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Structural analysis of Factor VIII antigen in von Willebrand disease

(von Willebrand factor heterogeneity/hemostasis/radioactive peptide maps)

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ABSTRACT **The Factor VIII antigen molecules in the plasma of patients with classical type 1 and variant type 2A von Willebrand disease were compared to the Factor VIII antigen molecules in normal plasma. Factor VIII antigen was isolated from plasma by solid-phase immunoprecipitation and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis; the stained Factor VIII antigen bands were removed, radioiodinated, and subjected to tryptic digestion. Computerized analysis of autoradiographs revealed that the two-dimensional peptide maps of the different Factor VIII antigens were remarkably similar. The results suggest that the Factor VIII antigen molecules in these two forms of von Willebrand disease are probably identical to the Factor VIII antigen molecules present in normal plasma. It is thus likely that the differences observed in plasma Factor VIII antigen in classical and variant von Willebrand disease are not due to qualitatively abnormal molecules but rather represent quantitative shifts in the metabolism of normal Factor VIII antigen molecules.**

Von Willebrand disease is a congenital hemorrhagic disorder in which an abnormality of von Willebrand factor (VIII:VWF) is associated with decreased adhesion of platelets to the subendothelium. The disorder is heterogeneous, and several clinical forms of the disease have been recognized. The classical form of the disease is transmitted as an autosomal dominant trait, generally with a mild hemorrhagic syndrome. Two subtypes of the classical form are well recognized. Type 1 von Willebrand disease is usually associated with a concordant decrease in plasma Factor VIII procoagulant activity (VIII:C), Factor VIII antigen (VIII:Ag), and VIII:VWF. Analysis of VIII:Ag by crossed immunoelectrophoresis reveals a normal polydisperse distribution of molecules (1, 2). Type 2 or "variant" von Willebrand disease is associated with discordant decreases in the components of the plasma Factor VIII complex with a more pronounced deficit in the VIII:VWF level. Analysis of the VIII:Ag present in the plasma of patients with type 2 disease by crossed immunoelectrophoresis reveals an abnormal pattern with a marked reduction of the larger, slower moving, less anodal molecular forms (3–5).

A new subtype of the variant form (type 2B) recently has been described (6). This clinical group shows the type 2 abnormal pattern on crossed immunoelectrophoresis but is characterized by enhanced ristocetin-induced platelet agglutination compared to normal. Variant type 2A von Willebrand disease is characterized by decreased ristocetin-induced platelet agglutination.

A relatively rare form of von Willebrand disease is apparently transmitted as a recessive trait and is associated with a severe hemorrhagic syndrome in homozygous patients. Trace amounts of VIII:Ag in these patients also lack the larger, less anodal molecular forms (7).

The molecular basis for the heterogeneous nature of von Willebrand disease is not well understood. The disorder has been assumed to be associated with a quantitative and or a qualitative abnormality of the Factor VIII/von Willebrand factor protein. VIII:VWF which is necessary for normal platelet function and is measured *in vivo* by the bleeding time and *in vitro* by ristocetin-induced platelet agglutination is the functional attribute of VIII:Ag (8). Several studies have suggested that the factor VIII:Ag molecules in type 2 variant von Willebrand disease may be qualitatively abnormal analogous to hemoglobinopathies (4, 5, 9). Recent studies suggest that the abnormality in von Willebrand disease is due to an abnormal distribution of polymeric forms of VIII:Ag (10).

In order to determine whether VIII:Ag in von Willebrand disease is structurally abnormal, the plasma VIII:Ag molecules in normal pooled plasma, in plasma from four patients with type 1 von Willebrand disease, and in plasma from one patient with type 2A von Willebrand disease have been isolated, iodinated, and trypsinized and the radioactive peptide maps have been compared.

METHODS

Antisera. Monospecific rabbit anti-human VIII:Ag was prepared as described (11).

Immunoisolation of VIII:Ag from Normal and von Willebrand Plasma. This was performed essentially by the method of Kessler (12). One milliliter of a 10% suspension of formaldehyde-fixed *Staphylococcus aureus* Cowan strain I (CalBiochem–Behring) was pelleted. The bacteria were resuspended in 1 ml of 0.5% Triton X-100 (Sigma) in buffer B [50 mM Tris, pH 7.4/150 mM NaCl/5 mM EDTA/0.02% sodium azide/0.4 mM phenylmethylsulfonyl fluoride (Sigma)/1 mM benzamidine (Sigma)/1 μM pepstatin (Protein Research Foundation, Osaka, Japan)] and incubated for 30 min at 20°C with end-over-end rotation. The bacteria were then washed three times with 1 ml of the above solution by centrifugation at 8000 × *g* for 1 min. The washed bacteria were incubated for 30 min with the anti-VIII:Ag (0.1 ml) and 0.8 ml of 0.05% Triton X-100 in buffer B, washed again three times with 1 ml of buffer B, resuspended in either 2.5 ml of normal pooled plasma or von Willebrand plasma, and incubated for 1 hr at 20°C. Then, the bacteria were washed eight times with phosphate-buffered saline (until *A*280 of the wash reached background) and eluted by boiling for 3 min in 2% $NaDodSO_4$/6 M urea containing the protease inhibitors used above. The bacteria were pelleted and the supernatant was removed and frozen.

Two-Dimensional Immunoelectrophoresis. This was performed by the method of Converse and Papermaster (13). In

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Abbreviations: VIII:VWF, von Willebrand factor; VIII:C, Factor VIII procoagulant activity; VIII:Ag, Factor VIII antigen. "Factor VIII complex" refers to all three functions or activities of circulating plasma Factor VIII.

the first dimension the sample was electrophoresed unreduced in $NaDodSO_4$/3% acrylamide/0.5% agarose gels. The gel was sliced longitudinally and electrophoresed through a layer of agarose containing 0.5% Lubrol PX into a second layer of agarose containing 0.15% anti-VIII:Ag.

Plasma VIII:Ag. This was purified from normal plasma as described (14).

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Gel electrophoresis was performed in 3% acrylamide/0.5% agarose gels by the method of Weinstein *et al.* (15).

^{125}I-Labeling and Peptide Mapping. Radioiodination of fixed and stained proteins within $NaDodSO_4$ gel slices, tryptic digestion, and peptide mapping were done as described (16). Cellulose thin-layer chromatography plates (20 × 20 cm) were used.

Factor VIII Function Studies. VIII:C was measured with a one-stage assay for partial thromboplastin time. VIII:Ag was measured by a modification of the Laurell quantitative immunoelectrophoresis method (17). VIII:VWF was assayed by using the ristocetin system and formalin-fixed platelets (18). Crossed immunoelectrophoresis was performed as described by Laurell (19).

Peptide Map Comparisons. Computer analysis was used to compare the separate radioactive VIII:Ag peptide maps. The radioautograph on Kodak XRP-1 x-ray film was scanned with a high-speed raster-scanning microdensitometer (Optronix). The resulting two-dimensional density assay was entered into a PDP 11/70 computer. A program to analyze two-dimensional gels originally written by W. Lutin (20) for a PDP 10 computer was modified for the PDP 11/70 and used in these experiments. A density contour map was generated to allow comparison with the original radioautograph. This allowed more precise separation of peptides than apparent on the original and facilitated comparison of the separate radioautographs. The program quantitates peptide spots by fitting each peptide peak to a two-dimensional gaussian function. Peaks with nongaussian shapes are approximated as a sum of gaussians by combining overlapping gaussians. By using this program, overlapping peptide spots can be separated from one another and their relative volumes can be estimated. The volume fit is accurate to within approximately 2% for isolated spots (20). After the fitting of the peptide patterns by two-dimensional gaussians, the generated gaussian functions are plotted as a density contour map for comparison to the original density pattern.

RESULTS

Von Willebrand Patients. The four patients with type 1 von Willebrand disease had prolonged bleeding times, concordant decreases of VIII:C, VIII:Ag, and VIII:VWF levels (Table 1), and normally distributed but quantitatively decreased plasma VIII:Ag (Fig. 1). The patient with type 2A had a more pronounced decrease in VIII:VWF compared to VIII:Ag and had

Table 1. Laboratory data for five patients with von Willebrand disease

Type	Patient	Data* VIII:C	VIII:Ag	VIII:VWF
1	L	43	18	29
	W	37	24	20
	T	47	20	34
	M	31	43	33
2A	R	28	67	13
Normal values		>50	>50	>45

* Shown as % of normal.

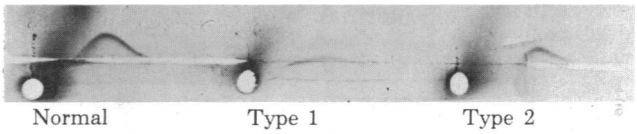

FIG. 1. Crossed immunoelectrophoresis of pooled normal plasma, plasma from type 1 von Willebrand disease, and plasma from type 2A von Willebrand disease. Positive pole is toward the right in the first dimension and toward the top in the second dimension. Anti-VIII:Ag concentration was 0.1%.

an abnormal distribution of plasma VIII:Ag molecules characterized by a marked reduction of the larger, less anodic (slower moving), molecular forms.

Immunoisolation of VIII:Ag. VIII:Ag in normal and von Willebrand plasma was isolated by using monospecific rabbit anti-VIII:Ag bound to killed staphylococci (which bind to the Fc regions of immunoglobulins) (12). The immunoisolates were analyzed by a $NaDodSO_4$/polyacrylamide gel system which clearly differentiated VIII:Ag from fibronectin. The VIII:Ag bands in the immunoprecipitates from normal and von Willebrand plasma barely entered the gels and moved to the same position as a purified nonimmunoprecipitated VIII:Ag derived from normal pooled plasma (Fig. 2). When removed from unstained gels and reduced with dithiothreitol, the normal and von Willebrand VIII:Ag bands moved into the gels with apparent molecular weights of 200,000 (not shown).

The major contaminating bands in the $NaDodSO_4$ gels of the normal and von Willebrand VIII:Ag immunoprecipitates comigrated with immunoglobulin markers. In order to deter-

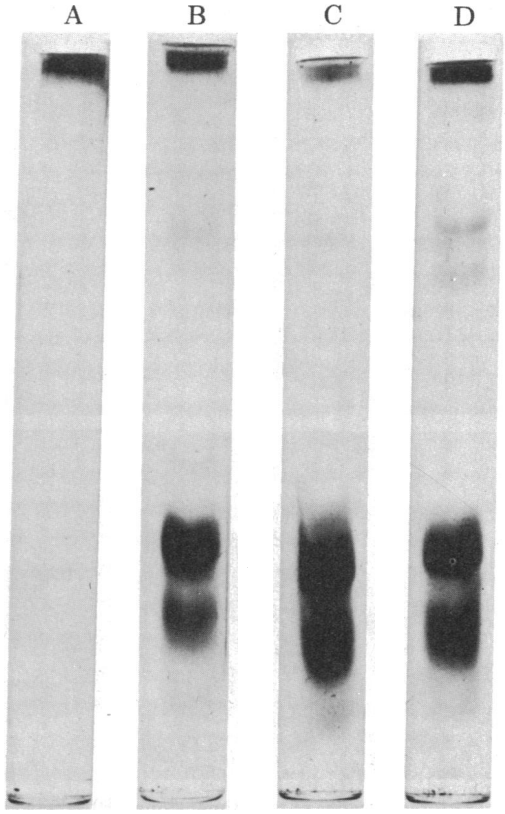

FIG. 2. Immunoisolation of plasma VIII:Ag on unreduced $NaDodSO_4$/polyacrylamide gels. Lanes: A, chromatographically purified normal plasma VIII:Ag marker; B, immunoisolated type 1 von Willebrand disease; C, immunoisolated type 2A von Willebrand disease; D, immunoisolated normal plasma VIII:Ag.

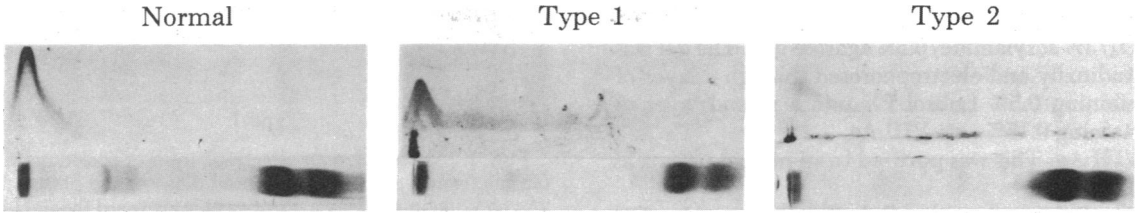

FIG. 3. Two-dimensional immunoelectrophoresis of VIII:Ag immunoprecipitates from normal plasma, type 1 von Willebrand disease plasma, and type 2A von Willebrand disease plasma. First dimension (horizontal) was NaDodSO$_4$/polyacrylamide gel electrophoresis of eluted immunoprecipitate (unreduced). Top of gel is toward the left. The gel was sliced in half lengthwise and one slice was stained in Coomassie blue. The second slice was subjected to second-dimension immunoelectrophoresis (vertically) through Lubrol agarose into agarose containing 0.15% anti-VIII:Ag.

mine whether the other minor contaminants were related immunologically to the VIII:Ag system, two-dimensional immunoelectrophoresis of the normal and von Willebrand Factor VIII immunoprecipitates was performed (Fig. 3). Only the high molecular weight band comigrating with the purified plasma VIII:Ag marker clearly reacted with the monospecific anti-VIII:Ag. The type 2A von Willebrand disease VIII:Ag reacted weakly in this system and appeared to spread out over a broader area near the top of the gel; however, no smaller VIII:Ag molecules were detected in the gel. No immunoprecipitate was detected when fibronectin was analyzed in the same system.

Two-Dimensional Tryptic Peptic Maps of Normal and von Willebrand VIII:Ag. In order to determine the structural relationship among the several VIII:Ag preparations, the fixed and stained reduced proteins in the NaDodSO$_4$ gels were removed, radioiodinated, and subjected to tryptic digestion. Autoradiographs of representative two-dimensional peptide maps are shown in Fig. 4. The peptide maps of VIII:Ag immunoisolated from normal pooled plasma, type 1 von Willebrand disease plasma, and type 2A von Willebrand disease plasma were remarkably similar. No unique normal VIII:Ag or von Willebrand VIII:Ag peptides were detected. All of the peptide spots present in normal VIII:Ag were present in type 1 and type 2A von Willebrand VIII:Ag, although the relative density of some of the peptide spots varied. The cause of the variability in the extent of labeling of different peptides in separate peptide map studies of the different proteins was not determined. A similar variability of density of labeled peptides with the Elder technique (16) has been noted with other proteins such as erythrocyte spectrin (21). All of the peptides present in the type 1 protein were identified in the type 2A protein. Peptide maps of VIII:Ag in the different patients with type 1 von Willebrand disease were identical. Peptide maps of equal mixtures of normal and von Willebrand plasma VIII:Ag as well as mixtures of type 1 and type 2 von Willebrand VIII:Ag revealed overlapping peptide patterns. The peptide maps of

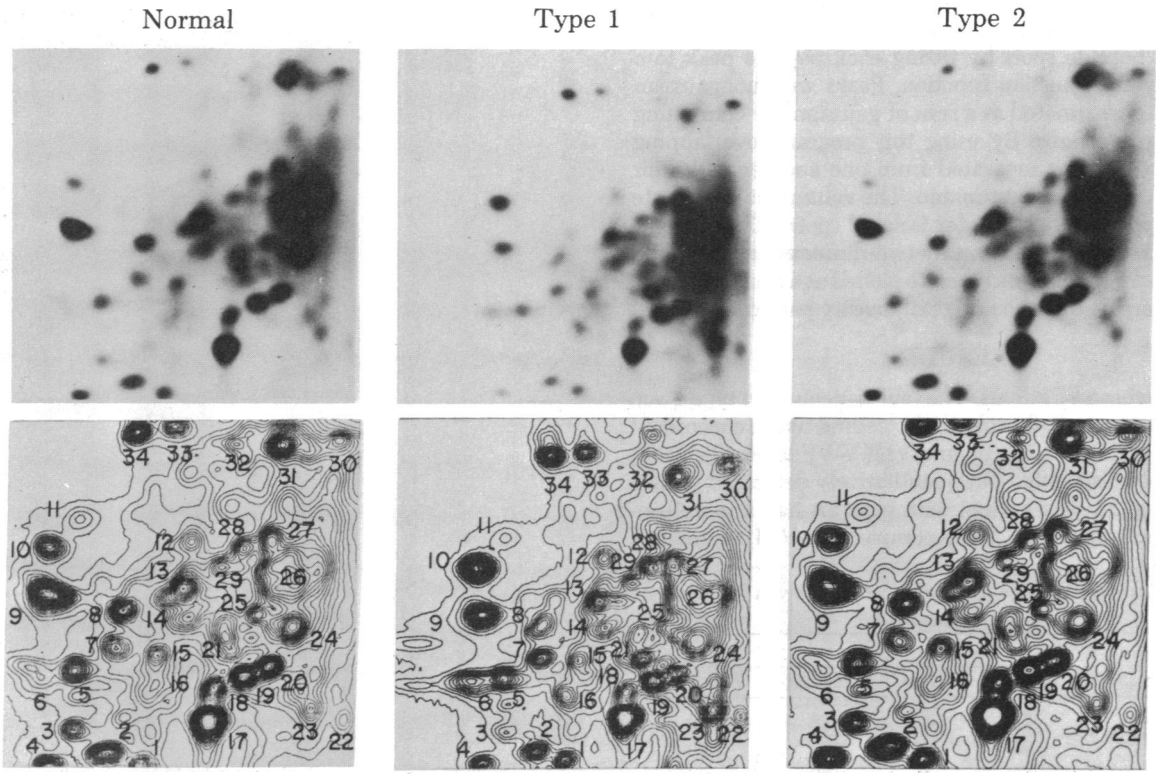

FIG. 4. Autoradiographs of two-dimensional ^{125}I-labeled tryptic peptide maps of immunoisolated VIII:Ag from normal plasma, type 1 von Willebrand disease plasma, and type 2 von Willebrand disease plasma. The tryptic peptides were applied at the lower right and subjected to high-voltage electrophoresis in the horizontal direction (anode on the right). Separation in the second dimension was by thin-layer chromatography (origin at the bottom). The right and bottom margins of the radiographs have been cropped for clarity, removing the streaked regions. Below each radioautograph is shown the corresponding computer-plotted density contour map. The peptides have been arbitrarily numbered to facilitate comparison.

chromatographically purified normal plasma VIII:Ag and immunoisolated plasma VIII:Ag were identical.

DISCUSSION

The proteins analyzed in these studies were quite large in the reduced state, with molecular weights of ≈200,000. It is thus possible that a single amino acid substitution could have been missed by this technique, particularly if present in a relatively lightly labeled peptide. The possibility has also to be considered that a mutant nontyrosine, and therefore unlabeled, peptide would be undetected by this method. Despite these reservations the remarkable structural similarity of the isolated proteins from these five separate, unrelated patients raises the strong possibility that the plasma VIII:Ag molecules in type 1 von Willebrand disease and type 2A von Willebrand disease are identical with normal plasma VIII:Ag. The observations suggest that von Willebrand disease is primarily a disorder of quantitative rather than qualitative aberrations in circulating VIII:Ag molecules.

It is not surprising that VIII:Ag in the plasma of patients with type 1 von Willebrand disease is identical to normal VIII:Ag. This might be predicted from the crossed immunoelectrophoretic pattern (Fig. 1) where the abnormality appears to be primarily quantitative in nature—i.e., a normal distribution of lesser amounts of the multiple molecular forms. The apparent identity of the VIII:Ag in variant von Willebrand disease plasma with normal VIII:Ag is further substantiated by the recent studies demonstrating that the abnormality in von Willebrand disease reflects an abnormal distribution of polymeric forms (10, 22). The distribution of the VIII:Ag polymers is slightly different in the two subtypes of variant von Willebrand disease (types 2A and 2B).

It is possible that, when larger numbers of patients are studied, specific examples of discrete protein molecular abnormalities may be uncovered. Patients with type 2B variant von Willebrand disease and an enhanced ristocetin response may be a particularly informative group to analyze for structural abnormalities (10). With highly sensitive immunologic techniques it is now possible to detect small amounts of VIII:Ag in the severe recessive form of von Willebrand disease (7). This heterogeneous group well may contain subsets of patients with amino acid-substituted VIII:Ag molecules.

Our observations on the apparent identical structure of VIII:Ag in two different types of von Willebrand disease raise questions regarding the normal physiological relationships within the Factor VIII system. VIII:Ag in normal plasma is heterogeneous and exists as a polydisperse series of molecular forms of different molecular weights (22–25). The basis for this molecular heterogeneity is not fully understood. The molecular dispersity may represent polymeric subunit combinations (25). Ekert and Chavin (26) suggested that VIII:Ag molecules exist in polymeric forms that spontaneously dissociate and exist in equilibrium with a pool of partially dissociated subunits. The fact that the low and high molecular weight forms appear to be indistinguishable on the basis of primary structure as illustrated in our studies strengthens the possibility that these molecular forms are composed of different sized multimers of identical subunits. The multimeric composition of the VIII:Ag present in type 2A and type 2B variant von Willebrand disease supports this premise (10).

Transfusion experiments using radiolabeled VIII:Ag in human volunteers demonstrate that the higher molecular weight forms disappear faster (27). These observations raise the intriguing possibility that there may be a continuous conversion of the higher molecular weight forms (possessing von Willebrand factor activity) into lower molecular forms (lacking von Willebrand activity) (28). Similar observations after transfusion in patients with von Willebrand disease suggest *in vivo* conversion of high molecular weight VIII:Ag into lower molecular weight VIII:Ag (9).

The mechanisms that regulate the conversion of slower moving, higher molecular weight forms of VIII:Ag into faster moving, lower molecular weight forms are not understood at present. The quantitative character of the abnormalities in these two common types of von Willebrand disease suggests that the altered synthetic or metabolic rates as well as compartmental shifts of normal population subsets of molecules might be the basis for the clinical disorder. A working hypothesis for these potential relationships is shown in Fig. 5. Under normal circumstances, endothelial cells synthesize and release into the plasma a polydisperse population of VIII:Ag molecules which contains a significant percentage of higher molecular weight forms (unpublished data). VIII:Ag_H (cathodal high molecular weight forms) are converted in the circulation to VIII:Ag_L (anodal low molecular forms) (27). The hypothesis allows for the possible physiologic recombination of VIII:Ag_L to form VIII:Ag_H. The resulting polydisperse population is then catabolized.

In type 1 von Willebrand disease there is primarily an altered synthetic or metabolic rate such that there are low levels of circulating plasma VIII:Ag molecules. However, the conversion rate of high molecular weight forms is normal and thus the molecules that are present are normally polydispersed. In type 2A von Willebrand disease the primary abnormality could be reflected in an accelerated conversion rate of VIII:Ag_H to VIII:Ag_L or there might be a direct defect in endothelial synthesis leading only to VIII:Ag_L release. It is of interest that there appears to be an absolute increase in the smaller molecular weight multimers in the plasma of patients with type 2A variant von Willebrand disease (10, 22). This is compatible with an accelerated rate of conversion of high molecular weight forms to low molecular weight forms. In addition, the intraplatelet VIII:Ag in patients with type 2A von Willebrand disease has greater amounts of the larger molecular weight multimers compared to the plasma. This suggests that intracellular sequestration may protect the multimeric VIII:Ag complex from rapid conversion of high molecular weight forms to low molecular weight forms.

This hypothetical model is testable by using radiolabeled

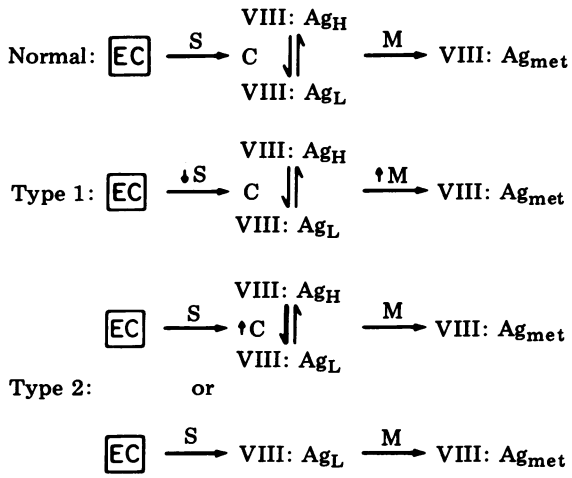

FIG. 5. Hypothesis for relationship of VIII:Ag forms in normal and in von Willebrand disease. EC, endothelial cell: VIII:Ag_H, cathodal high molecular weight forms; VIII:Ag_L, anodal low molecular weight forms; VIII:Ag_{MET}, metabolized products; S, synthesis rate; C, conversion rate; M, catabolic rate.

fractions of VIII:Ag, including high and low molecular weight forms, for transfusion into patients with various types of von Willebrand disease. Synthetic studies using cultures of venous endothelium from different patients with von Willebrand disease would also add important information.

The observation that the VIII:Ag molecules are probably identical in these two forms of von Willebrand disease imposes significant restraints on any future theoretical models used to explain these puzzling hemostatic disorders. It is obvious that more needs to be learned regarding the factors that control the assembly of the identical subunits into VIII:Ag molecular families.

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