The Defect in Hemophilic and von Willebrand's Disease Plasmas Studied by a Recombination Technique

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ABSTRACT Factor VIII in preparations from normal plasma is a large glycoprotein of greater than 2 million molecular weight which elutes in the exclusion volume of 4% agarose gels at an ionic strength of 0.15. Recent studies have demonstrated that the factor VIII in canine and bovine plasma is a macromolecular complex composed of a large inert carrier protein and a noncovalently bound small fragment which contains the procoagulant active site. This complex is dissociable in 0.25 M $CaCl_2$, and conditions for its recombination have been reported. The present study reports the dissociation characteristics of normal human factor VIII preparations in 0.25 M $CaCl_2$ and the ability to achieve quantitative recombination of the dissociated fragments of normal human and bovine factor VIII after the removal of Ca^{2+}. The recombination technique was used to characterize further the defect in hemophilia and von Willebrand's disease. Void volume preparations from human hemophilia A^-, canine hemophilia A, and human von Willebrand's plasma, with no factor VIII procoagulant activity, were mixed with the small active fragment prepared from the normal plasma of their respective species. Chromatography of the three mixtures in agarose gel showed that the fractions from the human hemophilic plasmas contained a molecule that bound the small active normal fragment, but neither the fractions from canine hemophilia A plasmas nor the fractions from the human von Willebrand's plasmas demonstrated evidence of such material. These data suggest that there is present in human hemophilia A plasma a normal functional carrier molecule which is absent or nonfunctional in the plasma of hemophilic dogs and humans with von Willebrand's disease.

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INTRODUCTION

Factor VIII (antihemophilic factor, AHF)[1] is a trace glycoprotein (1) which in plasma has a high temperature coefficient of solubility (2). Previous studies have indicated an apparent molecular weight of 2.0×10^6 by agarose gel chromatography (3) and 1.12×10^6 by sedimentation equilibrium (4). We have recently shown, by dissociation in the presence of 0.25 M calcium chloride, that normal canine and bovine factor VIII are large macromolecular complexes composed of at least two different molecules (5, 6). One is a large inactive carrier protein with gel chromatographic behavior indistinguishable from plasma factor VIII, and the other is a small fragment with factor VIII procoagulant activity. The active canine fragment has an apparent molecular weight of 100,000 and, in the absence of calcium ions, has been recombined with the large carrier molecule to give an active product with chromatographic behavior similar to that observed for canine factor VIII (7).

A rabbit antibody to human factor VIII detects factor VIII-like antigen in most or all individuals with hemophilia A (8–10). This antigen is deficient in most patients with von Willebrand's disease, although recent reports suggest that human plasmas from some variants of von Willebrand's disease do possess the antigen in normal amounts (11, 12). With the use of the rabbit antibody, the nonfunctional high molecular weight carrier molecule can be identified by immunoprecipitation and radioimmunoassay whereas the small active fragment possessing factor VIII activity cannot (13). Although the antibody to factor VIII raised in rabbits gives no precipitin reaction with the small active factor VIII fragment, it still neutralizes its factor VIII activity.

Human antibodies which occur in the plasmas of some hemophiliacs after transfusion therapy have also been shown to neutralize factor VIII activity, but they do not form a detectable immunoprecipitate with plasma or fac-

[1] *Abbreviation used in this paper:* AHF, antihemophilic factor.

tor VIII preparations. In addition these antibodies have been used to study cross-reacting material in hemophilic plasmas by using an inhibitor neutralization technique. Those plasmas which resulted in neutralization of the inhibitor were called "hemophilia A^+" while those which did not were called "hemophilia A^-." Both naturally occurring and acquired factor VIII inhibitors in the human have been shown to neutralize the procoagulant activity of the small factor VIII fragment (5, 13). The high molecular weight material present after dissociation in high molar salt also retains the ability to neutralize an acquired factor VIII human inhibitor (13).

Studies with rabbit antibodies raised to canine factor VIII preparations have failed to demonstrate antibody neutralizing ability in the plasma of dogs with severe classical hemophilia (14). Comparable studies in dogs with von Willebrand's disease have not been reported.

This paper reports the dissociation of human factor VIII preparations and the conditions required for recombination of both human and bovine preparations. The recombination technique is used to demonstrate the presence of a functional carrier molecule in human hemophilia A and the apparent absence of a functional carrier molecule in human von Willebrand's disease plasma. No evidence was found for the presence of a functional carrier protein in the plasma from the Chapel Hill strain of canine hemophilia.

METHODS

Chemicals. Chemicals were reagent grade unless otherwise specified. Water was deionized and then glass distilled. All blood was collected in silicone-treated glassware or plastic. Silicone (Siliclad, Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.) treatment of all glassware, including columns, was performed according to the manufacturer's instructions.

Blood collection. Canine normal and factor VIII-deficient plasmas were collected as previously described (7). Bovine blood was collected from adult cows at the time of slaughter by using heparin (Sodium Heparin, Eli Lilly and Company, Indianapolis, Ind.) and benzamidine (Benzamidine·HCl, Aldrich Chemical Co., Inc., Milwaukee, Wis.) (15). A small amount of bovine blood was collected into 1/8 vol of 0.11 M trisodium citrate to obtain plasma free of heparin for use in establishing a standard curve for the factor VIII assay. Human plasma was obtained by plasmapheresis using plastic bags containing 4% sodium citrate (PC-210, Fenwal Inc., Walter Kidde & Co., Inc., Bronx, N. Y.).

Buffers. Tris-buffered saline was 0.05 M Tris·HCl-0.15 M NaCl (pH 6.8). A Tris-calcium buffer was used routinely for calcium dissociation and contained 0.05 M Tris·HCl-0.01 M NaCl-0.25 M $CaCl_2$ (pH 6.8). All buffers were freshly prepared for each gel chromatographic experiment, filtered through Millipore membrane filters, degassed under reduced pressure, and equilibrated to the appropriate temperature.

Preparations from normal and pathologic plasmas. Factor VIII from normal human and normal animal plasmas was prepared by several fold concentration of fresh plasma using a hollow fiber concentrator (model DC-2, Amicon Corp., Lexington, Mass.), incubation at 0°C for 1 h, and collection of the heavy phase by centrifugation. Gel chromatography (Bio Gel A-15m, 200–400 mesh, 4% agarose beads, Bio-Rad Laboratories, Richmond, Calif.) is the final procedure, with the 3,000–5,000 times purified factor VIII appearing in the exclusion volume (16). In the case of normal plasma, the peak fractions containing factor VIII activity were pooled and 30 g dry polyethylene glycol (mol wt 3,700, Matheson, Coleman, and Bell, Cincinnati, Ohio) was added per 100 ml of solution and dissolved. After 1 h chilling at 0°C, the precipitate was collected by centrifugation at 10,000 *g* for 20 min at 4°C. The supernate was decanted, and the precipitate was quick frozen and stored at −20°C.

The same procedures were also used to process plasmas from hemophilic dogs (17) and from human donors with severe hemophilia A^-[2] and von Willebrand's disease; none of the fractions eluted in the chromatographic procedure contained factor VIII activity. Those fractions containing protein in or near the void volume were pooled and precipitated with 30% polyethylene glycol. The precipitates were quick frozen and stored at −20°C.

Factor VIII assays. A modification (18) of the partial thromboplastin time assay (19) was used. Hemophilic substrate is activated with kaolin. Equal volumes (0.1 ml) of activated hemophilic substrate, dilute partial thromboplastin (Thrombofax, Ortho Diagnostics, Raritan, N. J.), and test material are mixed and 0.1 ml of 0.044 M $CaCl_2$ is added. The clotting time is determined as the time from the addition of calcium to the formation of the first visible fibrin strands. Standard curves are constructed using dilutions of normal plasma for each species. "1 unit" of human, canine, or bovine factor VIII is defined as that amount present in 1 ml of normal human, canine, or bovine plasma, respectively. Assay of fractions eluted in 0.25 M Ca^{2+} were performed according to the technique described earlier (5).

Dissociation of normal human factor VIII preparations. Polyethylene glycol precipitates of human factor VIII preparations were reconstituted with Tris-calcium buffer and chromatographed at 4°C on 2.5 × 35-cm 4% agarose columns equilibrated with Tris-calcium buffer. 3-ml fractions were collected and after elution of the protein peak, the fractions were collected into tubes containing 15 mg crystalline bovine albumin (Pentex, Miles Laboratories, Inc., Elkhart, Ind.) to give a 0.5% albumin solution. The albumin was added to enhance stability and to serve as a coprecipitating agent in the polyethylene glycol step. All fractions were assayed for factor VIII activity. Void volume fractions containing carrier protein but little factor VIII activity and the later fractions containing the small active factor VIII fragment were pooled separately and dry polyethylene glycol was added (30 g/100 ml of solution). The precipitates were collected and stored as described above. Dissociation of bovine and canine factor VIII preparations were similarly carried out, and the carrier protein and the small active factor VIII fragment were stored individually at −20°C. The precipitates were reconstituted in Tris-buffered saline at 23°C before use.

Recombination of normal human or bovine carrier protein with its respective small active fragment. By means of a semiquantitative test for Ca^{2+} ions, precipitation with 0.1 M sodium oxalate, it was determined that polyethylene glycol

[2] E. Barrow. Personal communication.

precipitation of the factor VIII components after $CaCl_2$ dissociation effectively removed more than 99% of the calcium. The reconstituted precipitates were therefore sufficiently lacking in calcium to be assayed by the routine partial thromboplastin time assay, and dialysis was unnecessary to achieve quantitative recombination of components when mixed. The mixtures of normal human and bovine carrier molecules with their respective small active factor VIII fragments were chromatographed on 4% agarose. Eluates were monitored at 280 nm by an LKB Uvicord ultraviolet analyzer and recorder (LKB Instruments, Inc., Rockville, Md.). 1-ml fractions were collected and assayed for factor VIII activity.

Combination of normal small active factor VIII fragment with the void volume fractions from hemophilic and von Willebrand's disease plasmas. The normal canine small active fragment and the void volume fractions from canine hemophilic preparations were reconstituted and mixed. In similar fashion the void volume fractions from human hemophilic and von Willebrand's disease plasmas were reconstituted and mixed with the human small active factor VIII fragment from normal plasma. These three types of mixtures were each studied by agarose gel chromatography with the procedure described above for normal human and bovine recombination.

Immunodiffusion. A fluid phase antigen-antibody system was used to test for factor VIII-like antigen in normal human plasma and in plasma from individuals with hemophilia A^- and von Willebrand's disease. This procedure made use of a rabbit antibody to human factor VIII (Nordic Pharmaceuticals and Diagnostics, Tilburg, The Netherlands). Clean glass plates were precoated with 0.3% agarose (Seravac Agarose, Gallard Schlessinger Chemical Mfg. Corp., Carle Place, N. Y.) in distilled water and allowed to dry overnight. A mold was constructed by clamping a $\frac{1}{16}$-inch C-shaped plastic spacer between a precoated and an uncoated plate. The mold was filled with 1% agarose in Tris-buffered saline and allowed to cool at 4°C for 30 min before the removal of the uncoated plate. The coated plate was stored in a humidity chamber until used. 10-μl wells were singly filled, and diffusion was allowed to continue for 5 days at 23°C before photography with an immunodiffusion camera (Cordis Laboratories, Miami, Fla.).

RESULTS

Dissociation of normal human factor VIII preparations by $CaCl_2$. Chromatography on 4% agarose of human factor VIII preparations in the presence of Tris-buffered saline demonstrated the active molecule to be of large size and present at or near the void volume. Fig. 1 shows the results of chromatography of this same material in the presence of 0.25 M Ca^{2+}. The factor VIII activity is well separated from the void volume, of small molecular size, and associated with minimal protein. The void volume fractions on the other hand, still contain the bulk of the protein but the peak fraction had less than 0.5 units/ml of factor VIII activity. These results are similar to those previously reported for canine and bovine factor VIII (5, 6).

Recombination experiments. The carrier proteins from normal bovine preparations after dissociation with 0.25 M Ca^{2+} were mixed with the small active bovine

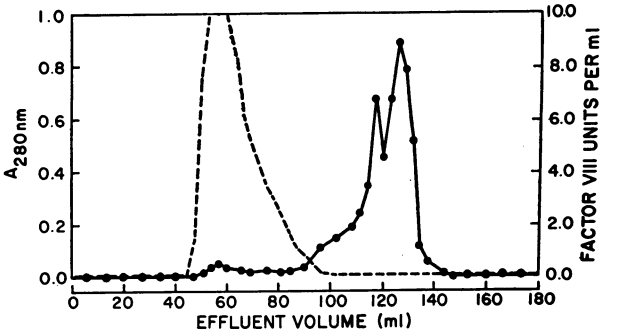

FIGURE 1 Chromatography of a human plasma factor VIII preparation on 4% agarose, illustrating dissociation at 4°C in 0.25 M $CaCl_2$, pH 6.8. A 10-ml sample (215 units of factor VIII) was applied to column. Bed dimensions were 2.5 × 33 cm, flow rate 20 ml/h (4.1·cm^{-2}·h^{-1}). 3.0-ml fractions were collected. Factor VIII concentration (●——●); A_{280} (- - -).

factor VIII fragment and chromatographed on 4% agarose (Fig. 2). Similar experiments were performed with the analogous human factor VIII fragments. In both species the small active factor VIII fragment recombined with the carrier protein, and the peak of the factor VIII activity was again found in the exclusion volume of the column. The second protein peak represents the added albumin. The recovery of factor VIII activity was variable with the greatest losses occurring during attempts to achieve recombination of the normal canine small active fragment with the hemophilic canine void volume preparations.

It may be seen that the added bovine crystalline albumin did not interfere with the recombination. As a

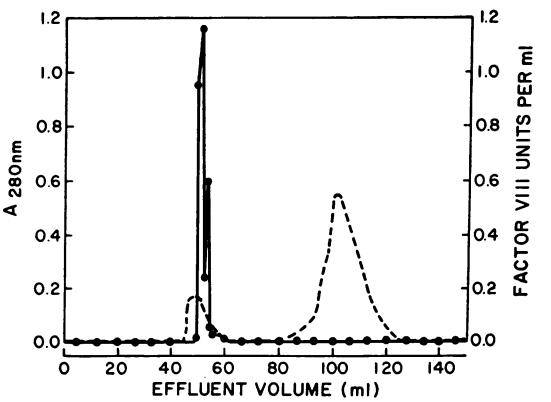

FIGURE 2 Recombination of normal bovine fractions at 23°C. 3 ml of a Ca^{2+}-free mixture of equal volumes of normal bovine carrier molecule and a fraction containing the normal bovine small active fragment (25 units of factor VIII) were applied to a 4% agarose column. Bed dimensions were 2.5 × 24 cm, flow rate 20 ml/h (4.1 ml·cm^{-2}·h^{-1}), eluant Tris-buffered saline pH 6.8. 3.0-ml fractions were collected. Factor VIII concentration (●——●); A_{280} (- - -).

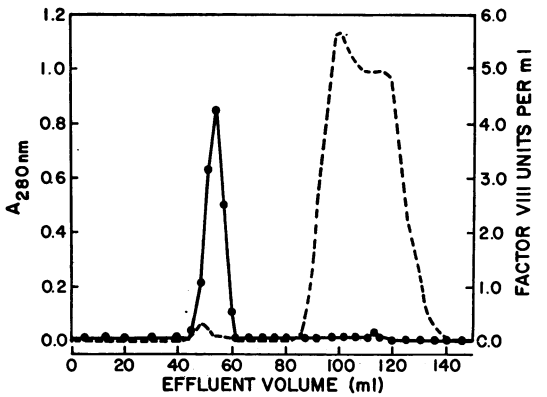

FIGURE 3 Combination of human hemophilia A⁻ carrier protein fraction and normal human small active fragment. A sample (4.8 ml) of a Ca^{2+}-free mixture of equal volumes of a hemophilia A⁻ carrier protein fraction and a fraction containing the normal human small active fragment (44 units of factor VIII) were applied to a 4% agarose column at 23°C. Bed dimensions, flow rate, eluant, fraction volumes as per Fig. 2. Factor VIII concentration (●——●); A_{280} (- - -).

control, the small active fragments from both human and canine factor VIII fractions were collected in 0.5% bovine crystalline albumin, precipitated, and chromatographed separately as above. No factor VIII activity was found in void volume fractions; therefore the albumin was not responsible for the appearance of factor VIII in the void volume in the recombination experiments.

Undissociated human hemophilia A⁻ void volume fractions were mixed with the small active factor VIII fragment from normal human plasma and subjected to chromatography on 4% agarose. When the eluted

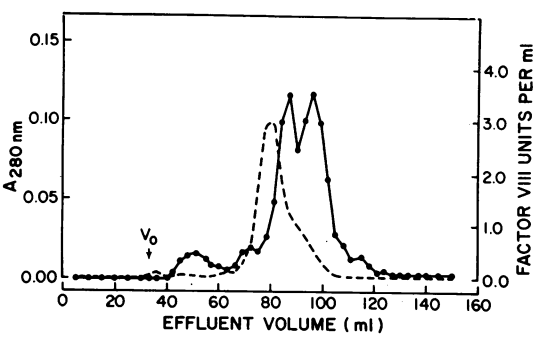

FIGURE 4 Chromatography on 4% agarose of a mixture of normal human small active fragment and a human von Willebrand's disease plasma fraction prepared analogously to normal factor VIII. A 3-ml sample of equal volumes of the von Willebrand's disease fraction and the normal human small active fragment (42 units of factor VIII) were applied to the column at 23°C. Bed dimensions 2.5 × 20 cm, flow rate 20 ml/h (4.1 $ml \cdot cm^{-2} \cdot h^{-1}$), eluant Tris-buffered saline pH 6.8. 3-ml fractions were collected. Factor VIII concentration (●——●); A_{280} (- - -).

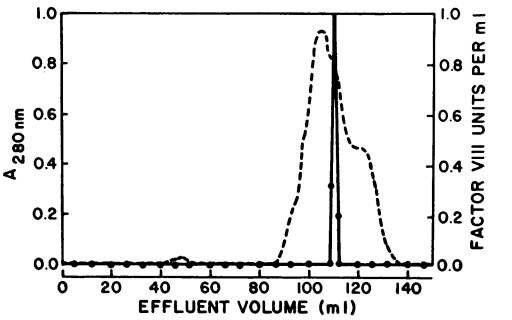

FIGURE 5 Chromatography on 4% agarose of a mixture of normal canine small active fragment and an undissociated canine hemophilic plasma fraction prepared analogously to normal factor VIII. A 2-ml sample (45 units factor VIII) was applied to the column at 23°C. Bed dimensions, flow rate, eluant, and fraction volumes as per Fig. 2. Factor VIII concentration (●——●); A_{280} (- - -).

fractions were assayed, the factor VIII activity was again quantitatively recovered in the void volume (Fig. 3).

Undissociated human von Willebrand's disease void volume fractions were then studied for their ability to bind the small active fragment from normal human plasma by using the same procedure described above. In contrast to the results obtained by using hemophilia A void volume fractions, the preparations from von Willebrand's disease plasma when mixed with the small active factor VIII fragment failed to show any activity in the void volume fractions after gel chromatography (Fig. 4). A small peak of activity preceded the bulk of the factor VIII activity which eluted as a late peak well separated from the void volume fractions.

Canine hemophilic void volume fractions were mixed with the small active fragment from normal canine

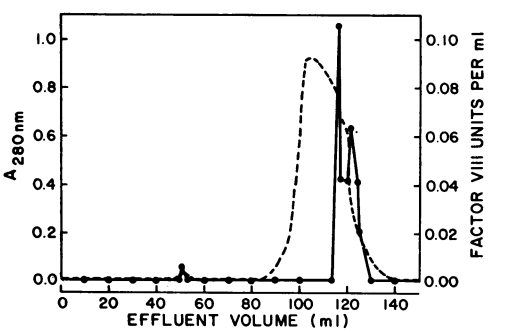

FIGURE 6 Chromatography on 4% agarose of a mixture of normal canine small active fragment and a canine hemophilic carrier protein fraction prepared analogously to the normal carrier fractions with 0.25 $CaCl_2$. A 2-ml sample (48 units factor VIII) was applied to the column at 23°C. Bed dimensions, flow rate, eluant, and fraction volumes as per Fig. 2. Factor VIII concentration (●——●); A_{280} (- - -).

plasma and immediately chromatographed on 4% agarose. The results are shown in Fig. 5. The small active factor VIII fragment did not bind with any large molecular weight protein in the void volume and continued to chromatograph as a small molecular weight entity. To rule out the possibility that the hemophilic protein failed to combine because the binding sites were already filled with abnormal inactive small fragments, the canine hemophilic void volume fractions were dissociated in 0.25 M $CaCl_2$. No factor VIII activity was found in any of the eluted fractions. The dissociated void volume protein was pooled, concentrated, and then mixed with the small active factor VIII fragment from normal canine plasma. The results of chromatography of this mixture on 4% agarose are shown in Fig. 6. The small active factor VIII fragment was not bound by the protein in the void volume fractions of the dissociated preparation.

Detection of factor VIII-like antigen. The human plasmas used in this study from normal, hemophilia A^-, and von Willebrand's donors were studied by immunodiffusion against an antibody prepared in rabbits to human factor VIII. An immunoprecipitin line formed between the rabbit anti-human factor VIII antibody and the normal and hemophilia A^- plasma but not between the antibody and the von Willebrand's disease plasma. Immunodiffusion of normal and hemophilic plasmas still gave a precipitin line with a 1 to 10 dilution of plasma.

Many of the experiments described were performed 10–15 times and all at least three times. Results presented are of typical experiments.

DISCUSSION

Normal human factor VIII, previously shown to undergo partial dissociation in 1 M NaCl (20), was found in these studies to achieve almost complete dissociation in 0.25 M Ca^{2+}, a result similar to that reported for canine (5) and bovine (6) factor VIII (Fig. 1). The calcium dissociated fragments of either normal human or bovine factor VIII were able to recombine, in the absence of calcium, (Fig. 2) producing a macromolecule in the same manner as previously reported for the dissociated fragments of canine factor VIII (7). Therefore, in the three species studied to date, the large molecular protein component of factor VIII has been shown to function as a carrier of the small fragment possessing the biologically active site. Other investigators (4, 15, 21–23) studying reduced human and bovine factor VIII preparations with sodium dodecyl sulfate polyacrylamide gel electrophoresis have shown that the factor VIII molecule is a polymer of repeating subunits of similar size linked together by sulfhydryl bonds. This model is consistent with a model of a small active fragment linked by electrostatic and hydrophobic bonds to a carrier molecule which is comprised of repeating subunits of similar size linked by sulfhydryl bonds.

Antibodies made to well characterized antigens have been used as probes for the identification of molecules that possess similar antigenic sites. This even applies to defective molecules whose alteration has caused loss of biological function but retention of antigenic characteristics. With the use of homologous antibodies to human factor VIII from patients with hemophilia A, in fluid phase, it has been shown that from 10 to 20% of individuals with classic hemophilia possess a material which, although lacking factor VIII procoagulant activity, is able to neutralize the antibody (24, 25). Thus, the defective material retains antigenic determinants to this specific antibody. These data were interpreted initially to suggest that 80–90% of hemophilic plasmas are deficient in factor VIII (hemophilia A^-) while 10–20% possess a defective factor VIII molecule (hemophilia A^+). In earlier studies, using similar techniques to study canine hemophilic plasma, McLester and Wagner were unable to show the presence of antibody neutralizing material (14).

Heterologous antibodies to factor VIII, after absorption to achieve apparent monospecificity, have been demonstrated to have factor VIII neutralizing ability and an ability to form immunoprecipitates with factor VIII preparations. This information has led to a large number of reports in which heterologous antibody, usually raised in rabbits, has been used as a biological probe to further characterize factor VIII (26–30). Results of these investigations suggest that in all or most individuals with hemophilia A a molecule is present in their plasmas with antigenic determinants for the heterologous antibody. Thus, despite the fact that these plasmas possess little or no measurable factor VIII activity, they do possess a factor VIII-like antigen in concentrations similar to those found in normal plasma. These data have been generally accepted as showing that the defect in human hemophilia A is the result of synthesis of a defective factor VIII having antigenic determinants in common with the normal molecule, but very low in factor VIII activity. With the use of this heterologous antibody, it has been shown that the specific antigenic determinants as well as factor VIII activity are missing or greatly reduced in plasma from patients with severe von Willebrand's disease. These associated findings have led to the presumption that these specific antigenic determinants are on the "von Willebrand factor" molecule or are closely associated with it. These specific antigenic determinants have been referred to as "AHF-like antigen," "($VIII_{AGN}$)," and "von Willebrand antigen." Recent use of heterologous antibodies with crossed immunoelectrophoresis (31, 32), radioimmunoassays (13), and solid phase immunochemical techniques (33) has produced data that are consistent with our earlier reports that factor VIII is composed of more than one

molecule. It should be noted that the normal and hemophilia A^- human plasmas used in our study, but not the von Willebrand's disease plasma, were shown to contain the factor VIII-like antigen.

The technique employing recombination of the small active factor VIII fragment with the void volume fractions of pathologic plasmas gives further insight into the characteristics of these deficient plasmas. This technique extends the information concerning the presence or absence of a molecule as well as some idea of its functional capabilities. If a small molecule is produced in hemophilia A^-, analogous to the normal small active fragment, it lacks biological activity and ability to neutralize a naturally occurring human inhibitor. Furthermore, if it is produced, it is either not tightly bound, or some of the binding sites of the carrier molecule in hemophilia A^- are unoccupied, as recombination of the carrier molecule with added normal small active fragment is possible (Fig. 3). Thus, the defect in human hemophilia A^- could be not the synthesis of a defective factor VIII molecule as suggested by the immunochemical data but rather a defective or absent small factor VIII fragment and the production of a functionally normal carrier protein. Similarly, plasma of patients with hemophilia A^+ could contain normal carrier molecules with the binding sites for the small fragment occupied by defective molecules which have low factor VIII activity but retain the ability to neutralize the naturally occurring human inhibitor. The production of normal carrier protein may explain why all hemophilia A plasmas demonstrate AHF-like antigen with the heterologous rabbit antibody.

Canine hemophilia A although indistinguishable by all other modes of comparison with human hemophilia A (34) is different by the recombination technique. In the Chapel Hill strain of canine hemophilia there appears to be an absence of a material that can function as a carrier molecule (Fig. 5). This could mean that there is deficient synthesis of the carrier, that the carrier is abnormal and has lost its ability to bind the small factor VIII fragment from normal plasma, or that the carrier is saturated with a defective small fragment. To test the latter possibility, void volume preparations of hemophilic plasma were subjected to dissociating conditions of 0.25 M Ca^{2+}. The exclusion volume fractions following agarose gel chromatography were then combined with the normal small active factor VIII fragment from normal canine plasma preparations. No return of activity to the void volume following agarose chromatography of the mixture occurred (Fig. 6).

Unless we were unable to remove a defective small fragment because it was too tightly bound to the carrier molecule, this experiment suggests that saturated carrier is probably not an explanation for the failure of recombination experiments in this particular strain of canine hemophilia. Further explanation must await testing of other canine hemophilia strains and additional immunochemical data on canine hemophilia.

The combination studies with von Willebrand's disease preparations show the lack of both a functional carrier molecule and a molecule with a normal active site. However, we are not able to conclude from existing data that these individuals completely fail to synthesize any carrier related protein. It is possible that this disease represents a nonsense coding and that a molecule of unknown size is produced, which lacks the antigenic sites to the rabbit antibody and is also incapable of binding the small active factor VIII fragment from normal plasma. Humans with von Willebrand's disease, unlike humans with hemophilia A, can synthesize or release factor VIII only when preparations containing factor VIII-related antigen (35), and thus presumably the carrier protein, are administered. Although these data are not complete they suggest that the carrier molecule may be the material which stimulates the appearance of factor VIII activity in these patients after transfusion. Further transfusion studies must be done to confirm these preliminary observations.

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REFERENCES

1. Kass, L., O. D. Ratnoff, and M. A. Leon. 1969. Studies on the purification of antihemophilic factor (factor VIII). I. Precipitation of antihemophilic factor by concanavalin A. *J. Clin. Invest.* **48**: 351–358.
2. Brinkhous, K. M. 1954. Plasma antihemophilic factor biological and clinical aspects. *Sang.* **25**: 738–741.
3. Ratnoff, O. D., L. Kass, and P. D. Lang. 1969. Studies on the purification of antihemophilic factor (factor VIII). II. Separation of partially purified antihemophilic factor by gel filtration of plasma. *J. Clin. Invest.* **48**: 957–962.
4. Legaz, M. E., G. Schmer, R. B. Counts, and E. W. Davie. 1973. Isolation and characterization of human factor VIII (antihemophilic factor). *J. Biol. Chem.* **248**: 3946–3955.
5. Owen, W. G., and R. H. Wagner. 1972. Antihemophilic factor: separation of an active fragment following dissociation by salts or detergents. *Thromb. Diath. Haemorrh.* **27**: 502–515.
6. Griggs, T. R., H. A. Cooper, W. P. Webster, R. H. Wagner, and K. M. Brinkhous. 1973. Plasma aggregating factor (bovine) for human platelets: a marker for study of antihemophilic and von Willebrand factors. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 2814–2818.
7. Cooper, H. A., T. R. Griggs, and R. H. Wagner. 1973. Factor VIII recombination after dissociation by $CaCl_2$. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 2326–2329.

8. Zimmerman, T. S., O. D. Ratnoff, and A. E. Powell. 1971. Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand's disease. *J. Clin. Invest.* **50**: 244–254.
9. Hoyer, L. W. 1972. Immunologic studies of antihemophilic factor (AHF, factor VIII). III. Comparative binding properties of human and rabbit anti-AHF. *Blood J. Hematol.* **39**: 481–489.
10. Meyer, D., J. M. Lavergne, M. J. Larrieu, and F. Josso. 1972. Cross-reacting material in congenital factor VIII deficiencies (haemophilia A and von Willebrand's disease). *Thromb. Res.* **1**: 183–196.
11. Holmberg, L., and I. M. Nilsson. 1972. Genetic variants of von Willebrand's disease. *Br. Med. J.* **3**: 317–320.
12. Holmberg, L. and I. M. Nilsson. 1973. Two genetic variants of von Willebrand's disease. *N. Engl. J. Med.* **288**: 595–598.
13. Rick, M. E., and L. W. Hoyer. 1973. Immunologic studies of antihemophilic factor (AHF, factor VIII). V. Immunologic properties of AHF subunits produced by salt dissociation. *Blood J. Hematol.* **42**: 737–747.
14. McLester, W. D., and R. H. Wagner. 1965. Antibody to antihemophilic factor and its lack of reaction with hemophilic plasma. *Am. J. Physiol.* **208**: 499–507.
15. Schmer, G., E. P. Kirby, D. C. Teller, and E. W. Davie. 1972. The isolation and characterization of bovine factor VIII (antihemophilic factor). *J. Biol. Chem.* **247**: 2512–2521.
16. Owen, W. G., and R. H. Wagner. 1972. Antihemophilic factor. A new method for purification. *Thromb. Res.* **1**: 71–88.
17. Graham, J. B., J. A. Buckwalter, L. J. Hartley, and K. M. Brinkhous. 1949. Canine hemophilia: observations on the course, the clotting anomaly, and the effect of blood transfusions. *J. Exp. Med.* **90**: 97–111.
18. Margolis, J. 1958. The kaolin clotting time. A rapid one-stage method for diagnosis of coagulation defects. *J. Clin. Pathol. (Lond.).* **11**: 406–409.
19. Langdell, R. D., R. H. Wagner, and K. M. Brinkhous. 1953. Effect of antihemophilic factor on one-stage clotting tests. A presumptive test for hemophilia and a simple one-stage antihemophilic factor assay procedure. *J. Lab. Clin. Med.* **41**: 637–647.
20. Weiss, H. J., L. L. Phillips, and W. Rosner. 1972. Separation of sub-units of antihemophilic factor (AHF) by agarose gel chromatography. *Thromb. Diath. Haemorrh.* **27**: 212–219.
21. Marchesi, S. L., N. R. Shulman, and H. R. Gralnick. 1972. Studies on the purification and characterization of human factor VIII. *J. Clin. Invest.* **51**: 2151–2161.
22. Shapiro, G. A., J. C. Anderson, S. V. Pizzo, and P. A. McKee. 1973. The subunit structure of normal and hemophilic factor VIII. *J. Clin. Invest.* **52**: 2198–2210.
23. Bennett, B., W. B. Forman, and O. D. Ratnoff. 1973. Studies on the nature of antihemophilic factor (factor VIII). *J. Clin. Invest.* **52**: 2191–2197.
24. Denson, K. W. E., R. Biggs, M. E. Haddon, R. Borrett, and K. Cobb. 1969. Two types of hemophilia (A^+ and A^-): a study of 48 cases. *Br. J. Haematol.* **17**: 163–171.
25. Hoyer, L. W., and R. T. Breckenridge. 1968. Immunological studies of antihemophilic factor (AHF, factor VIII): cross-reacting material in a genetic variant of hemophilia A. *Blood J. Hematol.* **32**: 962–971.
26. Lechner, K. 1972. Inactive factor VIII in hemophilia A and Willebrand's disease. *Acta Haematol. (Basel)* **48**: 257–268.
27. Kernoff, P. B. A., and C. R. Rizza. 1973. The specificity of antibodies to factor VIII produced in the rabbit after immunization with human cryoprecipitate. *Thromb. Diath. Haemorrh.* **29**: 652–660.
28. Hoyer, L. W. 1973. Specificity of precipitating antibodies in immunological identification of antihaemophilic factor. *Nat. New Biol.* **245**: 49–51.
29. Gralnick, H. R., B. S. Coller, and S. L. Marchesi. 1973. Immunological studies of factor VIII in haemophilia and von Willebrand's disease. *Nat. New Biol.* **244**: 281–282.
30. Barrow, E. M., and J. B. Graham. 1974. Blood coagulation factor VIII (antihemophilic factor): with comments on von Willebrand's disease and Christmas disease. *Physiol. Rev.* **54**: 23–74.
31. Bouma, B. N., J. A. van Mourik, J. J. Sixma, S. de Graaf, and J. Hordijk. 1973. von Willebrand factor and factor VIII. *In* IV International Congress Thrombosis and Haemostasis. 249. (Abstr.)
32. Holmberg, L., and I. M. Nilsson. 1973. On the structure of the antihemophilic factor. *In* IV International Congress Thrombosis and Haemostasis. 255. (Abstr.)
33. Zimmerman, T. S., and T. S. Edgington. 1973. Factor VIII coagulant activity and factor VIII-like antigen: independent molecular entities. *J. Exp. Med.* **138**: 1015–1020.
34. Graham, J. B., and E. M. Barrow. 1957. The pathogenesis of hemophilia: an experimental analysis of the anticephalin hypothesis in hemophilic dogs. *J. Exp. Med.* **106**: 273–292.
35. Bennett, E. 1973. Abnormality of separate subunits of factor VIII? *Lancet.* **1**: 885–886.