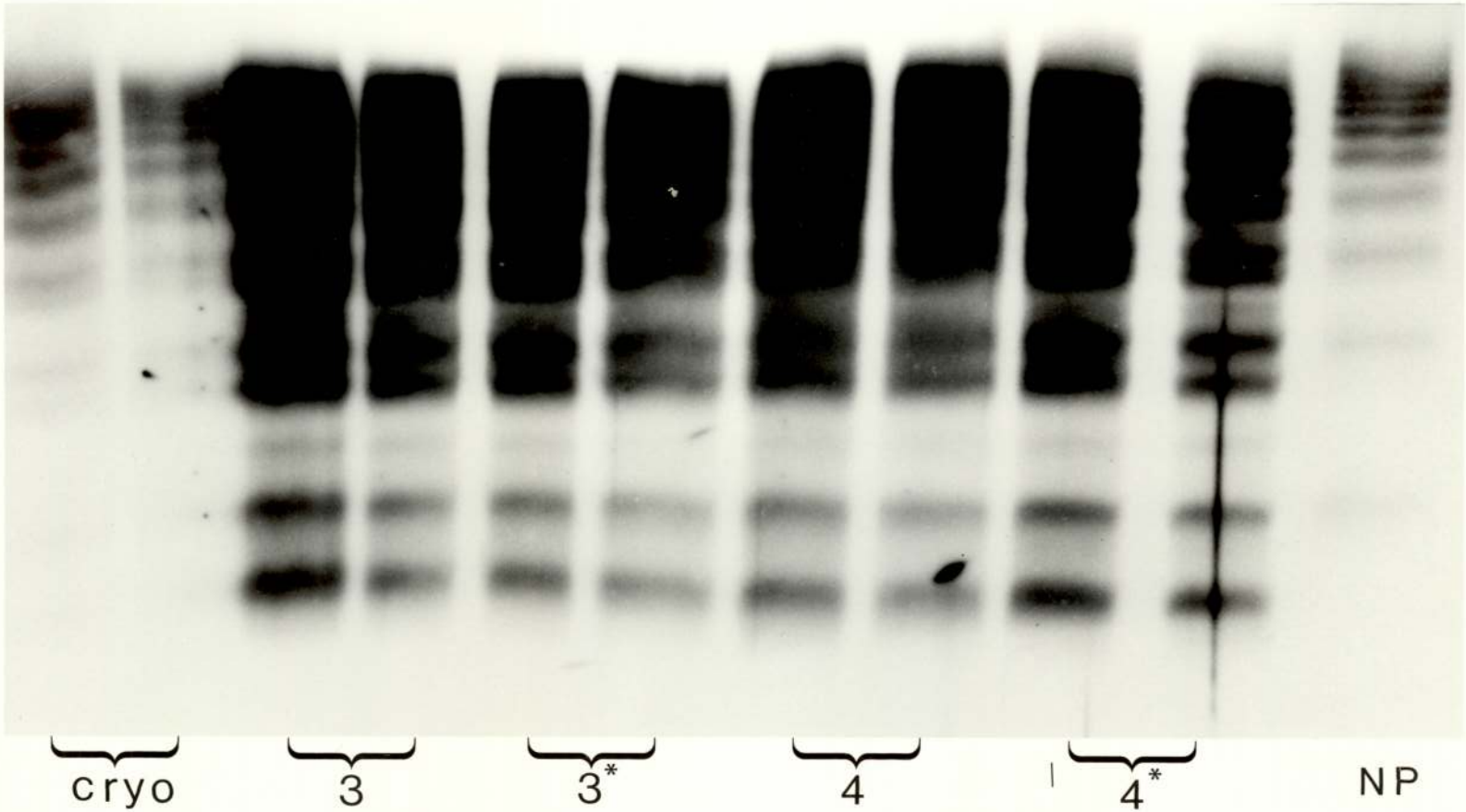
1*
2
2*
NP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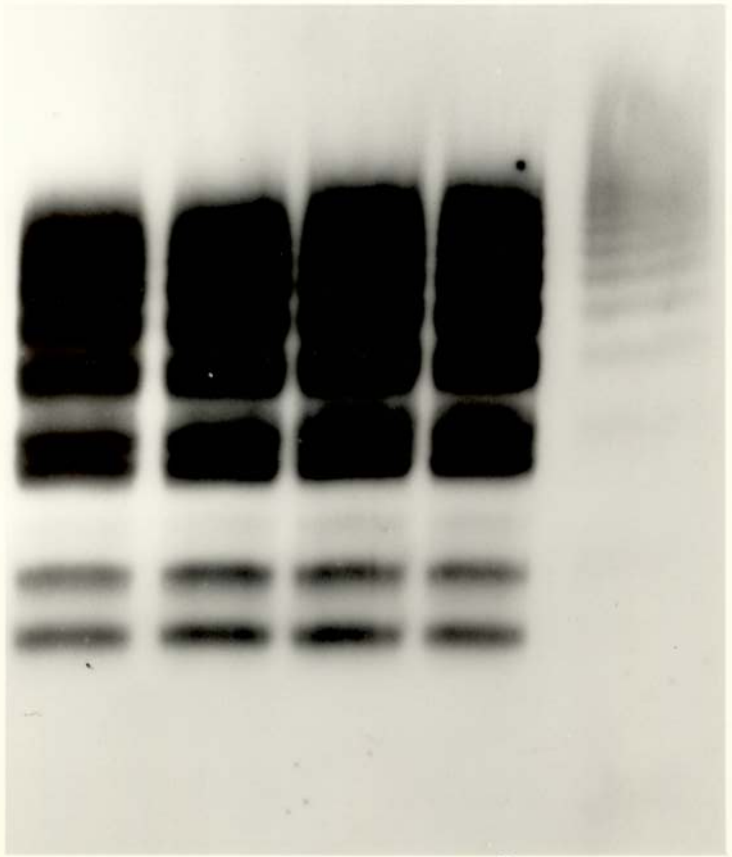
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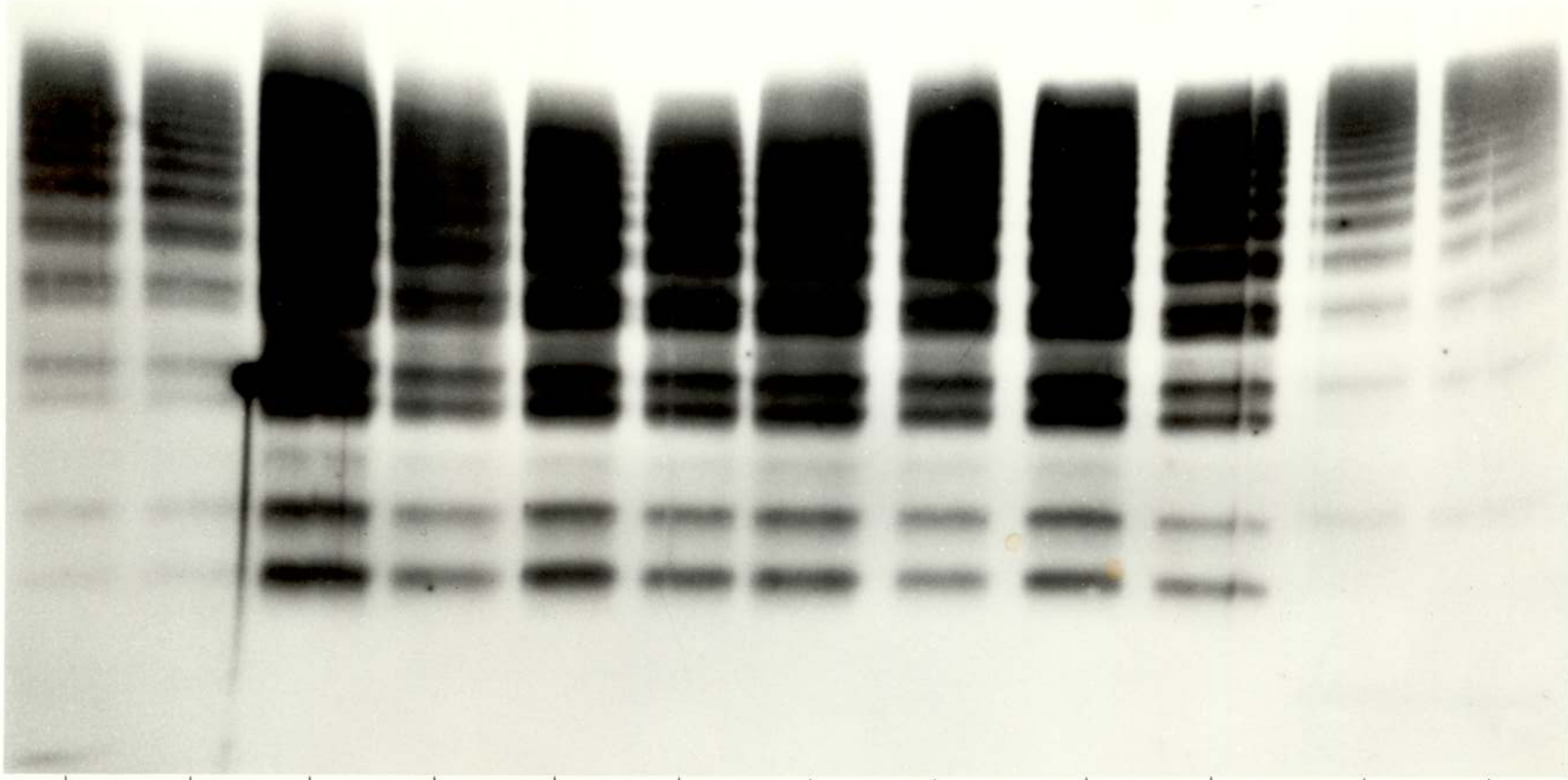
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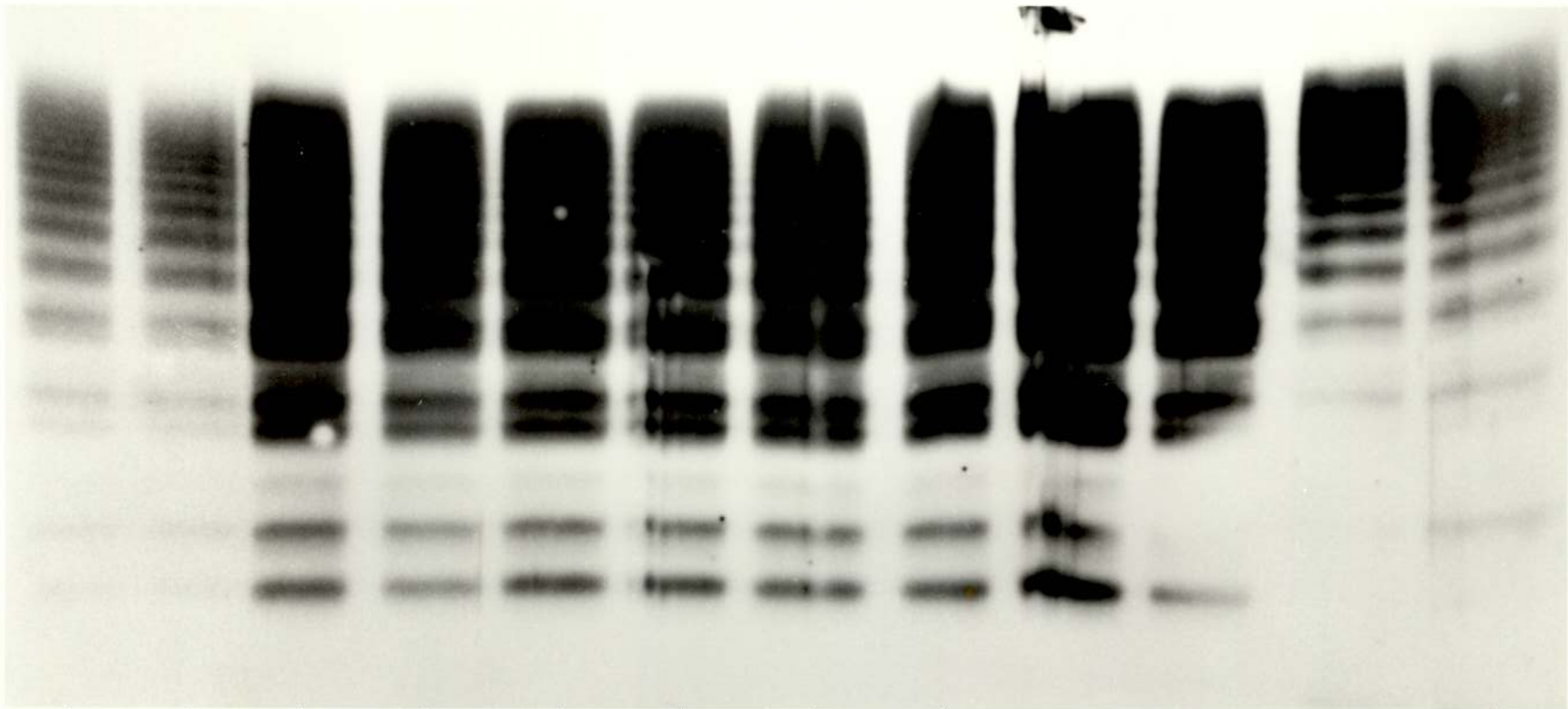
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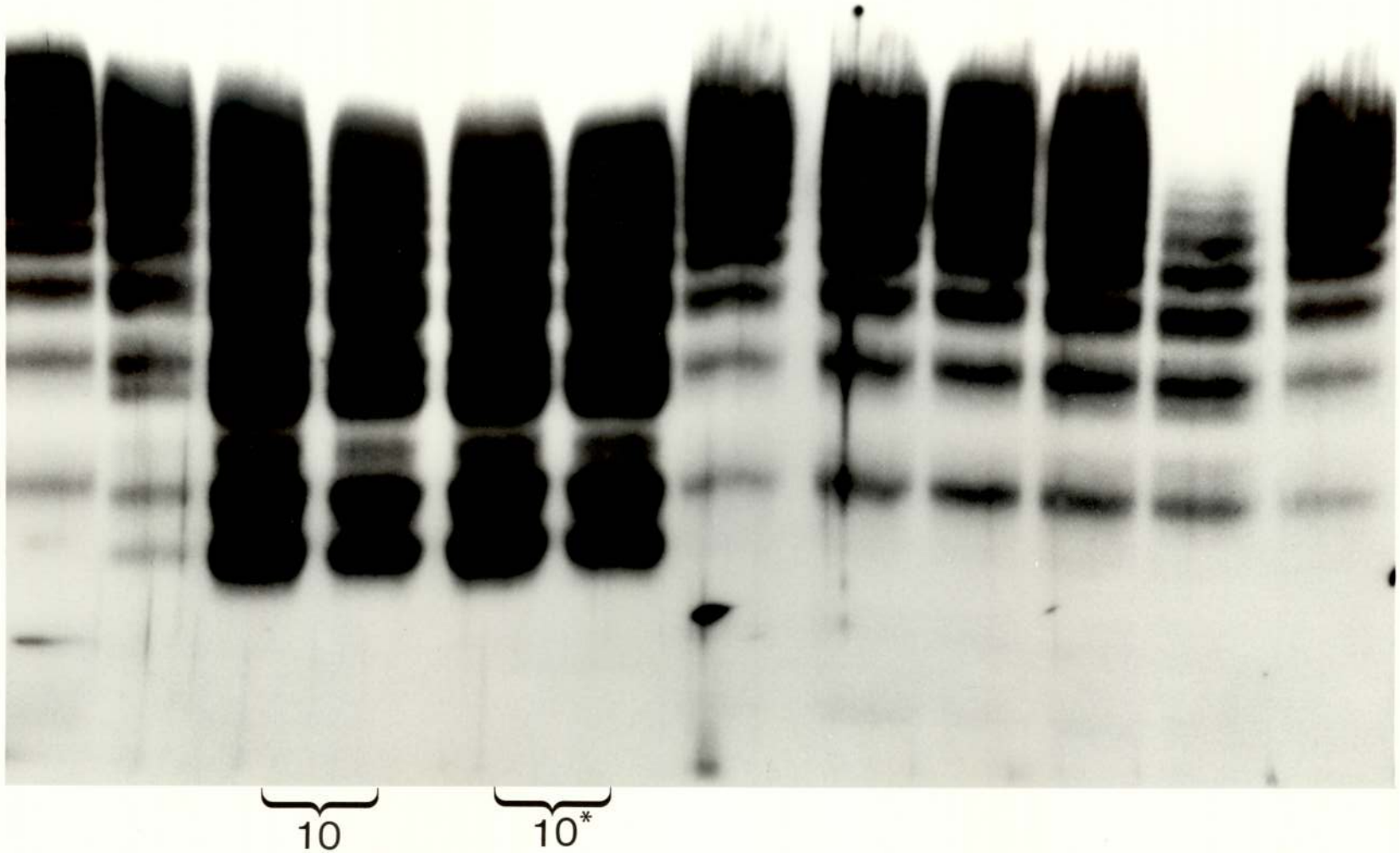
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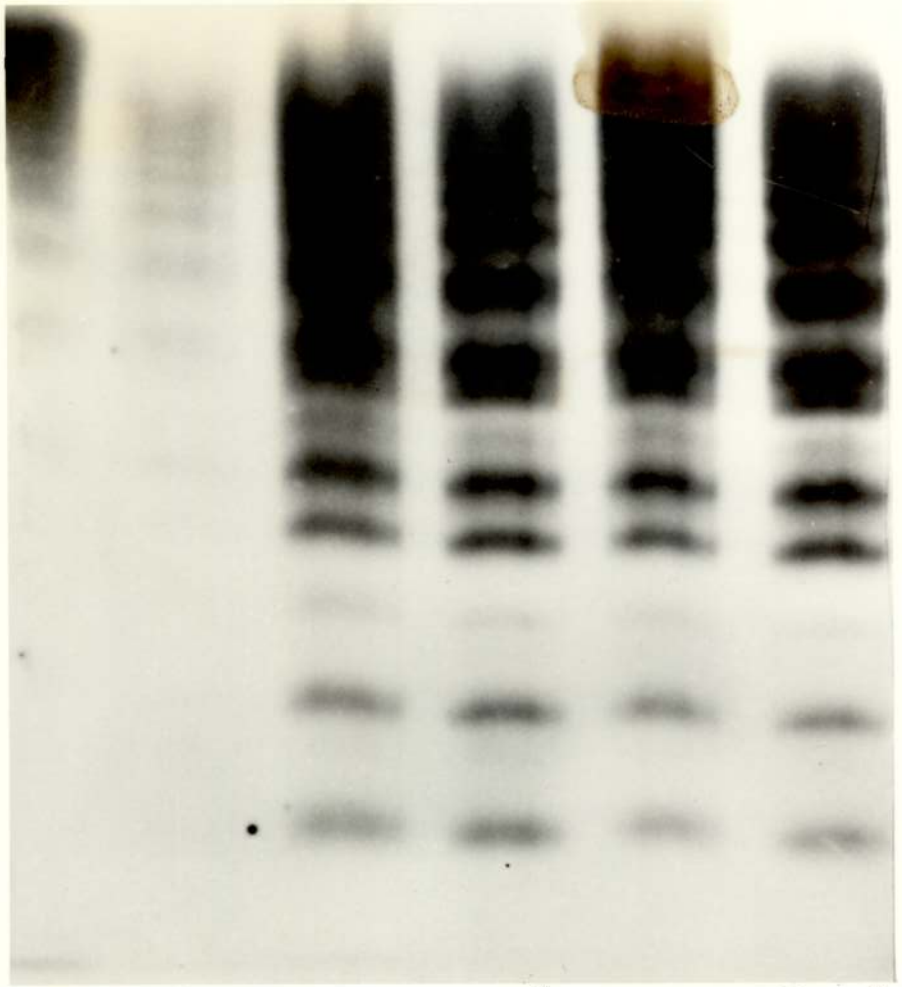
10*


NP
cryo
11
11*
12
12*

7.8. Comparison of an ELISA for vWf and vWf:Ag of normal plasma collected in anticoagulants containing protease inhibitors.

The forgoing sections have outlined various structural and functional tests for vWf. Clearly degradation by storage/ heating can compromise such assays. Likewise some authors (168,169,170) have suggested that proteolysis, particularly of the protein of vWd sub-types, may severly compromise assays. Suggestions that a number of proteolytic enzymes, plasmin, trypsin, chymotrypsin, calcium-activated proteases and an elastase-like protease derived from neutrophils, can cause proteolytic cleavage of vWf and produce an apparent loss of ristocetin activity with an increase in vWf:Ag appeared after the start of present studies (168,169, 170). However it was considered important to evaluate such possibilities for our own assay procedures. In order to evaluate the effect of in vitro proteolysis of vWf on vWf:Ag and vWf levels, blood from normals were collected into anticoagulants containing proteolytic inhibitors or different concentrations of EDTA.

Blood for plasma studies was collected in several different anticoagulant protease inhibitor mixtures. The blood was collected at a final ratio of 9 parts blood to 1 part anticoagulant/mixture at the following final concentrations; 1) 0.38% sodium citrate, 2) 0.38% sodium

citrate/5mM EDTA/1mM Leupeptin, 3) 0.38% sodium citrate/ Apoprotinin (10 trypsin inhibitor units (TIU), 4) 0.38% sodium citrate/Apoprotinin (10TIU)/1mM Leupeptin, 5) 0.38% sodium citrate/1mM Leupeptin, 6) EDTA (2mg/ml) and 7) heparin. The plasma was separated by centrifugation at 2500xg for 15 minutes at 4°C. The supernatant plasma was removed, aliquoted, and vWf:Ag and ELISA vWf measured.

In a complementary series of experiments we attempted to prevent the action of the calcium-dependent proteases proir to vWf evaluation. Thus one volume of EDTA was added to 9 volumes of heparinised normal plasma to give a final concentration of EDTA of 0.01, 0.05, 0.1, 0.25, 0.50, 0.75, 1.0, 1.5 and 2.0mg/mL. The total plasma calcium, vWf:Ag and vWf were measured using an ELISA assay as cofactor assays cannot be done in the presence of EDTA and heparin. The experiments were performed on three separate occasions and the mean results are shown in Tables 15 and 16.

7.8.1. Results of an ELISA for vWf and vWf:Ag of normal plasma collected in anticoagulants containing protease inhibitors.

Inclusion of 1 mmol/L Leupeptin, 5mmol/L EDTA and 10TIU/L Apoprotinin in the anticoagulant did not result in any significant changes in vWf:Ag levels in normal plasma (Table 15). Plasma taken into EDTA gave higher vWf:Ag and

vWf levels when assayed against a NIBSC standard. However, similar results were obtained when EDTA plasma was assayed against an EDTA normal plasma pool of 20 adult males (results not shown). Heparinised plasma gave a lower vWf:Ag of 0.90U/ml as compared to the 3.8% citrate only anticoagulant, but the same vWf level of 1.00U/ml. The addition of 1 mmol/L Leupeptin or a combination of Apoprotinin and Leupeptin resulted in a lower vWf level of 0.80U/ml as compared to 1.00U/ml for 3.8% citrate alone.

EDTA at a final concentration of 0.25mg/ml significantly altered the vWf:Ag level from 1.00 to 1.20U/ml. The vWf:Ag increased as the final concentration of EDTA increased giving a vWf:Ag value of 1.80U/ml at an EDTA concentration of 2mg/ml (Table 16). Overall, an increase in EDTA did not significantly influence the ELISA for vWf. However, at an EDTA concentration of 0.1mg/ml a low vWf value of 0.74U/ml was obtained.

Protease inhibitors confer no apparent protection and therefore no advantage for assay purposes. On the other hand using the conventional vWf:Ag assay use of EDTA as anticoagulant apparently and dramatically increases assay value. The monoclonal ELISA assay however did not reveal such increases. Thus in normal plasma the use of protease inhibitors is not justified but assay of samples taken into

EDTA anticoagulant against a standard taken into sodium citrate is contraindicated.

Table. 15. Comparison of an ELISA for vWf:Ag and vWf of normal plasma taken into anticoagulant containing different protease inhibitors.

Anticoagulant	vWf:Ag(U/ml)	vWf(U/ml)
Citrate (3.8%)	1.20	1.00
EDTA (2.0mg/ml)	1.40	1.20
Heparin	0.90	1.00
Cit/EDTA*/Leu	1.25	1.00
Cit/Apo/Leu	1.00	0.80
Cit/Apo	0.95	1.00
Cit/Leu	1.00	0.80

Cit = Sodium Citrate (3.8%). Leu = Leupeptin (1mmol/l).
Apo = Apoprotein (10IU). EDTA* (5mmol/l).

Table. 16. Comparison of ELISA vWf:Ag and vWf activities in different concentrations of EDTA.

EDTA(mg/ml)	vWf:Ag(U/ml)	ELISA vWf(U/ml)	Total Calcium(mmol/L)
0.00	1.00	1.00	2.33
0.01	1.00	0.94	2.14
0.05	1.00	0.94	2.02
0.10	0.90	0.74	1.89
0.25	1.20	0.81	1.55
0.50	1.20	0.83	0.97
0.75	1.30	0.92	0.46
1.00	1.30	0.92	0.00
1.50	1.30	1.00	0.00
2.00	1.80	1.00	0.00
Buffer Control	1.20	0.86	2.30
Normal Range	0.50-1.50	0.50-1.50	2.25-2.75

Buffer Control = 9 volumes Heparin to 1 volume of buffer.

7.9. Botrocetin and ristocetin cofactor activity of vWf using monoclonal antibodies that inhibit vWf activity.

Monoclonal antibodies to vWf have been used to assay vWf (165). Such antibodies have also been used to define the functional properties of vWf. Monoclonal antibodies which inhibit platelet aggregation, platelet adhesion, vWf binding to platelets or inhibit VIIIR:RCo activity (165) and skin bleeding time have been described (178). The specificity of these monoclonal antibodies inhibitory to vWf, provides evidence for the role of vWf in these interactions. In contrast some monoclonal antibodies to vWf enhance both ristocetin cofactor activity (177) and botrocetin cofactor activity (179).

In order to study these possible enhancing or inhibitory effects of monoclonal antibodies to vWf on botrocetin and ristocetin activity, equal volumes of normal plasma and one of the following monoclonal antibodies, (RF/1 and RF/2) kindly supplied by Dr A Goodall, (Ed3 and Ed4) kindly supplied by Dr C Prowse and 21/42 kindly supplied by Dr D E Joshua, were incubated separately at a final concentration of 10ug/ml at 37°C for 1 hour. Sufficient monoclonal antibodies to try different concentrations were not available. The concentration of 10 ug/ml was chosen because of the data published by these

groups on their monoclonal antibodies. Only sufficient monoclonal antibody was available to perform one experiment. A buffer control of equal parts of Owren's buffer and normal plasma was also included and treated identically. After incubation the ristocetin and botrocetin cofactor activities were measured on all the samples.

7.9.1. Effect of monoclonal antibodies on VIIIR:RCo and VIIIR:BCo activity.

When the 5 monoclonal antibodies (see above 7.15) to human vWf were assessed for their effect on VIIIR:RCo and VIIIR:BCo, three showed no VIIIR:RCo inhibition (21/42, Ed 3 and Ed 5) while two others (RF/2 and RF/1-RF/2) although completely inhibiting VIIIR:RCo activity did not inhibit VIIIR:BCo. All the monoclonal antibodies, except, RF/2 and RF/1-RF/2, enhanced VIIIR:BCo. Polyclonal (rabbit) antiserum (anti-vWf:Ag) completely inhibited VIIIR:RCo but had no effect on VIIIR:BCo activity (Table.17).

How some of these monoclonal antibodies produce apparent enhancement of activities has not been investigated as part of the studies reported in this thesis. However, as none of the antibodies either inhibit or enhance both VIIIR:RCo and VIIIR:BCo it seems that these two functional sites are separate epitopes on vWf.

Table.17. Comparison of botrocetin and ristocetin cofactor assays in normal plasma incubated with monoclonal antibodies directed against vWf.

MAb Antibody	VIIIR:RCo(U/ml)	VIIIR:BCo(U/ml)
Buffer Control	0.30	0.35
Ab 21/42	0.38	2.00
Ab RF/2	0.00	0.30
RF/1 & RF/2	0.00	0.30

MAb Antibody	VIIIR:RCo(U/ml)	VIIIR:BCo(U/ml)
Buffer Control	0.51	0.56
Ed 3	0.51	1.64
Ed 5	0.51	1.00
Polyclonal anti-vWf:Ag	0.00	0.55

MAb RF/2 = Monoclonal antibody kindly supplied by Dr A Goodall, Royal Free Hospital, London, for use in the ELISA vWf assay.

MAb 21/42 = Monoclonal antibody obtained from Dr D E Joshua, Royal Prince Alfred Hospital, Sidney, Australia.

MAb Ed 3 & 5 = Monoclonal antibodies obtained from Dr C Prowse, Edinburgh Blood Transfusion Service, Edinburgh, Scotland.

7.10. Evaluation of concanavalin A binding of vWf:Ag in patients with von Willebrand's disease.

The structure and function of the carbohydrate portion of vWf is important for its biological function. The lectin concanavalin A (Con A) precipitates vWf from normal plasma but to a reduced extent from plasma with atypical vWd (171). A carbohydrate deficiency or abnormality has also been demonstrated in some type II vWd (167). In an attempt to establish a reliable and convenient method for screening for vWf carbohydrate abnormalities an ELISA method has been used to quantitative the binding of vWf to Con A. These experiments were a prelude to the investigations on an unusual vWd variant described in section 7.11. In this study concanavalin A binding and the migration index (MI) were determined on 4 type IIA vWd, 2 type IID vWd and 20 normal plasmas.

7.10.1. Concanavalin A binding of vWf experiment.

The binding of Con A was dependent on the time of incubation at 37°C and the concentration of Con A used (Table 18). It was found that binding of vWf:Ag to Con A in normals repeatedly reached a plateau after 60-120 minutes incubation at 37°C at a final concentration of 2mg/ml. Furthermore, a higher degree of binding of vWf:Ag to Con A was achieved using 2mg/ml Con A as compared to 1 mg/ml. At a final concentration of 1mg/ml Con A the

binding of vWf:Ag to Con A reached a plateau at 90-120 minutes. Thus, for all routine experiments, 2mg/ml Con A and an incubation of 2 hours at 37°C was used.

The measurement of vWf:Ag binding to Con A of 20 normal plasmas was (mean = 82%, 1SD = 5.7%), with a normal range of 70-94% binding. The mean binding for vWf:Ag in cryoprecipitate was 95% with 1SD=6% on 5 different preparations.

Table 19 presents the results of vWf:Ag binding to Con A in 4 type IIA and 2 type IID vWd patients. In 3 out of 4 type IIA and one type IID vWd, the percentage of vWf:Ag binding to Con A was below the lower limit of normal (70-94%).

Table. 18. Typical Concanavalin A binding results for normal plasma vWf:Ag by ELISA.

Con A (1mg/ml final concentration).

Time (mins)	Binding (%)
30	33
60	33
90	81
120	80

Con A (2mg/ml final concentration)

Time (mins)	Binding (%)
30	68
60	85
90	88
120	89

The mean value = 82%, Standard deviation = 5.7%.

Normal Range (2mg/ml for 2 hours at 37°C) = 70-94% (n=20).

Table.19. Concanavalin A binding of plasma vWf:Ag in patients with von Willebrand's disease.

vWd	Migration index	% Con A binding
IIA	1.33	60
IIA	1.30	50
IIA	1.66	70
IIA	1.30	30
IID	1.37	51
IID	1.37	70
Cryo (n=5)	NT	82
Normals (n=20)	0.88-1.10	70-94

For the vWd patients the above results are means of duplicate assays. Cryo = Cryoprecipitate, mean value of 5 different experiments, SD = 6.0%. NT = Not tested.

7.11. Factor VIII studies on an unusual variant form of vWd.

Following the diagnosis of severe vWd in a 1 year old propositus (III_2) girl, family studies were undertaken to establish the inheritance pattern (Tables 20 and 21).

The father's vWf, showed a severe impairment in ristocetin activity whereas botrocetin activity was essentially normal (Table 20). He was therefore studied in more detail to see if he was similar to the patient described by Howard et al (6). Thus the possible relationship between the biological vWf activities (identified by ristocetin and botrocetin) and any structural abnormality in the vWF:Ag of the father's plasma or platelets was examined. For analysis of platelet vWf, the father's platelets were washed as previously described (64), stored at -20°C and then thawed and solubilized using lysing buffer (10mM Tris-HCL, 1mM EDTA, 2mM SDS and 1% Triton X-100). Multimeric analysis was performed on the platelet lysate and also on an additional plasma sample EDTA (2mg/ml), was added to prevent potential cleavage of vWf by calcium-activated protease, from the father.

In a separate experiment, plasma from the father (II_3), was incubated with equal volumes of buffered saline with or without the monoclonal antibodies, RF/2 and 21/42 for 1

hour at 37°C. The former was found to inhibit ristocetin-induced platelet aggregation whereas the latter apparently enhanced botrocetin activity in normal plasma (Table 17). After incubation VIIIR:RCo and VIIIR:BCo were measured.

7.11.1. Results of factor VIII studies on an unusual variant form of vWd.

The family tree is shown in Fig 15 and the results of the investigations are given in Tables 20 and 21. The propositus (III_2) has a markedly prolonged bleeding time (BT) and partial thromboplastin time (PTT). All the other family members have normal bleeding times and coagulation tests.

The father's (II_3) PRP did not aggregate in response to ristocetin at a final concentration between 1.25-1.5 mg/ml. The presence of an inhibitor was excluded as a mixture of the patients plasma and normal platelets all aggregated normally in response to ristocetin after 10 minutes incubation at 37°C. In contrast, botrocetin induced a hyperactive aggregation response in the patient's platelets.

The propositus (III_3) and her father (II_3) have absent plasma VIIIR:RCo while the mother (II_4) and son (III_1) have similar reduced levels. In contrast, the father (II_3) has a

normal plasma VIIIR:BCo of 0.90 U/ml and the mother a lower normal value of 0.65 U/mL. The grandmother (I_2) also has a borderline plasma VIIIR:RCo and VIIIR:BCo.

The father (II_3) of the propositus showed 0% binding of vWf:Ag to concanavalin A while his wife (II_4), with type I vWd, showed reduced Con A binding of 45%. In this study, normal vWf:Ag shows binding of 70-94% while patients with type IIA vWd varied from 30-70%. Of the 2 patients with type IID vWd studied, Con A binding was 50% and 70%. This data is presented for other patients in 7.10.

There was an absence of plasma vWf:Ag in propositus (III_2) but normal vWf:Ag multimer pattern in other family members. The lowest multimer of the father's (II_3) platelet vWf:Ag does not have a normal doublet appearance as seen in his wife (type I vWd) (Fig 16).

Studies with monoclonal antibodies demonstrated that the father's plasma vWf behaves abnormally in that the monoclonal antibody 21/42 did not enhance plasma botrocetin activity as it does with normal plasma (Table 22). It is of interest that the RF/2 monoclonal antibody and a combination of the monoclonal antibodies RF/1 and RF/2 (normally employed in ELISA assays for vWf) did not inhibit the botrocetin activity (Table 22). An assay for vWf activity (Table 20) using these monoclonals showed a

value for vWf activity similar to the patient's botrocetin activity. Both values were essentially normal.

This suggests that the botrocetin site of this patients vWf is abnormal. Furthermore the ristocetin and botrocetin sites are separate but must nevertheless be close together. Finally as the RF/1 and RF/2 antibodies interact with the patient's biological active vWf site the patient must have retention of epitopes on this site. Nevertheless the ristocetin reactive site cannot be properly expressed whereas the botrocetin site apparently remaining intact.

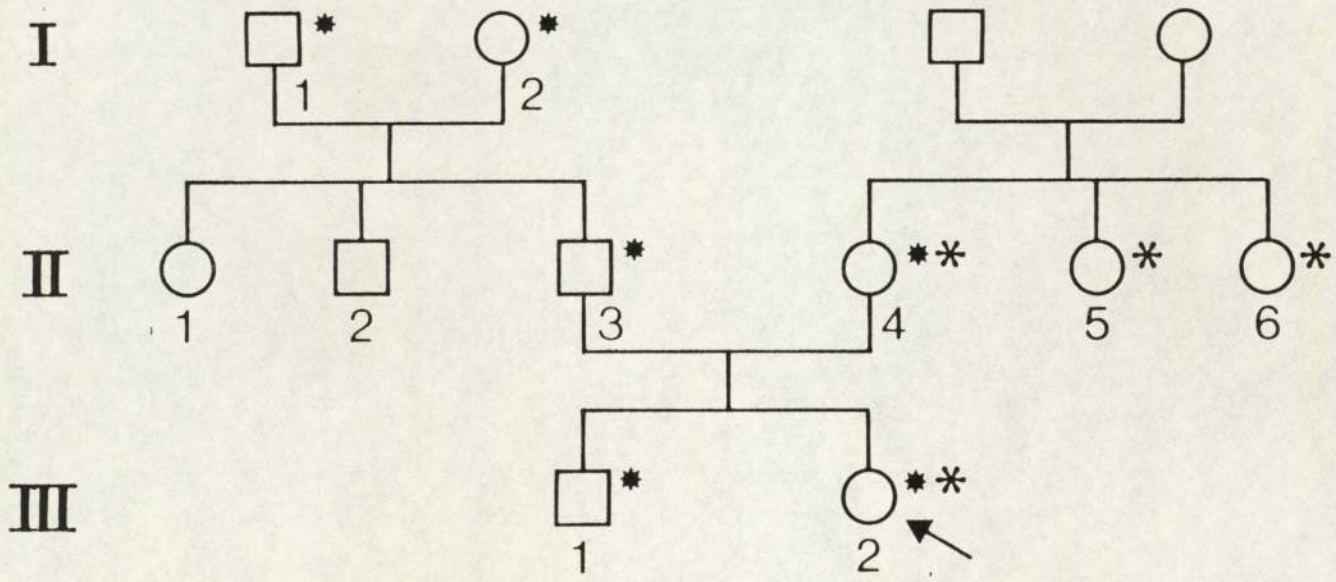

I
II
III
1
2
1
2
3
4
5
6
1
2
Males
Females
* History of easy bruising
* Available for study and have been tested

Table. 20. Family study of an unusual variant form of vWd.

Investigations	Father(II_3)	Mother(II_4)	Son(III_1)	Daughter(III_2)
BT(min)	4	5	4	>20
PT(sec)	14	13	14	15
PTT(sec)	30	30	31	62
VIII:C(U/ml)	1.70	1.30	2.10	0.01
vWf:Ag(U/ml)	0.61	0.18*	0.34*	0.00
vWf(ELISA)(U/ml)	0.66	0.21	0.36	0.00
VIIIR:RCo(U/ml)	<0.05	0.25	0.23	<0.05
VIIIR:BCo(U/ml)	0.90	0.65	NT	<0.05
Con A (% binding)	0.00	45	NT	NT
2DCIE		Ab		
Plasma Multimers	N	N	N	NT
Platelet Multimers	Ab			
Platelet Aggregation:				
Ristocetin 1.25mg/ml	Absent	N	N	Absent
1.50mg/ml	Absent	N	N	Absent
Botrocetin 10ug/ml	Hyperactive	N	N	NT

Normal ranges: BT = <7mins, PT = 12-15 secs, PTT = 30-37 secs,
VIIIR:RCo & VIIIR:BCo = 0.5-1.5U/ml, ELISA vWf = 0.64-1.30U/ml.
NT = Not tested. * = Non-parallel. Ab = Abnormal.

Table 21. Family study of grandparents of an unusual variant form of vWd.

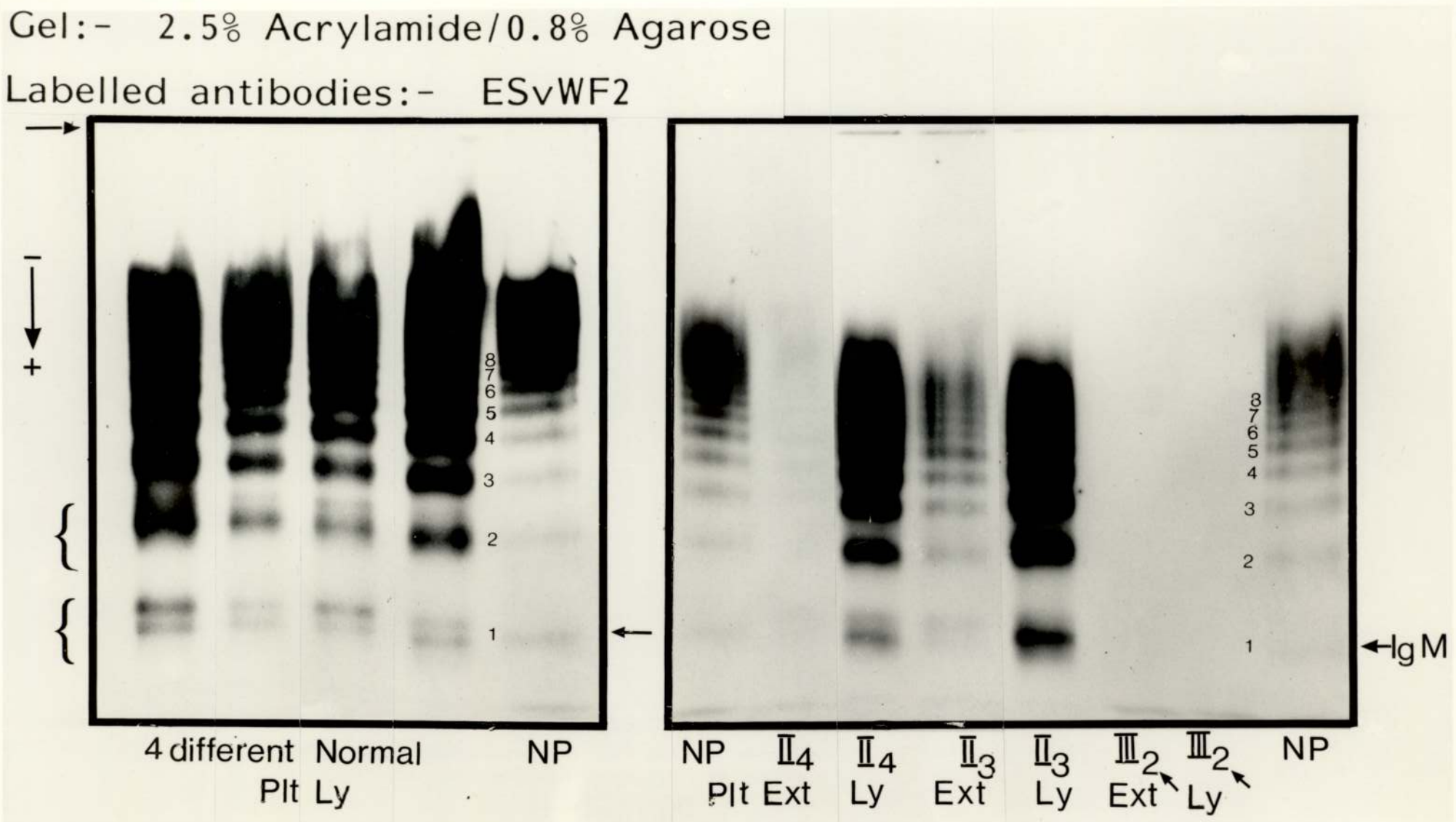
Investigations	Grandfather(I_1)	Grandmother (I_2)
BT(min)	NT	NT
PT(sec)	15	16
PTT(sec)	34	32
VIII:C(U/ml)	2.0	1.0
vWf:Ag(U/ml)	1.90	0.39
vWf(ELISA)(U/ml)	NT	NT
VIIIR:RCo(U/ml)	1.30	0.55
VIIIR:BCo(U/ml)	1.00	0.55
Con A (%binding)	64	40
2DCIE	N	Sl Ab
Plasma Multimers	N	N
Platelet Multimers	NT	NT
Platelet aggregation	NT	NT

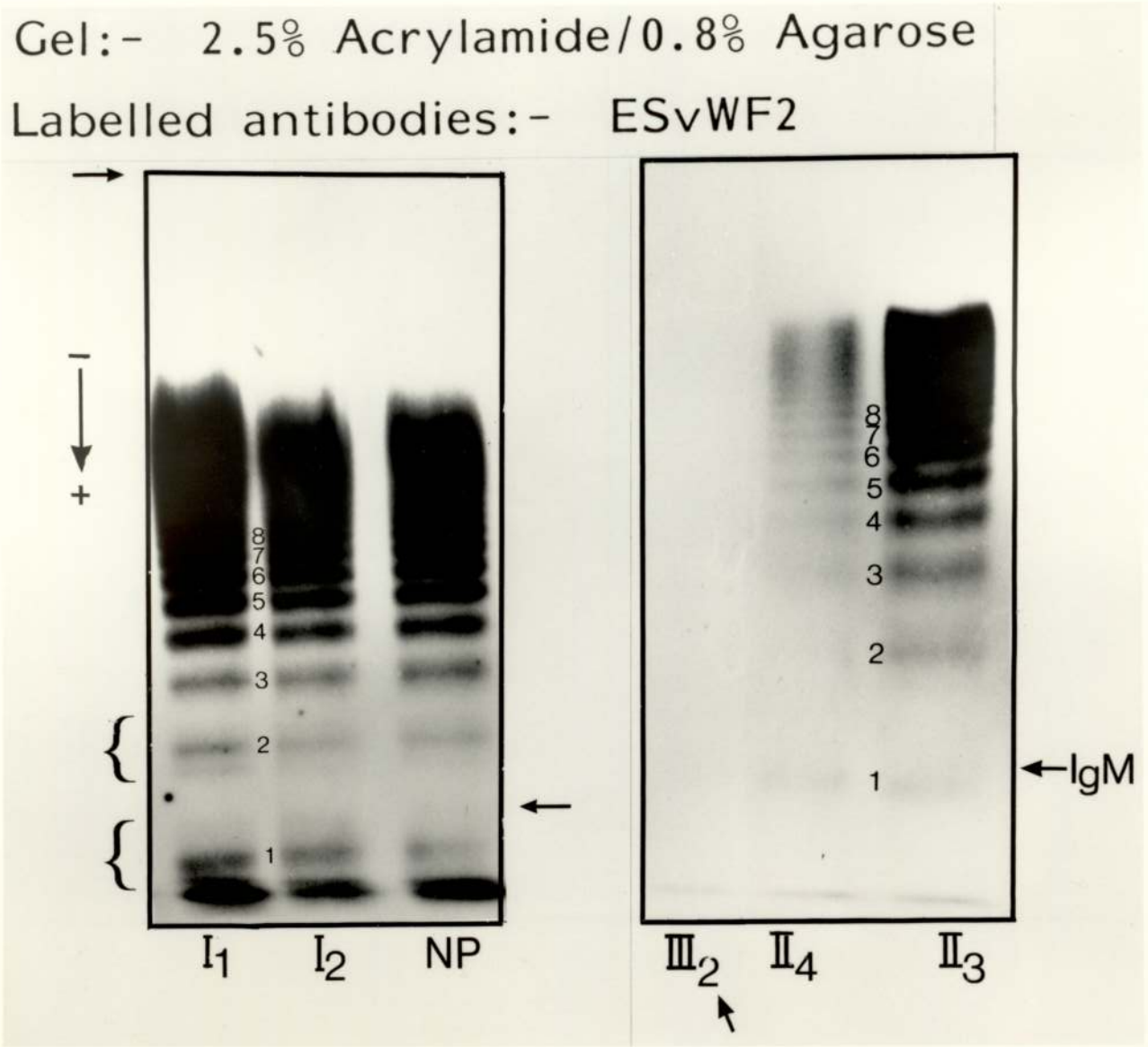
NT = Not tested. Sl Ab = Slightly abnormal. N = Normal.

Table. 22. Studies with monoclonal antibodies on the father (II_3) of propositus (III_2).

Plasma	VIIIR:RCo(U/ml)	VIIIR:BCo(U/ml)
Patient (II_3)/No MAb	<0.05	0.90
Patient (II_3)/MAb-RF/2	<0.05	NT
Patient (II_3)/MAb-21/42	<0.05	0.40
Normal Plasma/MAb-21/42	0.38	2.00
Normal Plasma/MAb-RF/2	<0.05	0.30
Normal Plasma/buffer	0.30	0.35

NT = Not tested. MAb = Monoclonal antibody.


Gel:- 2.5% Acrylamide/0.8% Agarose
Labelled antibodies:- ESvWF2
−
+
8
7
6
5
4
3
2
1
IgM
4 different Plt
Normal Ly
NP
NP Plt
II4 Ext
II4 Ly
II3 Ext
II3 Ly
III2 Ext
III2 Ly
NP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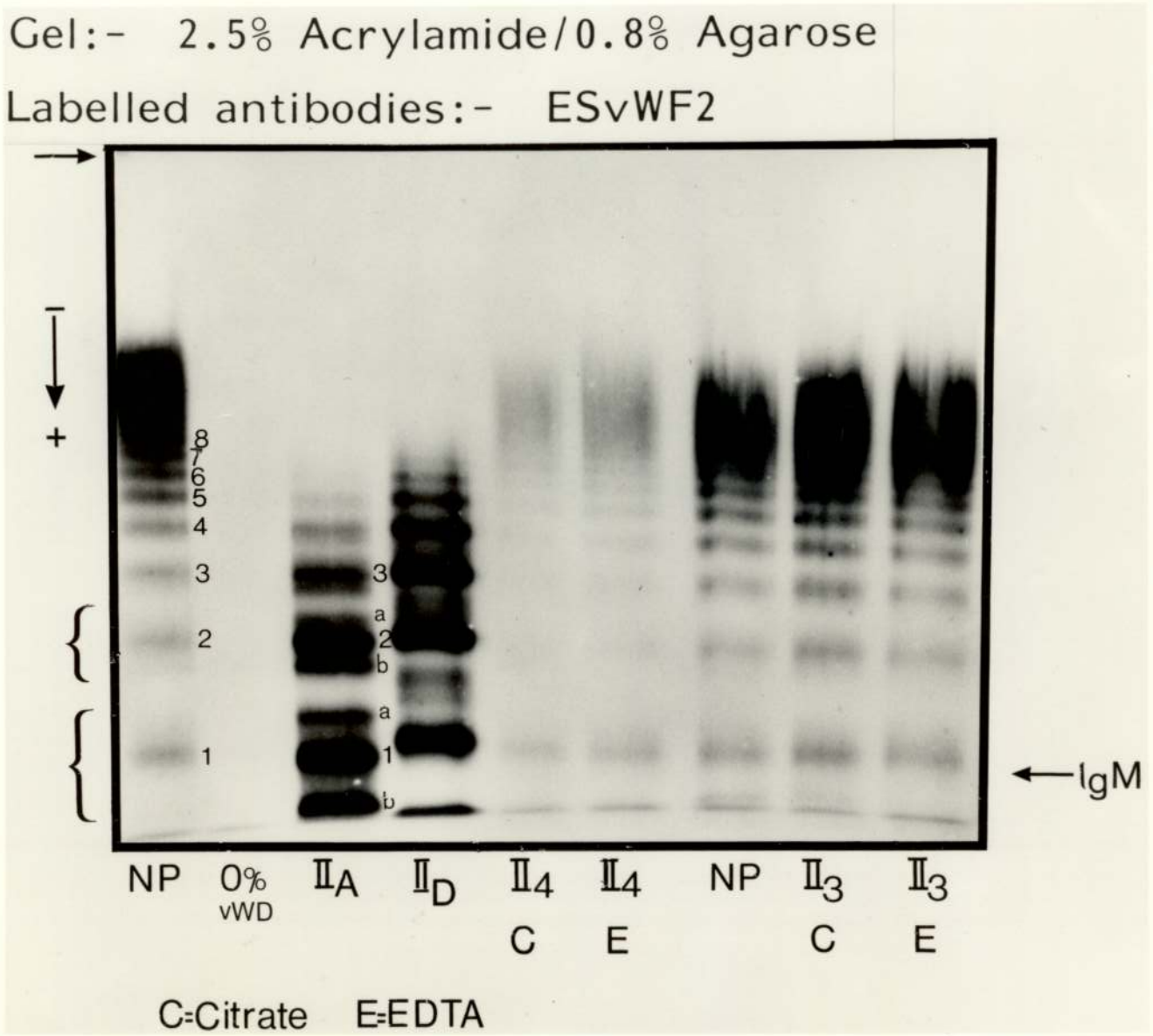
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C
II 4
E
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II 3
E
C=Citrate E=EDTA

Summary of findings in experiments to compare the activities of ristocetin and botrocetin.

1. Gel filtration studies.

Ristocetin activity was restricted to the higher molecular weight multimers of plasma vWf that eluted nearest the void volume, whereas botrocetin activity was associated with high, intermediate and low molecular weight multimers of plasma vWf.

2. Binding of vWf to platelets

Ristocetin-induced platelet binding of vWf involves the large molecular weight multimers of vWf. In contrast, botrocetin-induced platelet binding of vWf requires both large and small molecular weight multimers.

3. Comparison of ristocetin and botrocetin cofactor and an ELISA for vWf activity in vWd patients

A good correlation was found between VIIIR:RCo and VIIIR:BCo assays and an ELISA for vWf (using monoclonal antibodies that inhibit ristocetin cofactor) in type I and type II vWd patients. However, the fact that two patients have been found with discrepancies between plasma VIIIR:RCo and VIIIR:BCo activities suggests that the two assays are not always providing the same information and raises the possibility of two separate binding sites on the vWf protein for these materials.

4. DDAVP infusion experiments.

In these experiments the plasma vWf:Ag, VIIIR:RCo and VIIIR:BCo was raised to twice the pre-infusion level in normals. In type I vWd, DDAVP restored the vWf:Ag, VIIIR:RCo and VIIIR:BCo to normal levels while in type IIA vWd a threefold response in vWf:Ag but not VIIIR:RCo and VIIIR:BCo activities was observed. In the uraemic patients responses to DDAVP were very variable.

5. RIPA and BIPA experiments.

Platelet-rich plasma from patients with Bernard-Soulier disease (platelets lacking membrane glycoprotein Ib) showed a reduced response to ristocetin and botrocetin. Two patients with Glanzmann's thrombasthenia (platelets lacking membrane glycoproteins IIb/IIIa) gave an essentially normal responses with ristocetin and botrocetin. This suggests that the GPIb and not GP IIb/IIIa on the platelet is important for both botrocetin and ristocetin-induced interaction between platelets and plasma vWf.

6. Factor VIII concentrates.

In most of the factor VIII concentrates tested, VIIIR:BCo activity was higher than vWf:Ag and VIIIR:RCo. In addition, the vWf:Ag levels were higher than VIIIR:RCo activity. Heat treatment caused a general reduction in vWf:Ag and VIIIR:RCo, although in some batches no differences was demonstrated. The effect of heat on VIIIR:BCo activity was variable. However neither concentration nor heat treatment caused significant alteration in structural or functional properties of the vWf protein.

Furthermore full spectrum of vWf multimers was found in the concentrates by SDS agarose-acrylamide electrophoresis. Heating of the concentrates did not change the multimeric pattern. This finding confirms the need to measure functional activities in addition to the multimeric profile.

7. In vitro proteolytic studies.

The ELISA for vWf using monoclonal antibodies that inhibit ristocetin cofactor activity, unlike ristocetin cofactor assays, can be used with a variety of different anticoagulants that prevent vWf proteolysis. No effect of various anticoagulants, including EDTA, was found using the ELISA test. However EDTA as an anticoagulant did alter ristocetin cofactor assay and is therefore contraindicated.

8. Experiments with monoclonal antibodies directed against vWf:Ag.

These experiments suggest that ristocetin and botrocetin epitopes on vWf:Ag are distinct but studies of the unusual variant vWd (see section 10 below), suggests they are in close proximity to each other.

9. Con A binding of vWf:Ag.

Most of the type IIA vWd patients had a reduced or borderline Con A binding of vWf:Ag. These results suggest a possible carbohydrate deficiency in the type II vWd tested.

10. Unusual vWd variant.

A patient with an unusual and indeed unique variant form of vWd, retained botrocetin cofactor activity but had no ristocetin cofactor activity. Despite absence of ristocetin cofactor the plasma vWf:Ag multimers were normal. This patient's platelet vWf:Ag multimers however were abnormal. The patients vWf molecule interacts with RF/1 and RF/2 monoclonal antibodies that inhibit ristocetin cofactor activity but not botrocetin cofactor activity suggesting a non-functioning "Ristocetin" epitope is present.

DISCUSSION.

DISCUSSION.

Botrocetin has been proposed as an alternative agent to ristocetin for evaluating vWf function (5). Previous studies have shown important differences between the vWf-platelet interaction induced by these agents (6,135). These experiments suggested that different regions of vWf and platelet may be involved in the interactions induced by the two agonists. This study was designed to examine whether the biological activities of vWf as detected by botrocetin and ristocetin are functions of the same or different sites on the vWf protein.

One of the initial difficulties encountered was the purification of a standard batch of botrocetin free of fibrinogen clotting activity. The protein concentration of botrocetin varied with different batches of crude venom (1-6g/l) but standardisation was achieved by pooling different batches of crude venom and purifying as described. Using this procedure the average protein concentration obtained was 4-5g/l and the platelet aggregating activity of botrocetin remained stable for approximately 6 months at 4°C and 1 year at -70°C. In addition, the ultra-centrifugation step in the purification procedure was essential in order to remove sub-cellular particles which otherwise inhibited the platelet aggregating activity of the purified venom. Several

preparations of purified venoms failed to aggregate normal platelets in the presence of normal vWf when lower centrifugation speeds were used.

In the preliminary experiments when the optimum concentration of purified venom for botrocetin-induced platelet aggregation was being determined, the optimum final protein concentration of the purified venom in the test system for the same individual varied from 8-16 ug/ml. By pooling different batches of the crude venom prior to purification, a standardised solution of botrocetin (final protein concentration of 16ug/ml) was obtained which agglutinated platelets in all normals tested. This contrasts with the lower protein content of 6ug/ml previously reported by Howard and Firkin (135) and the 5ug/ml by Yoshihiro (176). However, 16ug/ml of botrocetin was the lowest final concentration which consistently induced maximum platelet aggregation in normal platelet rich plasmas (Fig 13). This finding is also supported by the platelet binding of vWf experiments which, although limited to a maximum botrocetin concentration of 10ug/ml, clearly demonstrated that a final concentration of botrocetin greater than 6ug/ml is required for maximum binding of vWf (Fig 7 and Table 5). Differences in the crude venom is the most likely reason for the observed differences in protein concentrations of botrocetin produced by different workers. This indicates

that individual standardisation of each batch of botrocetin is essential.

The majority of published work on botrocetin and ristocetin compares and contrasts the vWf cofactor activities. In using these cofactor assays it was important to achieve good standardisation and the rate of platelet agglutination was determined by changes in either platelet numbers or concentrations of botrocetin. The botrocetin cofactor assay, like the ristocetin cofactor assay, therefore required a constant number of platelets in the test system. Furthermore, the assay also required a constant botrocetin concentration so that the only rate-limiting variable was the test plasma vWf concentration. To achieve standardisation between assays a stable lyophilised formaldehyde-fixed platelet preparation was used and a standard botrocetin solution with a protein concentration of 7.5ug/ml. Brinkhous has shown, however, that the concentration of venom can be determined by taking that which causes platelet agglutination in 7-9 seconds with undiluted human plasma in a tilt-tube technique (5).

The best botrocetin bioassay results were obtained when donor platelets were washed immediately and then fixed with formaldehyde. Thus the use of 1 day old platelets from the local blood transfusion service gave inconsistent platelet agglutination. Furthermore in some lyophilised platelet preparations, even after reconstitution and centrifugation, the supernatant was found to be cloudy. Further washing of these platelets improved their ability for agglutination, emphasising that platelets must be washed adequately after fixing in formaldehyde.

Having standardized and optimised various techniques we consistantly found ristocetin and botrocetin were similar in their ability to induce platelet agglutination in normals and nearly all of the vWd patients. The only exception to this was the variant vWd that is reported in section 7.11, who had no ristocetin-induced platelet agglutination but had retained botrocetin-induced platelet agglutination (Table 20). This patient had no history of bleeding (he had played rugby but had not had any dental extractions). These observations indicate that agglutination of platelets by ristocetin and botrocetin is probably mediated by platelets interacting with different sites on vWf.

The results of a study by Howard et al, 1984 (135), on platelet agglutination by botrocetin and ristocetin also

suggest the possibility that botrocetin and ristocetin act at different sites on the vWf protein and platelet membrane (135). They reported that platelet-rich plasma from patients with Bernard-Soulier syndrome, who showed qualitative and quantitative abnormalities of platelet glycoprotein Ib, shows a total lack of platelet aggregation in the presence of ristocetin but a partial response with botrocetin (135). In the two Bernard-Soulier patients in this study abnormalities in aggregation were certainly present but in only one of these was there a difference between ristocetin and botrocetin and such differences were only marginal (Table 11). Clearly glycoprotein Ib is important for the ristocetin-vWf-platelet interaction but may be only partially involved in the botrocetin-vWf-platelet interaction. However two patients with thrombasthenia (Glanzmann's disease), in which platelet glycoprotein IIb and IIIa is deficient, gave similar ristocetin and botrocetin-induced platelet aggregation results (Table 12). This would seem to exclude platelet glycoprotein IIb and IIIa for the vWf binding site. Further evidence to support this conclusion comes from the reported effect of monoclonal antibodies directed against glycoprotein IIb/IIIa on ristocetin and botrocetin-induced platelet agglutination (176). Thus monoclonal antibodies directed against glycoprotein Ib blocked both ristocetin and botrocetin-induced platelet aggregation, whereas the anti-GPIIb/IIIa monoclonal antibodies had no effect.

Previous studies have compared botrocetin and ristocetin cofactor activity in normal subjects and a small number of vWd patients (5). It has been possible to study a total of 66 vWd patients comprising of 31 type 1 vWd, 26 type IIA vWd, 2 type IIC vWd, 5 type IID and two unusual vWd patients supplied by Dr M Howard and Dr Y Sultan (Tables 6,7 and 8). A good correlation was found between VIIIR:RCo and VIIIR:BCo, and an ELISA for vWf for type I and type II vWd patients. However, an unusual vWd (type B), (supplied by Dr Howard) gave a discrepant plasma VIIIR:RCo of <0.10U/ml as compared to the VIIIR:BCo of 0.40U/ml. Another unusual vWd plasma also gave discrepant and in this case reversed ristocetin and botrocetin cofactor activities; the VIIIR:RCo was 1.60U/ml whereas the VIIIR:BCo was 0.30U/ml. The patient also had a multimer pattern similar to that seen in type IIB vWd (Table 9).

A new unusual asymptomatic vWd variant with normal vWf plasma multimers but absent ristocetin and preserved botrocetin activity has also been described in the present study (Table 20). This patient had some similarities with the patient described by Howard. However, the patient described by Howard differed by having a prolonged bleeding time, a two-peaked 2DCIE pattern but normal SDS gel multimer pattern. Howard's patient also had reduced vWf binding with concanavalin A, whereas the new variant had absent binding of vWf to concanavalin A. The normal multimeric pattern of the plasma vWf may suggest a

specific error of the carbohydrate side-chain of the plasma vwf that is associated with alteration of the ristocetin detected site rather than the botrocetin site. These very unusual patients also indicate that there can be finer structural features of the vWf protein revealed by ristocetin which are not necessarily correlated with the gross multimeric analysis.

The hypotheses proposed by Howard and Firkin et al (6) for the vWf abnormality responsible for the unusual results reported was 'failure of the vWf subunits to form normal polymers in the intermediate molecular weight region, giving rise to an increased concentration of very low molecular weight polymer'. This hypotheses was to some extent supported by crossed-immunoelectrophoresis experiments, which showed an unusual arc composed of two peaks with increased concentration of very low molecular weight polymers present in the anodal peak, and by the minimal blocking of ^{125}I-FVIII binding to platelets in the presence of ristocetin at high vWf:Ag concentration. Alternatively, a specific error of the carbohydrate side chain was proposed, evidenced by a reduced Con-A binding, thus causing an inability of the vWf to form a normal sequence of polymers and a subsequent normal ristocetin site. Only the latter explanation would apply to the variant reported in this thesis.

Conflict exists in reports concerning the role of carbohydrate in the structure and function of vWf.However, Federici et al (91) have reported that carbohydrate protects vWf from disaggregation occurring caused by proteolytic attack but does not play a direct role in maintaining its multimeric structure or ristocetin cofactor activity. This would seem to be supported by the evidence from the two unique vWd variants with absent ristocetin but preserved botrocetin activity. In contrast, Gralnick demonstrated that a 60% release of the D-galactose was associated with a demonstrable loss of large multimers and a 70% decrease in ristocetin cofactor activity (88).

The correlation between the bleeding time and plasma vWf activity (as here measured by both ristocetin and botrocetin) was poor (Tables 6 and 7). Although the first description of vWd by von Willebrand reported that all the affected members had prolonged bleeding times, subsequent studies are conflicting. Gralnick (168) also found a poor correlation between plasma vWf activity (as measured by ristocetin only) or vWf:Ag levels and the bleeding time. However, an excellent correlation between the level of platelet vWf activity and the bleeding time was found, whereas the correlation between platelet vWf:Ag and bleeding time was significant but not as strong. Other studies have described subsets of type I vWd with low or abnormal platelet vWf and prolonged bleeding times which

shortened after DDAVP therapy, but did not become normal. These data clearly demonstrate the importance of platelet vWf in relation to the bleeding time and that platelet vWf may act as the initial link between platelets and the vessel wall. Analysis of platelet vWf was beyond the scope of this study but would appear to be essential in determining its importance in the bleeding time in vWd.

In these patients where discrepant ristocetin and botrocetin cofactor measurements occur the existence of two distinct functional sites on the vWf protein may be deduced. This is further supported, although not confirmed by the work of Brinkhous and Howard (136,135) who have reported that some type IIA vWd patients, who lack the largest vWf multimers, have decreased levels of ristocetin cofactor activity but normal botrocetin activity.

Measurement of vWf:Ag levels by EIA and ELISA, in type IIA vWd, gave discrepant results (Table 7). This discrepancy can be explained by the abnormal charge which exists on the vWf which not only gives an abnormal 2DCIEP migration index but also a higher vWf:Ag as measured by EIA. The ELISA assay is not influenced by the charge on the vWf molecule and is therefore not affected. However, some type IIA patients show a non-parallel dose response curve in the ELISA assay possibly due to the different epitope distribution on the vWf molecule.

In order to investigate if ristocetin and botrocetin detected activities are distributed on vWf proteins of different molecular sizes, normal plasma was chromatographed on Sephacryl S-400 columns and the multimeric structure determined in the eluting fractions together with ristocetin and botrocetin activity (Table 4, Fig 5 and 6). The earliest eluting fractions, with the higher molecular forms of vWf, contained only high molecular weight multimers on SDS gel electrophoresis with more botrocetin than ristocetin activity. As the elution volume increased firstly there were fractions containing vWf with both activities, but the later smaller molecular forms had only botrocetin cofactor activity.

Further evidence of this type of distribution was obtained by experiments in which washed normal platelets were incubated with normal plasma in the presence of different concentrations of ristocetin (Table 5). We confirmed, using SDS gel electrophoresis, that ristocetin removes the largest vWf multimers (Fig 9). Parallel experiments with botrocetin, however, showed that it removed large, intermediate and low molecular weight multimers. Thus platelet vWf interaction in the presence of ristocetin and botrocetin involves different molecular species of vWf. This agrees with the work reported by Howard (60), who used crossed immunoelectrophoresis after interaction of normal washed platelets and plasma in the

presence of botrocetin to show that large and small multimers are removed.

The availability of monoclonal antibodies that inhibit ristocetin-induced platelet agglutination have allowed the development of an ELISA assay which correlates with ristocetin cofactor assay results (Goodall et al) (165). In this thesis good correlation between this ELISA assay and both botrocetin and ristocetin activity was obtained (Table 8). The surprising finding was that the unique vWd patient, with no ristocetin but considerable botrocetin cofactor activity, had an ELISA assay result similar to the botrocetin activity rather than the ristocetin cofactor assay (Table 20). A series of monoclonal antibodies were obtained in addition to the Royal Free monoclonal antibodies (RF1& RF2) and incubation studies undertaken with this patients plasma prior to determining residual ristocetin and botrocetin cofactor activities. Despite the ELISA assay result the RF1 and RF2 monoclonal antibodies did not alter the botrocetin activity (Table 22). This suggests that the epitope detected by these monoclonal antibodies is on or close to the ristocetin cofactor site, which in this patient is biologically inactive. However, another monoclonal antibody, 21/42, which normally enhances botrocetin activity, did not produce this effect in this patient. This suggests that the site is not entirely normal

or that the vWf configuration is altered in relationship to this site.

Recently new information about the functional domains of vWf responsible for interacting with the platelet glycoprotein Ib has been obtained (196). The techniques used included monoclonal antibody inhibition of the interaction of tryptic fragments of vWf with platelets and Western blotting techniques. Clearly, the 2 patients in presented in this thesis have abnormal vWf GPIb binding sites which need further study using the same techniques.

Evidence that the vWf domain mediating botrocetin-induced platelet agglutination lies within a tryptic fragment (52/48 kD) and is therefore close to or identical with that which mediates ristocetin-induced binding and spontaneous binding of vWf to platelet GPIb has recently been reported by Yoshihiro et al (176). This indicates that the domain for binding to GPIb is present on all multimers. The large multimers as a result of their increased valency, have a higher affinity. In the absence of the large multimers, botrocetin may provide a stronger stimulus for vWf binding than does ristocetin and thus inducing platelet agglutination in the absence of large multimers. This may explain the higher botrocetin activity observed in some type IIA vWd patients and in the elution studies of normal plasma presented in this thesis. However, this

hypothesis does not seem apply to the two unusual patients presented in this study who both had apparently normal plasma vWf multimers. In addition, they showed that anti-GPIb monoclonal antibodies blocked agglutination of platelets by ristocetin and botrocetin, indicating that both agents induce binding of vWf to the GPIb receptor. Further corroboration is provided by the observation in patients with Bernard-Soulier syndrome.

The 2 two unique patients that have retained botrocetin but no ristocetin activity offer the opportunity to study the interrelationship of these 2 apparently separate functional sites on vWf. VWf from these patients could be purified by first preparing cryoprecipitate from the plasma followed by gel filtration. This semipurified vWf could then be digested enzymes (eg trypsin and S aures V8 protease) to produce vWf fragments. Similarly, normal vWf could be treated in parallel. Fragments of the digests of vWf could be separated by molecular size using SDS-PAGE electrophoresis. Western blotting could then be used to transfere theses fragments proir to detection by labelled monoclonal antibodies directed against ristocetin and botrocetin binding sites. These would include the Royal Free monoclonal antibodies (RF1 and RF2) used in this thesis.

An alternative approach would to be to extract the patients DNA and to use cDNA probes to map the patients

DNA to try and determine any abnormal sequences and provide information about the encoding for the vWf sites detected by ristocetin and botrocetin.

In summary botrocetin and ristocetin activity assays correlate well with each other in normal patients and those with type I vWd. However discrepancies between the assays can occur in some variant vWd patients. Evidence on two patients in particular has been presented to show that botrocetin cofactor activity can exist without ristocetin cofactor activity, and in one patient that ristocetin cofactor activity can exist with a much reduced botrocetin cofactor activity. Different molecular species of vWf have different amounts of ristocetin and botrocetin cofactor activity and this is further confirmed by determining which plasma vWf multimers interact with platelets in the presence of these agents. The experiments with monoclonal antibodies suggest that these sites although separate are close together. As both these vWf sites interact with the same platelet site, there is some possible biological advantage that ensures the different vWf molecular forms will all interact to ensure haemostasis.

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APPENDIX.

Reagents.

Owren's buffer, pH 7.35.

0.029M Sodium barbitone

0.125M Sodium chloride

0.215M Hydrochloric acid (215ml N/10)

Make up to 1 litre of distilled water.

Buffered saline, pH7.4.

800 ml 0.145M (0.87%) sodium chloride

200 ml Owren's buffer, pH 7.4

Keep at 4°C.

Phosphate buffered saline (0.01M), pH7.2.

0.345g $NaH_2PO_4.H_2O$

2.68g $Na2HPO_4.12H_2O$

8.474g NaCl

1 Litre of distilled water

Acetate buffer, pH 6.0.

0.1M Sodium acetate (97 vol)

0.1N Acetic acid (3 vol)

0.1mM $MnSO_4.4H_2O$

0.1mM $MgCl_2.6H_2O$

1.0mM $CaCl_2$

0.15M NaCl

34% Bovine albumin (Sigma Chemical Ltd).

34g of bovine albumin (fraction V) was layered onto 100 ml of buffered saline in a conical flask. The powder was allowed to dissolve undisturbed at 4°C for 48 hours. After 48 hours the pH should be 5.5-6.0. Aliquots were stored at -20°C.

Ristocetin (Lundberg, Copenhagen)

A 100mg vial of ristocetin sulphate was reconstituted with 5ml of buffered saline and mixed well until thoroughly dissolved. This gave a stock concentration of 20 mg/ml which was stored in 0.3ml aliquots at -20°C. For use, the ristocetin aliquots were thawed and diluted in isotonic saline to the required concentration.

Snake Venom, Bothrops jararaca (South American Pit Viper), Sigma Chemical Ltd, Poole, Dorset, No V-5652.

The powder was stored at -20°C until purified.

Paraformaldehyde, 40% w/v, BDH.

Siliconised Glassware.

The glass tubes were immersed in dichlorodimethylsilane (2% solution in carbon tetrachloride) and the chloroform allowed to evaporate at room temperature. Afterwards the tubes were rinsed in distilled water and dried. This procedure was repeated after the tubes were washed with detergent.

Appendix 2. Details of type I vWd patients studied (Table 6).

Patient No	BT (mins)	VIII:C U/ml	vWf:Ag U/ml	vWfBCof U/ml	vWfRiCof U/ml	Multimers present				vWd Type
						High	Intermediate	Low	Triplet	
1	NT	0.14	0.11	0.28	<0.10	+	+	+	N	I
2	>15	0.20	0.16	<0.10	<0.10	R+	R+	R+	N	I
3	10	0.25	0.16	<0.10	<0.10	+	+	+	N	I
4	>15	0.23	0.16	<0.10	<0.10	+	+	+	N	I
5	4	0.10	0.66(L)	<0.10	0.14	R+	R+	R+	N	I
6	NT	0.18	<0.10	0.11	0.11	+	+	+	N	I
7	7	0.48	0.44	0.11	<0.10	R+	R+	R+	N	I
8	>12	0.12	0.13	<0.10	<0.10	R+	R+	R+	N	I
9	>12	0.26	0.27	<0.10	<0.10	R+	R+	R+	N	I
10	>25	<0.10	0.01	<0.10	<0.10	Abs	Abs	Abs	Abs	III**
11	15	0.14	0.21	0.27	0.28	R+	R+	R+	N	I
12	>10	0.02	0.02	0.12	<0.10	MR	MR	MR	N	III**
13	4	1.00	0.40	0.26	0.47	+	+	+	N	I
14	5	1.00	0.37	0.24	0.25	+	+	+	N	I
15	NT	0.04	0.02	0.11	<0.10	MR	MR	MR	N	I
16	NT	0.18	0.05	0.11	0.11	+	+	+	N	I
17	NT	1.00	0.50	0.27	0.46	+	+	+	N	I
18	NT	0.00	0.02	<0.10	<0.10	Abs	Abs	Abs	Abs	III**
19	NT	0.95	0.91	<0.10	0.48	+	+	+	N	I
20*			0.07	<0.10	<0.10	*	*	*	*	I
21*			0.28	<0.10	0.12	*	*	*	*	I
22			0.09	0.11	<0.10	MR	MR	MR	N	I
23			0.09	0.23	<0.10	MR	MR*	MR	N	I

= Normal, MR = Markedly reduced, R+ = Present but reduced, L = Laurell immunoelectrophoresis, NT= Not tested,
= Present, 20* & 21* Duplicate samples (Nos 5 & 6 on Table 8). Abs = Absent, III** = Based on family studies.

Appendix 2. Details of type IIA vWd patients studied (Table 7).

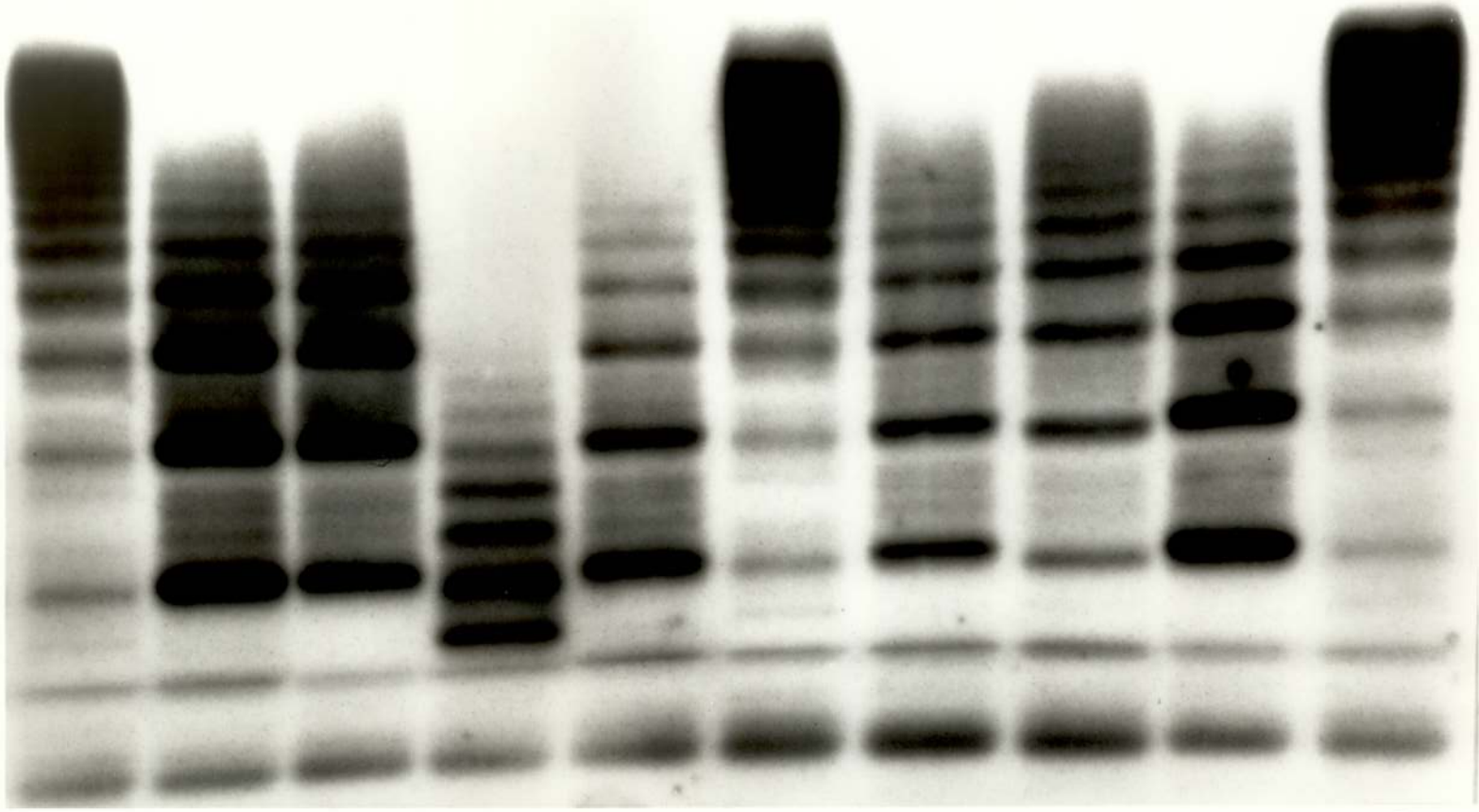
Patient No	BT (mins)	VIII:C U/ml	vWf:Ag U/ml	vWfBCof U/ml	vWfRiCof U/ml	Multimers present				vWd Type
						High	Intermediate	Low	Triplet	
1	>18	0.75	0.75	<0.10	<0.10	-	-	+	Ab	IIA
2	10	0.37	0.48	<0.10	<0.10	-	-	+	Ab	IIA
3	NT	0.61	0.75	<0.10	<0.10	-	-	+	Ab	IIA
4*	NT	0.64	0.63	<0.10	0.26	-	-	+	Ab	IIA
5	NT	0.32	0.28	<0.10	<0.10	-	-	+	Ab	IIA
6	4	0.00	0.78	<0.10	<0.10	-	-	+	Ab	IIA
7	3	0.72	1.76	<0.10	<0.10	-	-	+	Ab	IIA
8	NT	0.48	0.80	0.12	0.18	-	-	+	Ab	IIA
9	NT	0.32	0.25	<0.10	<0.10	-	-	+	Ab	IIA
10	6	0.30	1.07	<0.10	<0.10	-	-	+	Ab	IIA
11	11	0.42	0.76	<0.10	<0.10	-	-	+	Ab	IIA
12	8	0.60	1.52	<0.10	<0.10	-	-	+	Ab	IIA
13	NT	NT	0.35	0.11	<0.10	-	-	+	Ab	IIA
14	NT	NT	0.09	<0.10	0.12	-	+/-	+	Ab	IIA
15	NT	NT	0.22	<0.10	<0.10	-	+	+	Ab	IIA
16	NT	NT	0.36	0.40	0.29	-	+	+	Ab	IIA
17	NT	NT	0.12	<0.10	0.10	-	-	+	Ab	IIA

T = Not tested, - = Absent, + = Present, Ab = Abnormal , 4* = Duplicate sample (No 17 on table 8)

Appendix 2. Details of vWd patients studied (Table 8).

Patient No.	vWf:Ag U/ml	vWfRiCof U/ml	ELISA vWf U/ml	vWfBCof U/ml	Multimers present				vWd Type
					High	Intermediate	Low	Triplet	
1	0.16	<0.10	0.05	0.08	R+	R+	R+	N	I
2	0.16	<0.10	0.04	<0.08	R+	R+	R+	N	I
3	0.19	0.14	0.15	0.05	R+	R+	R+	N	I
4	0.015	<0.10	0.01	0.11	R+	R+	R+	N	I
5	0.07	<0.05	0.02	0.07	R+	R+	R+	N	Ia
6	0.28	0.12	0.05	0.07	R+	R+	R+	N	Ib
7	0.12	0.07	0.08	0.07	R+	R+	R+	N	I
8	<0.05	<0.05	0.06	<0.05	MR	MR	MR	N	I
9	0.35	<0.05	0.20	0.11	-	-	+	Ab	IIA
10	0.65	<0.10	0.08	<0.10	-	-	+	Ab	IIA
11	0.28	<0.05	0.06	<0.05	-	-	+	Ab	IIA
12	1.76	0.05	0.11	<0.05	-	-	+	Ab	IIA
13	0.80	0.16	0.16	0.12	-	-	+	Ab	IIA
14	0.75	0.05	0.07	<0.05	-	-	+	Ab	IIA
15	0.48	<0.05	0.10	<0.05	-	-	+	Ab	IIA
16	0.75	<0.05	0.08	<0.05	-	-	+	Ab	IIA
17	0.63	0.26	0.07	<0.05	-	-	+	Ab	IIA
18	0.80(NP)	0.41	0.26	0.31	-	+	+	Ab doublet	IIC
19	0.74	<0.05	0.04	0.07	-	+	+	Ab doublet	IIC
20	0.90(NP)	0.10	0.21	0.31	-	+	+	****	IID
21	0.72	0.86	0.26	0.66	-	+	+	****	IID
22	0.90	0.16	0.09	0.50	-	+	+	****	IID
23	0.84	0.24	0.12	0.21	-	+	+	****	IID
24	0.84	0.24	0.21	0.21	-	+	+	****	IID

NP = Non Parallel, MR = Markedly reduced, R+ = Present but reduced, **** = Absent but minor extra bands between multimers 1 & 2, and 2 & 3. Ab = Abnormal


NP
1
2
4
5
NP
*
3
*
NP