

Effect of recombinant factor VIIa on the hemostatic defect in dogs with hemophilia A, hemophilia B, and von Willebrand disease

(animal models/factor VIII/factor IX/hemostasis/tissue factor)

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ABSTRACT Recombinant factor VIIa (rF.VIIa) is a two-chain procoagulant enzyme (M_r , $\approx 50,000$) active only when complexed with tissue factor in the extrinsic clotting system. We administered human rF.VIIa to hemophilic and von Willebrand disease (vWD) dogs to determine its hemostatic effectiveness and survival in the circulation. Hemophilia A dogs lacking factor VIII demonstrated an immediate increase in plasma rF.VIIa and prompt stoppage of hemorrhage at bleeding time (BT) sites. In seven studies in two dogs, the range of dose of rF.VIIa was 50–220 $\mu\text{g}/\text{kg}$, with an apparent 7- to 11-fold increase in plasma factor VII and a mean recovery in plasma of 34%. The $t_{1/2}$ was 2.8 ± 0.5 hr. The BT was normalized except in an animal given the minimum dose. In four studies in two hemophilia B dogs lacking factor IX, BT was normalized. The elevation in plasma factor VII was by a factor of 8–30, with a mean recovery of rF.VIIa in plasma of 44%. In two studies in a homozygous vWD dog lacking von Willebrand factor, which is needed for platelet function, BT was not corrected even though large doses of rF.VIIa were given. The human rF.VIIa protein was immunogenic for dogs. These studies indicate that factor VIIa corrects the hemostatic defect in dogs with hemophilia A and B, diseases primarily of the intrinsic clotting system, but does not correct the hemostatic defect in vWD.

The pathophysiology of hemophilia A and hemophilia B is determined by the lack of specific plasma procoagulant proteins, factors VIII and IX, respectively. In each disease, there is impairment of the intrinsic pathway of coagulation, with inadequate thrombin generation and defective hemostasis. Infusions of the missing protein correct the hemostatic defect except when an alloantibody has developed that neutralizes the antihemophilic factor (inhibitor hemophilia). It has long been recognized that the extrinsic pathway of coagulation involving tissue factor (TF) and factor VII may also be impaired in hemophilia A (1). It was demonstrated 50 years ago that the very slow thrombin generation in hemophilic blood can be corrected by addition of small amounts of TF—i.e., thromboplastin (2), the TF effect being dose dependent. If only a trace of TF is used, the clotting of both human and canine hemophilic plasma remains long (3). This is the basis of the dilute thromboplastin time test for hemophilia, in which factor VII appears to be incompletely activated to form factor VIIa (4). The prolonged dilute thromboplastin time in hemophilia, while not completely understood, may be due to impaired factor VII activation in this disease (5), possibly the result of lack of factor VIII or IX (6). Normally factor VIIa, complexed with TF, forms the active enzyme that converts factor X to Xa, which in turn converts prothrombin to thrombin in the presence of factor Va. The complex also converts factor IX to factor IXa (7),

thus representing a link between the intrinsic and extrinsic pathways. The mechanism of action of TF and factor VII has been reviewed (8, 9).

Additional evidence for a possible role of the extrinsic system in hemophilia came from infusing plasma concentrates containing the activated procoagulant enzymes, factors IXa, Xa, and VIIa, to “bypass” the defect in inhibitor hemophilia. Such concentrates had a hemostatic effect, but thrombosis was observed with higher dosage (10, 11). The purification of factor VII from plasma (12) allowed preliminary testing of isolated human factor VIIa in microgram amounts in inhibitor hemophilia. The results pointed to this procoagulant as an active bypassing agent (13). Recently, human factor VII has been cloned and expressed in mammalian cells (14), making it possible to produce this trace protein in larger amounts than heretofore. During purification, recombinant factor VIIa (rF.VIIa) is formed and is composed of two polypeptide chains (M_r , $\approx 20,000$ and $\approx 30,000$).

The studies reported here were conducted to test the hypothesis that when sufficient rF.VIIa is introduced into the circulation in hemophilia, the hemostatic defect can be corrected without causing systemic coagulation. This hypothesis is based on several prior findings. (i) No specific plasma inhibitor for factor VII has been identified (15–17); hence, rF.VIIa should not be cleared rapidly from the circulation. (ii) Lacking TF, the rF.VIIa will be inactive in the circulation. (iii) At the site of an injury, such as a bleeding-time (BT) wound, TF is generated as a cell-membrane receptor for factor VIIa, and the factor VIIa–TF complex forms promptly. The complex then initiates the extrinsic pathway of coagulation (1, 18, 19). (iv) The onset of extrinsic coagulation with rapid formation of fibrin in BT wounds is normal in hemophilia (20). Supplemental rF.VIIa in hemophilia could result in sufficient thrombin generation with fibrin formation and platelet activation to facilitate formation of a stable hemostatic plug.

MATERIALS AND METHODS

Hemophilic and von Willebrand Disease (vWD) Dogs. The subjects used for testing rF.VIIa were from the inbred Chapel Hill colonies of hemophilia A (21, 22), hemophilia B (23), and vWD (24) dogs. The hemophilic animals were either hemizygous or homozygous, and the vWD dog was homozygous. All were severe bleeders.

Factor VIIa Preparations. rF.VIIa was obtained from baby hamster kidney cell culture transfected with human factor VII genomic material as described (14). The rF.VIIa was purified from the culture medium by a four-step procedure

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Abbreviations: BT, bleeding time; mAb, monoclonal antibody; rF.VIIa, recombinant factor VIIa; PTT, partial thromboplastin time; APTT, activated PTT; TF, tissue factor; vWD, von Willebrand disease; vWF, von Willebrand factor.

including three ion-exchange chromatography steps and immunoabsorption on a Sepharose 4B column (Pharmacia) to which a monoclonal antibody (mAb) against human plasma factor VII was coupled. On SDS/gel electrophoresis, unreduced samples revealed a single band; reduced samples revealed two bands, corresponding to the light and heavy chains of factor VIIa. The specific activity was 39 units/ μ g. The rF.VIIa was provided frozen (-70°C) in 1- or 2-ml vials (1 ml contained 0.6 mg of protein, equivalent to 23,600 units of rF.VIIa).

Tests for Factor VII. A modified one-stage factor VII clotting assay with rabbit brain thromboplastin and a normal dog plasma standard were used (25). Factor VII-deficient plasma for substrate was prepared from normal human plasma by immunoabsorption using a mAb raised against plasma-derived factor VII. Factor VII antigen in plasma was determined by a double monoclonal ELISA, using two mAbs directed against different epitopes on the factor VII molecule. One mAb, the catching antibody, was bound to the microtiter plate and the test plasma was added. After addition of the second mAb, an immunoperoxidase-labeled anti-mouse IgG was used. The mAb did not cross-react with canine factor VII. Human plasma was used as a standard.

Other Coagulation Assays. Factor VIII:C assays were performed by a modified one-stage method (3) using a kaolin-activated procedure with canine hemophilia A plasma as substrate. Factor IX assays were done with the same procedure, using canine hemophilia B plasma as substrate. Partial thromboplastin time (PTT) determinations were performed essentially as described (3). The activated partial thromboplastin time (APTT) determinations were performed by using an APTT reagent (Pacific Hemostasis, Ventura, CA), following the manufacturer's directions. Dilute thromboplastin time tests (3) were performed with a rabbit brain thromboplastin reagent (Sigma Diagnostics) diluted to 1:25,000 in 0.154 M NaCl. The procedure was a three-part test consisting of 1 part each of undiluted citrated plasma and dilute thromboplastin incubated 3 min at 37°C . The clotting time was determined after the addition of 1 part 0.025 M CaCl_2 . von Willebrand factor (vWF) assays were done with botrocetin using the macroscopic tap-tube procedure with lyophilized human platelets (26, 27). A group of assays was performed to monitor for evidence of disseminated intravascular coagulation following infusions of rF.VIIa. Fibrinogen levels were determined by a syneresis method (28). Fibrinogen degradation products were measured by the Thrombo-

Wellcotest (Wellcome Diagnostics), following the manufacturer's instructions. The ethanol gelation test was performed by a standard method (29). Platelet counts were done by the Unopette microcollection method (Becton Dickinson).

Antibody Assays. Bleeder dog plasmas were assayed for antibodies against injected human factor VIIa with an ELISA method as follows: rF.VIIa was bound on the ELISA plate, test plasma was added, and a peroxidase-conjugated rabbit anti-canine IgG (Nordic, Tilburg, The Netherlands) was used as a detecting antibody. Normal dog plasma served as a negative control.

Infusions of rF.VIIa. rF.VIIa (2–15 ml) was infused via the jugular vein into hemophilia A, hemophilia B, and vWD dogs. Blood sampling was done immediately before infusion and at intervals up to 24 hr postinfusion. Citrated plasma samples were frozen in 0.2-ml aliquots at -70°C within 20 min of blood collection. The expected levels of plasma factor VIIa antigen were calculated on the basis of dose and an assumed plasma volume of 5% of body weight. Half-life ($t_{1/2}$) values of infused rF.VIIa were determined in hemophilia A dogs (Exps. 5–7) by linear-regression analysis after transformation of factor VII (units/ml) and factor VII antigen (ng/ml) values into natural logarithmic values. Blood samples were collected at 10 min, 1, 3, and 6 hr. The $t_{1/2}$ is $\ln 2/\text{slope}$ of the regression line. In normal dogs, the $t_{1/2}$ for factor VII was 2.3 ± 0.12 hr and for factor VII antigen it was 2.8 ± 0.04 hr.

Hemostatic Testing. A modified toenail BT test, as described (22, 30), was used for the hemophilic dogs. The saline BT test on the ear (31) was used for the vWD dog.

RESULTS

Response of Hemophilia A and Hemophilia B Dogs to Infused rF.VIIa. Seven infusions of rF.VIIa were given to two hemophilia A dogs (Table 1, Exps. 1–7). The dose of rF.VIIa varied from 49 to 219 μ g per kg of body weight. The expected and actual plasma levels of factor VIIa antigen immediately postinfusion indicate a mean retention in the circulation of $34\% \pm 13\%$ of the injected dose, with a range of 18% to 48%. The plasma levels of factor VII were elevated from 4- to 11-fold above preinjection values. The fall-offs of plasma factors VII and VII antigen are illustrated in Fig. 1A. At 24 hr, the levels of these factors had returned to preinfusion values. The $t_{1/2}$ for factor VII was 2.1 ± 0.6 hr and for factor VII antigen it was 2.8 ± 0.5 hr. The postinfusion BT was

Table 1. Infusion of recombinant human factor VIIa into hemophilia A, hemophilia B, and vWD dogs

Exp.	Dog			rF.VIIa dose		Plasma F.VII 10 min postinfusion			PTT, sec		Secondary BT, min	
	No.	Disease	Day*	Total, mg	μ g/kg	Expected antigen, ng/ml	Antigen, ng/ml	One-stage, units/ml	Base	10 min	Preinfusion	Postinfusion
1	1	Hem A	2	0.7	49	993	450	7.2	55	42	>15	11.0
2	1	Hem A	0	0.85	60	1,206	512	7.3	52	43	>15	3.0
3	1	Hem A	1	0.85	60	1,206	473	7.9	54	44	>15	3.0 [†]
4	1	Hem A	5	3.1	219	4,397	1210	9.4	52	46	>15	1.5
5	2	Hem A	0	1.17	64	1,277	228	3.7	64	48	>15	2.0
6	2	Hem A	1	2.78	51	3,016	545	8.1	58	30	>15	2.0
7	2	Hem A	2	3.55	193	3,859	1867	11.2	59	35	>15	1.5 [†]
8	3	Hem B	3	1.25	67	1,344	468	8.3	78	46	>15	2.5
9	3	Hem B	1	3.65	196	3,925	1810	14.0	49	34	>15	4.0 [†]
10	3	Hem B	0	3.7	199	3,978	1813	30.7	69	36	>15	2.0
11	4	Hem B	0	2.5	153	3,067	1490	22.9	60	37	>15	2.5
12	5	vWD	0	3.5	257	5,147	2855	42.5	33	30	ND	>15 [‡]
13	5	vWD	1	7.5	550	11,029	4925	64.0	33	25	ND	>15 [‡]

Hem, hemophilia; ND, not done.

*Day after first infusion for each dog.

[†]Secondary cuticle BT initiated 3 min before rF.VIIa.

[‡]Ear BT.

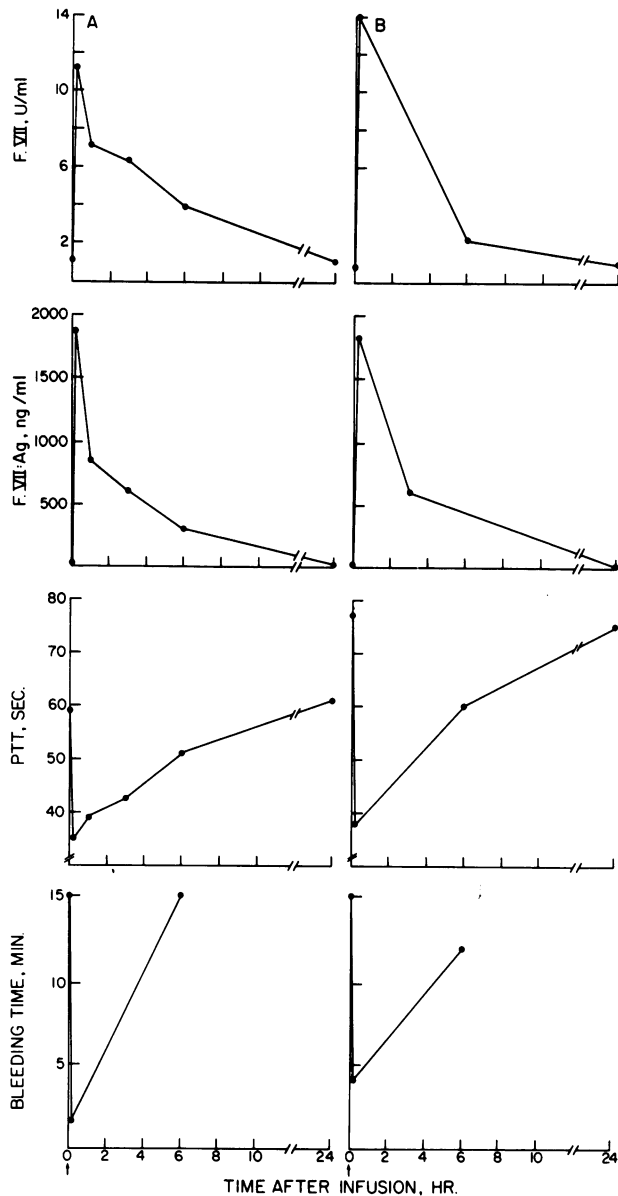


FIG. 1. Effect of infusions of rF.VIIa to hemophilia A (A) and hemophilia B (B) dogs on plasma factor VII, factor VII antigen, PTT, and toenail BT. Arrows indicate time of rF.VIIa administration. Illustrations of infusions in a hemophilia A dog (Table 1, Exp. 7; dose, 193 $\mu\text{g}/\text{kg}$) and a hemophilia B dog (Table 1, Exp. 9; dose, 196 $\mu\text{g}/\text{kg}$).

shortened to the normal range of <5 min except in Exp. 1, in which a low dose of rF.VIIa was administered. At 6 hr postinfusion (Fig. 1A), the BT had returned to basal levels, although in a few experiments a shortened BT was still noted. Both the activated and unactivated PTTs were determined at intervals before and after rF.VIIa infusions. The APTT values were affected very little by the infusions. In contrast, the PTT values were considerably shortened (Table 1). Some shortening of the PTT persisted at 6 hr but not at 24 hr (Fig. 1A). The various assays used as indicators of systemic intravascular clotting were unchanged from basal conditions, with one exception. In Exp. 4, conducted on the 5th day after the first rF.VIIa injection, the ethanol gelation test was negative for gelation but did demonstrate flocculation. A plasma sample collected on day 6 demonstrated low-titer anti-factor VII antibody. Plasma factor VIII remained at $<1\%$. Collectively, these experiments suggest an immediate correction of the hemostatic defect in hemophilia A dogs after

infusion of a sufficient amount of rF.VIIa without induction of a systemic clotting reaction.

Four infusions of rF.VIIa were given to two hemophilia B animals (Table 1, Exps. 8–11). The range of dose of rF.VIIa was 67–199 $\mu\text{g}/\text{kg}$. The postinfusion plasma levels of factor VII were increased 8.3–30.7 units/ml. The mean postinfusion recovery of rF.VIIa antigen in the plasma was $44\% \pm 6\%$. These values were higher than in the hemophilia A dogs with comparable doses of rF.VIIa. The correction of the hemostatic defect was similar to that observed in the hemophilia A dogs. Fig. 1B provides data illustrating the loss over time of the effect of rF.VIIa infusions. The basal values for the PTT and the APTT tests were longer in the hemophilia B dogs than in those with hemophilia A, and both tests were shortened after rF.VIIa infusions. The tests for systemic clotting were negative. Plasma factor IX was undetectable throughout the experiments. These data indicate that the hemophilia B dogs, like the hemophilia A animals, respond to rF.VIIa infusions by correction of the hemostatic defect and without evidence of systemic coagulation.

Effect of rF.VIIa on *in Vitro* Clotting of Hemophilic Plasmas.

In view of the shortening of both the PTT and the dilute thromboplastin time in hemophilia A and hemophilia B dogs given rF.VIIa, the effect of adding rF.VIIa *in vitro* on the prolonged clotting time of the hemophilic plasmas in these tests was determined. Fig. 2 illustrates the progressive shortening of hemophilia A and hemophilia B plasmas with increasing concentrations of rF.VIIa. The highest concentration of rF.VIIa corresponds to ≈ 70 units of factor VII per ml. Higher concentrations of rF.VIIa up to 500 units/ml reduced the PTT to a minimum of ≈ 20 sec for both plasmas. Fig. 3 depicts similar data on the effect of rF.VIIa on the dilute thromboplastin time. Hemophilia B plasmas consistently exhibited a longer clotting time with these tests than did hemophilia A plasmas. Semilogarithmic plots of the data (not shown) demonstrated a linear relationship between rF.VIIa concentration and clotting time in each of the tests.

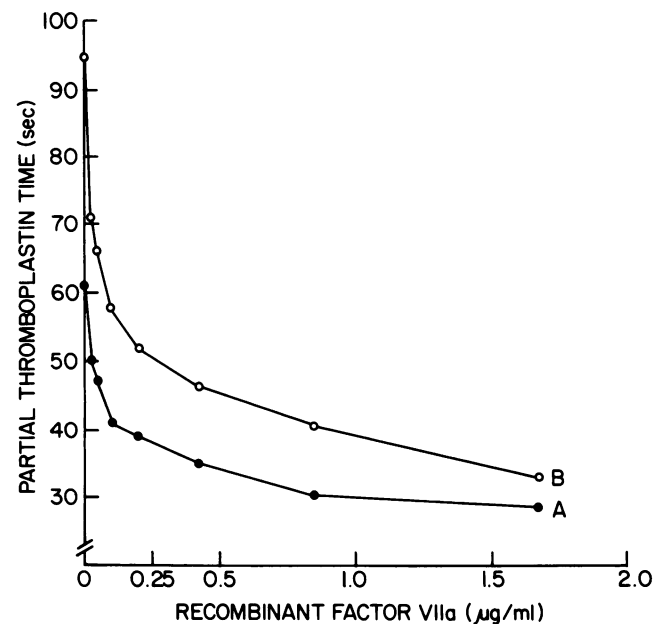


FIG. 2. Acceleration of the PTT of hemophilic plasmas on addition of rF.VIIa. The rF.VIIa (1 part) was added to hemophilic plasma (9 parts). The test mixture consisted of equal parts of partial thromboplastin reagent (Thrombofax, Ortho Diagnostics), citrated hemophilic plasma with rF.VIIa, and 0.025 M CaCl_2 . The values on the abscissa represent the concentration of rF.VIIa in the plasma. Curve A, canine hemophilia A plasma; curve B, canine hemophilia B plasma.

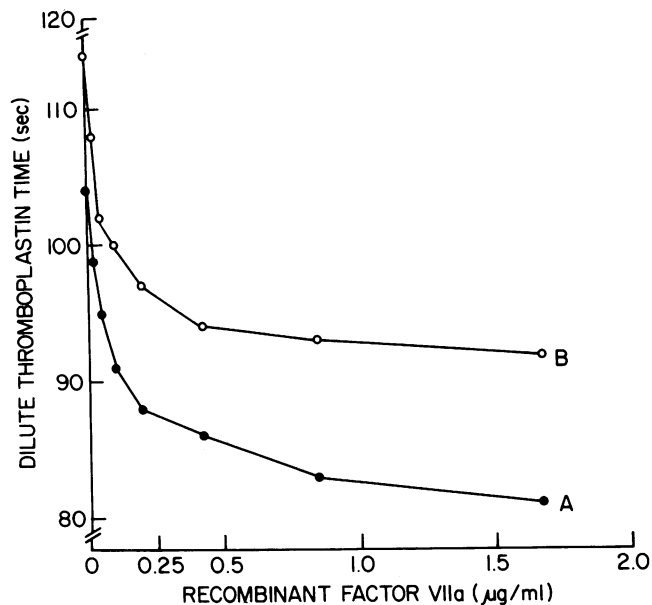


FIG. 3. Acceleration of the dilute thromboplastin time on addition of rF.VIIa to hemophilic plasmas. The rF.VIIa (1 part) was added to hemophilic plasma (9 parts). The test mixture consisted of equal parts of dilute rabbit brain thromboplastin reagent, citrated hemophilic plasma with rF.VIIa, and 0.025 M CaCl_2 . Curve A, canine hemophilia A plasma; curve B, canine hemophilia B plasma.

Response of a vWD Dog to Infused rF.VIIa. Two infusions were given to a vWD animal. The doses of rF.VIIa were considerably larger than those given to the hemophilic animals, and the increase in plasma levels of factor VII and factor VII antigen was correspondingly greater (Table 1, Exps. 12 and 13). The mean recovery of factor VII antigen immediately postinfusion was 40%. There was no corrective effect on the long BT.

Immunologic Response of Bleeder Animals to Human rF.VIIa. A transient urticarial-type reaction lasting for ≈ 30 min occurred on only one occasion in four of the five bleeder animals within 10 min following the injection of rF.VIIa (Table 1, Exps. 7, 10–12). Wheals appeared most prominently about the face.

Anti-human factor VII antibodies were detected in the plasmas of all animals 1–2 wk after the animals received the first dose of rF.VIIa. The response of the animals varied and appeared unrelated to the dose of rF.VIIa. Dog 2 was a low responder, with antibody being detected in the 2-wk sample only (titer, 1:100). The remaining dogs had a maximum titer of 1:1000. The antibody persisted for 10 wk in dogs 3 and 4, and for 22 and 29 wk in dogs 5 and 1, respectively.

DISCUSSION

The availability of rF.VIIa permitted the testing of its hemostatic properties over a wide range of dosages in the two hemophilic diseases, canine hemophilia A and B. The recipient's plasma levels of factor VII were elevated many times the physiological amounts. The secondary toenail BT test was used as the hemostatic test, since the primary toenail BT is normal in hemophilic dogs (3–5 min). The secondary BT was performed by shaving off the clot at the primary site to restart bleeding. In hemophilic subjects, the secondary BT is >15 min; in normal animals, it is <5 min (22). The design of the experiments was to test the effect of rF.VIIa in two ways: (i) infusion before the secondary BT was done—i.e., prevention of prolonged bleeding and (ii) infusion while the nail was actively bleeding—i.e., stanching of bleeding in progress. With each schedule the defective hemostasis in both types of

hemophilia was immediately corrected by infusions of similar doses of rF.VIIa. These events suggest that activation of factor IX by the rF.VIIa–TF complex is not essential for its hemostatic effect in hemophilia. The two hemophilic diseases are due to a deficiency of different proteins that act in a different manner in the intrinsic coagulation pathway. The $t_{1/2}$ of injected rF.VIIa in these experiments was in the 2- to 3-hr range, similar to that observed in inhibitor hemophilia A patients given clinical factor IX concentrates (32). The administration of rF.VIIa in excess seems to induce normal hemostasis in the hemophilic dogs, bypassing the activation of factor IXa and perhaps activation of factor VII as well.

Determination of plasma factor VII levels after rF.VIIa infusions can be made with either the factor VII bioassay or immunoassay procedures. Both the PTT and the dilute thromboplastin time tests appear to be suitable for monitoring changes in plasma rF.VIIa levels. The clotting times in these tests are prolonged in hemophilia A and B and are shortened by infusions of rF.VIIa in the hemophilic dogs (Fig. 1) or by addition of rF.VIIa *in vitro* to their plasmas (Figs. 2 and 3). The unactivated PTT was more sensitive to changes in plasma concentration of rF.VIIa than was the activated test. The sensitivity of the dilute thromboplastin time to rF.VIIa is probably due to the presence of TF in the thromboplastin reagent, giving rise to the TF–rF.VIIa complex. The question raised was whether the sensitivity of the PTT test to rF.VIIa could be due to some unrecognized source of TF in the reagents or whether it was independent of the availability of TF. A limited survey for TF was made in the PTT reagents. The lipid PTT reagent made from cow brain was tested in an ELISA procedure with a mAb specific for bovine TF (HTF1-7B8) (33), using Thrombotest (Nycomed, Oslo) reagent also made from cow brain as a positive control. The PTT reagent was negative for TF. A second mAb, an anti-human TF (34) that cross-reacts with canine TF, was used to test canine hemophilic plasmas. They were weakly positive. Further study of this question is needed.

Infusions of the human rF.VIIa protein into the dogs elicited an immediate urticarial response in 4 of the 11 experiments. Only one animal escaped this reaction. All of the animals developed antibodies against rF.VIIa. One of the animals was a low responder. Similar findings have been reported following administration of human factor VIII preparations to hemophilia A dogs (35).

The hemostatic defect in the vWD dog was not corrected by infusions of rF.VIIa in spite of the fact that very large doses were administered. The defect in these animals is severe and is due primarily to defective platelet adhesion and agglutination resulting from the absence of vWF. The vWD animal is a model for type III human vWD or homozygous type I vWD (36). vWF appears to be essential for the development of the platelet component of thrombi. Administration of a monoclonal anti-vWF has been shown to prevent development of an arterial thrombus in normal pigs (37), which correlates with the inability of homozygous vWD subjects to form a hemostatic plug. These data suggest that forced thrombin generation with rF.VIIa will not compensate for the lack of vWF and vWF platelet function. Thrombin activation of platelets is mediated through the glycoprotein IIb/IIIa receptor. vWF binds to both this receptor and to glycoprotein Ib. These and other findings suggest that the vWF–glycoprotein Ib pathway is an essential one for hemostatic plug formation (37, 38). rF.VIIa needs to be tested in other types of vWD in which some vWF is present. Under these conditions with limited vWF, rF.VIIa may promote hemostasis.

Note Added in Proof. Since this manuscript was prepared, Thim *et al.* (39) have published on the analysis of human factor VIIa.

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