Purified human factor VIII procoagulant protein: Comparative hemostatic response after infusions into hemophilic and von Willebrand disease dogs

(blood coagulation/antihemophilic factor/von Willebrand factor/animal models)

K. M. Brinkhous*, H. Sandberg†, J. B. Garris*, C. Mattsson†, M. Palm†, T. Griggs*, and M. S. Read*

*Department of Pathology and Center for Thrombosis, University of North Carolina, Chapel Hill, NC 27514; †KabiVitrum AB, Hematology Research and Development, S-112 87, Stockholm, Sweden

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ABSTRACT The procoagulant protein F.VIII:C is noncovalently bound to von Willebrand factor (vWF) to give the factor VIII macromolecular complex. New highly purified preparations of isolated human F.VIII:C, devoid of vWF and about 500,000-fold purified, were administered to hemophilia A and von Willebrand disease (vWD) dogs to determine their hemostatic effectiveness and survival in the circulation. Two preparations of F.VIII:C were used: peak 1, with active components of Mv, 185,000–280,000, and peak 2, with a single component of Mr, 170,000. In hemophilic dogs, with no plasma F.VIII:C but normal vWF, both preparations immediately elevated plasma F.VIII:C to expected levels, promptly stopped induced and spontaneous hemorrhages, and gave sustained plasma levels of F.VIII:C. The isolated F.VIII:C immediately complexed with endogenous vWF in hemophilic plasma and was eliminated exponentially, with a half-life (t1/2) of about 9 hr. Survival of peak 2 F.VIII:C was longer than that of peak 1 material. In contrast, F.VIII:C complexed to vWF in a therapeutic concentrate administered to hemophilic dogs was eliminated biexponentially with first-phase t1/2 of 3.2 hr and second-phase t1/2 of 9 hr. In vWD dogs with no vWF and reduced F.VIII:C levels, the isolated F.VIII:C produced supernormal levels of F.VIII:C without effect on induced bleeding. It was rapidly eliminated from plasma with a t1/2 of about 1 hr, as was the complexed F.VIII:C in the concentrate. These data indicate that isolated F.VIII:C promptly complexes with vWF and in this form is highly effective in controlling hemophilic hemorrhages with good survival in plasma. Without endogenous vWF with which to complex, the F.VIII:C is promptly eliminated.

Factor VIII macromolecular complex is composed of two different noncovalently bound proteins, factor VIII procoagulant protein (F.VIII:C) and von Willebrand factor (vWF). vWF constitutes the larger part of the complex and is composed of disulfide-linked subunits, each of Mv, 200,000. These subunits form heterogenous multimers ranging from Mv, 400,000 to Mv, 12 × 10^6 (1). vWF is involved in primary hemostasis and is reduced or defective in individuals with the autosomal bleeding disorder von Willebrand disease (vWD) (1). F.VIII:C is involved in secondary hemostasis and plays a central role in intrinsic blood coagulation. It participates as a cofactor in the proteolytic-cleavage interaction between factor IXa and factor X leading to the formation of factor Xa (2–8). The F.VIII:C protein is lacking or inactive in individuals with classic hemophilia, an X chromosome-linked recessive bleeding disorder. The procoagulant protein can be dissociated from the F.VIII:C-vWF complex with 0.25 M calcium chloride or 0.8–1.0 M sodium chloride and separated by size-exclusion chromatography (9–18) or by antigen–antibody affinity chromatography (19, 20). Recombination of the two proteins can be accomplished by decreasing the salt concentration (15, 21). Furthermore, when purified F.VIII:C is incubated with normal or hemophilic human plasma, it readily combines with the vWF (22). Because of the low concentration of F.VIII:C in plasma, it is only recently that highly purified F.VIII:C free of vWF has been obtained. Bovine F.VIII:C has been purified approximately 300,000-fold from plasma (23), and porcine F.VIII:C, 16,000-fold (25). Purified human F.VIII:C in one preparation had a specific activity of 2294 F.VIII:C units (u)/mg of protein, representing a 164,000-fold purification (26), while another preparation had 4900 u/mg, a 350,000-fold purification (27).

Recently a new procedure for preparation of human F.VIII:C free of vWF was described (28). The purified F.VIII:C had a specific activity of about 7000 u/mg, representing a 500,000-fold purification relative to plasma. The purified material contained a series of F.VIII:C active components with molecular weights ranging from 280,000 down to 170,000. These components were composed of one light peptide chain of Mv, 80,000 and one heavy chain of Mv, varying from 200,000 to 90,000. In this paper we present data on the hemostatic effectiveness of infusions of these different F.VIII:C active components in hemophilic and vWD dogs. Half-life (t1/2) survival studies of the purified F.VIII:C were also performed in normal, hemophilic, and vWD dogs.

MATERIALS AND METHODS

F.VIII:C Preparation. Purified F.VIII:C free of vWF was prepared as outlined previously (28). Starting material was the high-purity factor VIII concentrate, Octonativ (KabiVitrum AB, Stockholm, Sweden). The purification procedure started with an immunoaffinity chromatography step using a Sepharose CL-2B column containing immobilized antibodies against vWF. After the adsorption of the F.VIII:C-vWF complex, the F.VIII:C was dissociated from vWF with 0.5 M CaCl2. Further purification was obtained by HPLC on Mono Q gel (Pharmacia, Uppsala, Sweden): F.VIII:C was eluted in two fractions, having specific activities of 6710 u/mg and 6940 u/mg, respectively. The major and first-eluted fraction (peak 1) contained eight active components with molecular weights from 280,000 to 185,000. The F.VIII:C material in the minor and last-eluted fraction (peak 2) contained only one component of Mr, 170,000. The polypeptide complement of each fraction was assessed by NaDodSO4/PAGE (28).

Abbreviations: vWF, von Willebrand factor; vWD, von Willebrand disease; F.VIII:C, factor VIII procoagulant protein; F.VIII:CAG, factor VIII coagulant antigen; BT, bleeding time; u, unit(s).
components in peak 1 consisted of one peptide chain of Mr 80,000 and another of Mr varying between 200,000 and 105,000. The component present in peak 2 contained one peptide chain of Mr 80,000 and another of Mr 90,000. Limited proteolysis of the peak 1 material with thrombin resulted in disappearance of the peptide chains of Mr 200,000–105,000 in parallel with the formation of a Mr 90,000 peptide chain. Immunological studies and amino acid sequence determination of the components in peaks 1 and 2 showed that the Mr 80,000 peptide chains were the same in all components and that the other chains (Mr 90,000–200,000) were all more or less fragmented products of the Mr 200,000 chain. Thus, the material contains a series of fragmentation products all of which are active. This fragmentation did not take place during the purification, as the same components could be detected in the starting material by immunoblotting and with the purification performed in the presence of protease inhibitors. The two peptide chains in the F.VIII:C could be dissociated by exposure to EDTA, indicating one or several metal-ion bridges. When F.VIII:C was incubated with hemophilic or normal dog plasma and then was chromatographed on Sepharose CL-6B, the F.VIII:C was eluted in the void volume, indicating complexing with the plasma VWF.

F.VIII:C Assays. F.VIII:C assays were performed by a modified one-stage method (29), using a kaolin-activated procedure. Human hemophilic substrate was used for the studies with hemophilic dogs, and canine hemophilic substrate, with vWD dogs. The assay of F.VIII:C using the synthetic substrate S-2222 (Coaest; KabiVitrum) was performed by the method of Rosen (30). Plasma levels of factor VIII coagulant antigen (F.VIII:C) were determined by a solid-phase immunoradiometric assay, essentially as described by Holmberg et al. (31). Results are expressed as u/ml; a pooled normal human plasma was used for reference.

VWF Assays. These assays were done with both botrocetin and ristocetin, using the macroscopic tap-tube procedure with lyophilized human platelets (32, 33). Canine VWF is unreactive with ristocetin (33).

Transfusions of F.VIII:C Fractions. For studies on normal dogs, purified factor VIII:C was labeled with 125I (24) to a specific activity of 5.3 μCi/μg (1 Ci = 37 GBq) and 3.8 μCi/μg for peak 1 and peak 2 material, respectively. The biological activities of 125I-labeled peak 1 and peak 2 material were 50 and 90% of the initial activities. Two normal beagle dogs were injected with 4 μg of radiolabeled peak 1 material. Three weeks later the same dogs were injected with 4 μg of labeled peak 2 material. The F.VIII:C-derived 125I radioactivity in citrate-treated plasma samples was precipitated by trichloroacetic acid and immediately assayed by scintillation counting.

Both the hemophilia A dogs and the vWD dog were from the Chapel Hill inbred colonies (34, 35). Table 1 provides basic information about each animal. The purified F.VIII:C fractions (3–9 ml) were infused into a jugular vein, as was the factor VIII concentrate (14–35 ml). Citrate-treated plasma samples were frozen in 0.2-ml aliquots at −70°C within 20 min of blood collection. The t½ of purified F.VIII:C was determined by linear-regression analysis after transformation of F.VIII:C radioactivity (normal dogs) and F.VIII:C activity (bleeder dogs) values into natural logarithmic values (36). The slope of the line is −β and the half-life is 0.693/β.

Chromatography on Bio-Gel A-15m was used to determine whether infused F.VIII:C was complexed with VWF in vivo. Citrate-treated plasma samples were collected 30 min (bleeder animals) or 1 hr (normal animals) after infusion for chromatography.

Hemostatic Testing. For the hemophilic dogs, a modified toenail BT (bleeding-time) test (37) was used to test the hemostatic effectiveness of infused F.VIII:C preparations. The paw of the front leg was warmed by placing it in isotonic saline at 37°C and a toenail was trimmed to expose only the distal matrix. The bleeding nail was placed in isotonic saline at 37°C and the time until cessation of bleeding was recorded as the primary BT. The paw was then exposed to air. Rebleeding of variable duration occurred, with the formation of a soft red clot over the cut nail matrix. At 2–4 hr, the site was shaved to remove the clot and as little nail matrix as possible. The paw was again placed in saline at 37°C. A discrete stream of extruding blood was visible. Bleeding continued for 30 min or longer. This is recorded as a secondary BT >30 min. For normal dogs, the primary BT was 2–5 min, the secondary BT <5 min. For testing the hemostatic effect of infusions of F.VIII:C preparations, a primary BT test was performed about 4 hr before infusion. The secondary BT test was performed about 15 min after infusion. For the vWD dogs, the saline BT test on the ear (38) was used to determine hemostatic effectiveness of infused F.VIII:C.

RESULTS

F.VIII:C Administered to Normal Dogs. The experiments in normal dogs were designed to determine the rate of elimination of infused F.VIII:C and whether it circulated in plasma as isolated F.VIII:C or as a complex with VWF. The F.VIII:C-derived 125I radioactivity declined exponentially with time (data not shown), and the plasma t½ of peak 1 and 2 material was 7.6 and 10.9 hr, respectively. Addition of 40 μg of unlabeled peak 1 material caused no change in the rate of elimination. About 90% of the total radioactivity in 1-hr plasma samples was eluted in the void volume (Bio-Gel A-15m), suggesting that the infused human F.VIII:C had complexed in vivo with the canine VWF.

F.VIII:C Administered to Hemophilia A Dogs. The experiments in hemophilic dogs were designed to determine the survival of infused purified F.VIII:C in the circulation and the ability of isolated F.VIII:C to control hemophilic hemorrhage. For comparison a factor VIII commercial concentrate was also used. Table 2 (Exps. 1–3) provides the data on plasma after infusions of purified F.VIII:C, peaks 1 and 2. The dose given was calculated to give a plasma F.VIII:C level of 2 u/ml. The response was similar in three hemophilic

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Table 1. Hemophilia A and vWD dogs used in F.VIII:C infusion studies

<table>
<thead>
<tr>
<th>Animal</th>
<th>Disease</th>
<th>Age, months</th>
<th>Sex</th>
<th>Weight, kg</th>
<th>Plasma F.VIII:C* u/ml</th>
<th>Plasma vWF, a u/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hemophilia</td>
<td>6</td>
<td>F</td>
<td>13.2</td>
<td>&lt;0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>Hemophilia</td>
<td>24</td>
<td>M</td>
<td>16.8</td>
<td>&lt;0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>3</td>
<td>Hemophilia</td>
<td>12</td>
<td>F</td>
<td>15.6</td>
<td>&lt;0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>4</td>
<td>Hemophilia</td>
<td>12</td>
<td>F</td>
<td>17.2</td>
<td>&lt;0.01</td>
<td>0.002</td>
</tr>
<tr>
<td>5</td>
<td>vWD</td>
<td>42</td>
<td>F</td>
<td>7.7</td>
<td>1.0</td>
<td>0.23</td>
</tr>
</tbody>
</table>

* All animals had a history of hemorrhage.

† Pooled normal human plasma (1.0 u/ml) used as reference standard.

‡ Preinfusion canine sample (1.0 u/ml) used as reference plasma.
Table 2. Infusion of human F.VIII:C into hemophilic and vWD dogs

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Dog</th>
<th>Disease</th>
<th>Material</th>
<th>Total Dose</th>
<th>Expected F.VIII:C u/ml</th>
<th>Plasma F.VIII:C</th>
<th>F.VIII:C t1/2, hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>Hemophilia</td>
<td>Peak 1</td>
<td>1350 u/kg</td>
<td>2.05</td>
<td>V0 = 9.1</td>
<td>S-2222</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Hemophilia</td>
<td>Peak 2</td>
<td>1820 u/kg</td>
<td>2.17</td>
<td>V0 = 9.8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Hemophilia</td>
<td>Peak 3</td>
<td>1500 u/kg</td>
<td>1.92</td>
<td>V0 = 4.2</td>
<td>S-2222</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>Hemophilia</td>
<td>Octonativ</td>
<td>1750 u/kg</td>
<td>2.03</td>
<td>V0 = 4.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>vWD</td>
<td>Peak 1</td>
<td>900 u/kg</td>
<td>2.34</td>
<td>V0 = 1.4</td>
<td>S-2222</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>vWD</td>
<td>Octonativ</td>
<td>840 u/kg</td>
<td>2.18</td>
<td>V0 = 1.7</td>
<td></td>
</tr>
</tbody>
</table>

V0, void volume; V0 included volume; ND, not done.

*Pooled normal human plasma (1.0 u/ml) used as reference standard.

†Second-phase half-life (first-phase t1/2 was 3.2 hr; ref. 36).

§Increase in F.VIII:C values from preinfusion plasma samples.

Table 2: Infusion of human F.VIII:C into hemophilic and vWD dogs

The infusion of human commercial factor VIII concentrate (Table 3, Exp. 4) also promptly normalized the secondary BT.

F.VIII:C Administered to vWD Dog. One homozygous vWD dog was available for the transfusion studies (Table 1). The animal had no detectable plasma vWF and approximately 1.0 u of F.VIII:C/m, in terms of a human reference plasma (equivalent to about 0.3 u of F.VIII:C/m in relation to a dog reference plasma). A transfusion of purified human F.VIII:C was given first, followed by a transfusion of Octonativ concentrate 5 days later (Table 2, Exp. 5 and 6). Infusion of purified human F.VIII:C caused an increment in plasma F.VIII:C of about 1.34–1.78 u/ml, an amount considerably less than the expected value of 2.54 u/ml, suggesting that F.VIII:C not combined with vWF is rapidly lost from the circulation. Even though F.VIII:C levels were elevated to supernormal levels, there was no effect on the long BT (Table 3).

The comparable experiment with Octonativ in which the F.VIII:C was complexed with vWF demonstrated fairly good agreement between the expected F.VIII:C plasma value and that observed with the one-stage and F.VIII:C:Ag assays. The posttransfusion plasma vWF/ristocetin values were also close to those expected. However, there was no correction of the prolonged bleeding time. In this experiment, there was a delayed rise in plasma F.VIII:C, e.g., 24 hr, 0.85 u/ml, characteristic of the effect of infusions of cryoprecipitate and commercial concentrates on plasma F.VIII in vWD.

Survival Studies of F.VIII:C. The very different survival patterns of infused purified F.VIII:C in hemophilia and vWD are shown in Table 2 (last two columns) and in Fig. 2. F.VIII:C of peaks 1 and 2 administered to hemophilic dogs declined exponentially as it did in normal dogs, with similar t1/2 values. The t1/2 was longer with the peak 2 preparation. Peak 1 F.VIII:C administered to the vWD dog had a very short t1/2. These data indicate that the presence of plasma vWF in the recipient is needed for survival of infused purified F.VIII:C.

The survival patterns of F.VIII:C in the concentrate was likewise very different in hemophilia and vWD. In hemophilia, F.VIII:C declined in a biexponential way. The first-phase fall-off was 3.2 hr. The second-phase fall-off was about 9 hr, similar to that observed with peaks 1 and 2 of the purified F.VIII:C. In vWD, fall-off of F.VIII:C in the concentrate was very rapid, similar to that observed with purified F.VIII:C, again indicating the importance of endogenous vWF for F.VIII:C survival. The t1/2 for the vWF in the concentrate was also short (t1/2 of 1.3 hr for vWF/ristocetin and 3.2 hr for vWF/botrocetin).

DISCUSSION

The F.VIII:C preparations used in these in vivo studies in hemophilic and vWD dogs were made from a commercial
human factor VIII concentrate in which the F.VIII:C is complexed with vWF. The coagulant portion of the factor VIII complex, F.VIII:C, after dissociation from the vWF, was further purified by HPLC with Mono Q gel. Two F.VIII:C fractions were obtained: peak 1 with the higher molecular weight components (Mr 280,000–185,000) and peak 2 with a single active component (Mr 170,000). The peak 1 and peak 2 F.VIII:C fractions or preparations were tested separately. Preliminary studies were performed to determine whether the human F.VIII:C would recombine with vWF in both normal and hemophilic canine plasmas. Additional studies were performed with administration of human F.VIII:C to normal dogs to determine whether the coagulant protein would combine with the canine vWF in vivo and whether the human F.VIII:C would circulate in the dog. With the finding that cross-species recombination and survival both occurred, the transfusion experiments in the bleeder animals were carried out.

Data on the hemophilia A and vWD dogs pertinent to the transfusion studies are provided in Table 1. Four severe hemophilia A dogs, one male and three females, were used. Although classic human hemophilia occurs almost exclusively in males, the canine disease is produced in females by interbreeding carrier females and hemophilic males. The vWD subject used in this study was a severe homozygous vWD animal, a product of the breeding of a homozygous male and a heterozygous female. The hemophilic dog plasma lacked F.VIII:C but contained normal amounts of vWF. The vWD dog plasma lacked vWF but had circulating levels of uncomplexed F.VIII:C of about 1.0 u/ml when assayed with a human reference standard.

Data from the infusion of the purified F.VIII:C preparation into hemophilic dogs are presented in Table 2. A dose of F.VIII:C was selected so as to increase the plasma level of this procoagulant to about 2.0 u/ml. After infusion the plasma levels of F.VIII:C were immediately elevated to near expected levels. The three separate methods for assay of F.VIII:C, although very different (one a clotting procedure, another a chromogenic assay, and the third an immunoassay), gave comparable results. The infused human F.VIII:C, both peak 1 and peak 2, complexed with the canine vWF, since both were eluted in the void volume (Fig. 1) provides data on the fall-off of F.VIII:C in a hemophilic dog infused with the peak 2 preparation. The infused purified F.VIII:C was highly effective in stopping hemophilic hemorrhages (Table 3). Both peak 1 and peak 2 infusions resulted in a normal secondary BT and caused prompt cessation of bleeding from hemorrhages then in progress.

Data obtained from the infusion of purified F.VIII:C into the vWD animal are presented in Table 2 (Exp. 5). The dose was comparable to that used for the hemophilic dogs. With purified F.VIII:C there was an increment of plasma F.VIII:C of about 1.5 u/ml to give a plasma level of about 2.5 u/ml. This postinfusion F.VIII:C level was markedly less than the expected value. The F.VIII:C was recovered by chromatography in the included volume eluate, as would be expected since no vWF was present in either the recipient’s plasma or the infusate. This infusion of F.VIII:C was without effect on hemostasis (Table 3, Exp. 5). The lack of effect of this high level of F.VIII:C is of interest, since there has been speculation that with two deficiencies in vWD, vWF and F.VIII:C, there might be a beneficial effect on hemostasis with appropriate replacement therapy with either factor.

The difference in survival of purified infused F.VIII:C in hemophilic and vWD dogs was striking (Fig. 2). In hemophilic dogs, F.VIII:C complexed with endogenous plasma vWF survived for hours (t1/2 ≈ 9 hr). In the vWD dog with an absence of vWF, F.VIII:C was rapidly lost from the circulation (t1/2 ≈ 1 hr). It appears that endogenous vWF is required for the hemostatic effectiveness and survival of purified F.VIII:C. Fig. 2 also shows that purified F.VIII:C in peak 1, with active components of Mr 185,000–280,000, had a half-life somewhat shorter than did the peak 2 material, with only a Mr 170,000 component. This difference may indicate that the smaller the F.VIII:C molecule complexed to the endogenous vWF, the longer the half-life. Further data are needed to confirm this interpretation.

The results of infusing human F.VIII:C complexed with human vWF, Octonativ, into a hemophilic dog (Tables 2 and 3, Exp. 4) are included for comparison to the purified F.VIII:C experiments. The data obtained with the two preparations were similar in that there was an immediate increase in plasma F.VIII:C and prompt cessation of hemophilic hemorrhage with both.

The infusion of the human factor VIII complex into the vWD dog caused an immediate increase in the plasma F.VIII:C. The vWF infused was an integral component of the human factor VIII complex and therefore could be assayed.
with the ristocetin test. Expected levels of vWF/ristocetin, based on the dose of vWF in the infusate, were reached. As in human vWD (39), the concentrate did not normalize hemostasis, presumably because of the limited content of high molecular weight forms of vWF in this type of preparation. In addition, there was a secondary or late rise in F.VIII:C, a characteristic of infusions of factor VIII-complex preparations in vWD.

In hemophilia the immediate beneficial effects of administering purified F.VIII:C or the complex were much the same. However, the postinfusion fall-off of plasma F.VIII:C was dependent on the form in which it was administered. Purified F.VIII:C protein that was complexed in vivo with endogenous vWF followed a simple exponential loss pattern. In the concentrate, the human F.VIII:C was already complexed to the homologous vWF. It followed a biexponential loss pattern. Here the role of the endogenous vWF is less clear. Perhaps the more rapid first-phase fall-off ($t_{1/2} = 3.2$ hr) is due to the rapid loss of the infused vWF in the complex, carrying part of the F.VIII:C with it. The second phase ($t_{1/2} = 9$ hr) may represent the fall-off of F.VIII:C that had been transferred to the endogenous vWF carrier which is in a steady-state condition. This is suggested by the second-phase half-life, which is similar to that of the purified F.VIII:C complexed to the endogenous vWF in Exp. 2 ($t_{1/2} = 9$ hr) and the loss of ristocetin-sensitive vWF. The net effect of these two patterns of survivals is that higher levels of plasma F.VIII:C are sustained in hemophilic dogs with infusion of the purified form of F.VIII:C than with the F.VIII--vWF complex, dosage being the same. These data suggest that for maximum biologic effectiveness in hemophilic therapy it would be advantageous to use purified F.VIII:C, such as the preparations tested here or recombinant F.VIII:C. The latter also complexes with vWF (40). In the vWD dog, the F.VIII:C in the concentrate resulted in higher plasma levels of F.VIII:C which remained in the postinfusion plasma somewhat longer than did purified F.VIII:C. These findings suggest that preinfusion complexing of the coagulant protein to vWF has a very limited effect on F.VIII:C survival.

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