concentrations in the supernatant were measured by the o-cresolphthalein complexone method (Connerty & Briggs, 1966; Sysmex), a Bathocuproin method (Landers & Zak, 1958; Wako Fine Chemicals, Osaka, Japan) and a Formaldoxime method (Bartley *et al*, 1957; Machereynagel GmbH & Co., Dueren, Germany). The lowest limited values for detection in these assays using the spectrophotometer were 0·5 mg/dl, 1·0 μg/dl and 0·01 mg/dl respectively.

Results

Effect of cation-exchange resins with different function groups on factor VIII and V activity in plasma

The procoagulant activity of factor VIII as well as factor V depends on the presence of metal ions (Fay, 1988; Adams *et al*, 2004). To elucidate the metal ion (cation)-dependent function of both the coagulant factors, we first examined the possibility that cation-exchange resins affected factor VIII and V molecules in plasma. In the present study, three well-described sulfonated, carboxylated and iminodiacetate cation-exchange resins with different ion strengths were used (Table I). The results are shown in Fig 1 and summarized in Table II. The activity levels of factors VIII and V were measured in one-stage clotting assays. The three representative resins with sulfonated groups produced only marginal decreases in the activity of factor VIII (<5%) and V (*c.* 15%). The two resins with carboxylated groups produced a moderate decrease in factor VIII activity (*c.* 50%) and a substantial increase in factor V activity (*c.* 80%). However, the use of three representative resins with iminodiacetate groups resulted in the reduction to undetectable levels of activity of both the factors. The activity levels of factor VIII were similar to those obtained in a two-stage clotting assay (data not shown). Inactivation of factors VIII and V by the iminodiacetate resins was both dose- and time-dependent (Fig 2). In particular, the use of 5% (w/v) resin completely inhibited factor VIII activity after incubation for only 30 min (Fig 2A). Although factor VIII possesses a homologous structure similar to factor V (Vehar *et al*, 1984; Kane & Davie, 1986), this resin reduced factor VIII activity more rapidly and to a greater extent than that observed with factor V (Fig 2B). On the other hand, as expected, the activity of the other coagulant factors in the plasma samples were not significantly affected by treatment with the iminodiacetate resin (data not shown).

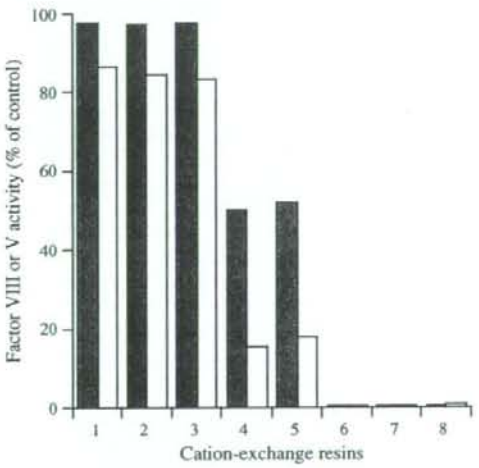

Fig 1. Activity of factor VIII and V in plasma incubated with cation-exchange resins. Normal plasma was mixed with 10% (w/v) cation-exchange resins at 22°C for 2 h. Resins 1–3 belong to the sulfonate group (Amberlite IR120B Na, Amberlite IR124 Na and Amberlite 200CT Na respectively), resins 4 and 5 belong to the carboxylate group (Amberlite IRC50 and Amberlite IRC76 respectively) and resins 6–8 belong to the iminodiacetate group (Amberlite IRC748, Muromac A-1 and Muromac B-1 respectively). The procoagulant activity of factor VIII (*filled bar*) and V (*open bar*) in plasma treated with each resin was measured in a one-stage clotting assay. The activity level of factor VIII or V in untreated plasma was considered to be 100%. Data represent the average values obtained from at least three separate experiments.

Table II. Effect of cation-exchange resins on factor VIII, factor V and von Willebrand factor (VWF) in plasma.

Functional group of resins	Factor VIII (%)		Factor V (%)		VWF (%)	
	Activity	Antigen	Activity	Antigen	Activity	Antigen
Sulfonate	>95	>95	85	92	>95	>95
Carboxylate	52	>95	16	81	>95	>95
Iminodiacetate	<0·5	88	<0·5	21	>95	>95

Normal plasma was incubated with 10% (w/v) resin for 2 h prior to measuring the activity and antigen of factor VIII, factor V and VWF. The values obtained for untreated plasma were considered to be 100%. Data represent the average values obtained from at least three separate experiments.

Effect of iminodiacetate resin on factor VIII- and V-deficient plasma

We next examined whether the action of iminodiacetate resin on factors VIII and V was mutually specific and independent. The iminodiacetate resin (10% w/v) was incubated with commercial factor VIII- or V-deficient plasma for 2 h at 22°C, followed by measuring factor V or VIII activity, respectively, by a one-stage clotting assay. Factor V or VIII activity in resin-treated factor VIII- or V-deficient plasma, respectively, decreased to <10% after a 1-h incubation, and to <1% after a 2 h incubation, similar to that obtained in resin-treated normal plasma (Fig 3). This indicates that the iminodiacetate resin inactivates factors VIII and V both specifically and independently.

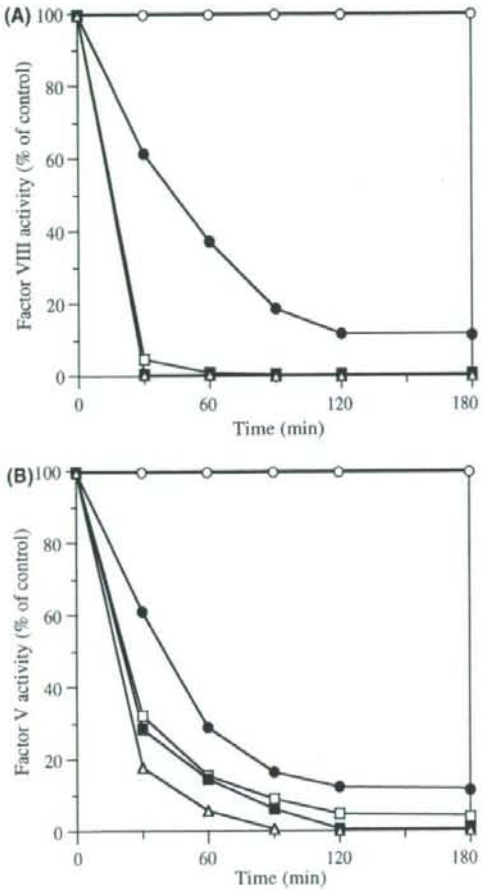

Fig 2. Inactivation of factors VIII and V in plasma by iminodiacetate resin. Varying amounts of iminodiacetate resin (%, w/v) were incubated with normal plasma at the indicated times prior to measuring the activity of factors VIII (A) and V (B) in a one-stage clotting assay. Symbols represent different concentrations of the resin: *open circles*, 0%; *closed circles*, 2·5%; *open squares*, 5%; *closed squares*, 7·5%; and *open triangles*, 10%. The initial activity of factor VIII or V at time zero was considered to be 100%. Data represent the average values obtained from at least three separate experiments.

Effect of iminodiacetate resin on factor VIII and V antigen in plasma

We postulated that the significant reduction of factor VIII and V activity was because of its direct adsorption to the iminodiacetate resin. To investigate this, we measured the levels of factor VIII and V antigen in plasma after incubation with 10% (w/v) resin by sandwich ELISA as described in Materials and methods (Table II). The level of factor V antigen

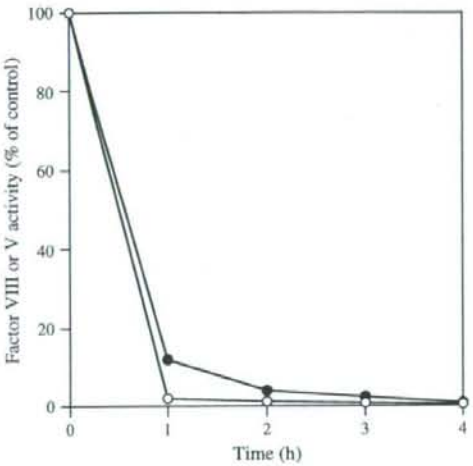

Fig 3. Inactivation of factor VIII in factor V-deficient plasma and factor V in factor VIII-deficient plasma by iminodiacetate resin. Iminodiacetate resin (10%, w/v) was mixed with factor V- and factor VIII-deficient plasma, following the measurement of the activity of factor VIII (*open circles*) or factor V (*closed circles*) in factor V- or factor VIII-deficient plasma, respectively, in a one-stage clotting assay. The initial activity of factor VIII or V at time zero was considered to be 100%. Data represent the average values obtained from at least three separate experiments.

in plasma treated with the iminodiacetate resin was moderately decreased (by *c.* 80%). Surprisingly, the level of factor VIII antigen was, however, decreased by only *c.* 10%. A control experiment showed that the levels of factor VIII and V antigen in plasma treated with the sulfonate or carboxylate resin were marginally decreased, if at all. In addition, fractions eluted from the treated resins by adding 1 N HCl contained negligible levels of factor VIII and V antigen. This indicates that factors VIII and V are not directly adsorbed to the resin, and are inactivated because of a different mechanism(s).

Effect of VWF on factor VIII inactivation by iminodiacetate resin

As VWF stabilizes the cofactor activity of factor VIII, qualitative and/or quantitative abnormalities of VWF in patients with von Willebrand disease result in reduced levels of factor VIII activity. To examine the possibility that the iminodiacetate resin depressed factor VIII activity through direct action on VWF, the levels of VWF activity and antigen in resin-treated plasma were measured. Levels in resin-treated plasma (>95%) were similar to those observed in untreated plasma (Table II). To confirm that the iminodiacetate resin affected factor VIII activity by direct action, a purified recombinant factor VIII was used instead of plasma. Human serum albumin (1% v/v) was added to factor VIII to minimize

Table III. Effect of iminodiacetate resin on the purified factor VIII and factor VIII/von Willebrand factor (VWF).

Factor VIII (%)	
Activity	<0·1
Antigen	<0·1
Factor VIII/VWF (%)	
Factor VIII	
Activity	<0·1
Antigen	>95
VWF	
Antigen	>95

Either recombinant factor VIII alone or plasma-derived factor VIII/VWF complex preparation was incubated with 10% (w/v) iminodiacetate resin for 2 h. The activity and antigen values of untreated factor VIII or VIII/VWF preparation were considered to be 100%. Data represent the average values obtained from at least three separate experiments.

non-specific binding to the resin. Incubation of recombinant factor VIII with 10% (w/v) resin for 2 h resulted in undetectable activity (<0·1%) (Table III). Unexpectedly, in contrast to the results obtained with resin-treated plasma, factor VIII antigen was also reduced to undetectable levels (<0·1%) in the resin-treated recombinant factor VIII. We further examined the effect of the resin on factor VIII complexed with VWF using plasma-derived factor VIII/VWF therapeutic concentrates. In these instances, factor VIII activity was reduced to undetectable levels by incubation with 10% (w/v) resin, whilst factor VIII antigen was preserved at >95% of the initial level in the factor VIII/VWF complex, similar to that obtained with resin-treated plasma. These results indicate that VWF might protect the factor VIII antigen from resin-induced degradation.

Deprivation of metal ions in factor VIII and factor VIII/VWF by iminodiacetate resin

The procoagulant activity and structure of factor VIII is dependent on divalent metal ions, in particular Ca^{2+}, Cu^{2+} and Mn^{2+} (Fay, 1988; Wakabayashi *et al*, 2001, 2005). As the property of the iminodiacetate resin involves cation exchange, we hypothesized that the resin-induced impairment of factor VIII function was associated with the metal ions present in the factor VIII molecule. To confirm this, the effects of the resin on the concentrations of metal ions, Ca^{2+}, Cu^{2+} and Mn^{2+}, contained in factor VIII and factor VIII/VWF were examined. The concentrations of Ca^{2+} and Mn^{2+} in recombinant factor VIII were decreased by >95% with resin treatment (Table IV). Furthermore, both metal ions were recovered by >95% in the fractions eluted from the treated resins by adding HCl, indicating that the resins adsorbed most Ca^{2+} and Mn^{2+} in factor VIII. Cu^{2+} was not detected in factor VIII. Similarly, resins reduced Ca^{2+} by *c.* 95% in plasma-derived factor VIII/VWF complex preparations, whilst Mn^{2+} in this product was decreased by only *c.* 40%. In contrast to factor VIII, 178 μg/dl of Cu^{2+} was detected in the untreated plasma-derived factor VIII/VWF preparation, and the concentration of Cu^{2+} was decreased by *c.* 65% by the resin treatment. Recovery of these ions adsorbed to the resins from the factor VIII/VWF complex was almost complete (Ca^{2+}, *c.* 95%) and substantial (Cu^{2+} or Mn^{2+}, *c.* 50%). This indicates an inactivation mechanism by the iminodiacetate resin that probably disturbs the structure of factor VIII by deprivation of metal ions, predominantly Ca^{2+}, from its cofactor protein.

Effect of addition of metal ions on resin-induced inactivation of factor VIII

Competitive inhibition experiments were conducted to further investigate the association between metal ion concentration and loss of factor VIII activity in resin-treated preparations. Recombinant factor VIII was mixed with increasing amounts of metal ions and was incubated with the resin for 2 h prior to measuring its activity. Addition of Ca^{2+} competitively inhibited the inactivation of treated factor VIII alone by *c.* 20% in a dose-dependent manner, saturating at 75 mM (Fig 4A), whilst addition of Mn^{2+} competitively inhibited by only <10%, with the saturable level at 100 mM (Fig 4C). Addition of Cu^{2+} did not block resin-induced inactivation even at the highest concentrations used (50 mM, Fig 4B). These findings confirmed the importance of Ca^{2+} compared with Mn^{2+} or Cu^{2+} in the

Table IV. Change in metal ion concentrations by treatment of purified factor VIII and factor VIII/von Willebrand factor (VWF) with iminodiacetate resin.

	Factor VIII			Factor VIII/VWF		
	Ca^{2+}, mg/dl(%)	Cu^{2+}, μg/dl (%)	Mn^{2+}, mg/dl (%)	Ca^{2+}, mg/dl (%)	Cu^{2+}, μg/dl (%)	Mn^{2+}, mg/dl (%)
Untreated	15·5 (100)	<1·0 (n.d.)	1·0 (100)	1·6 (100)	178 (100)	5·2 (100)
Treated	<0·5 (1·9*)	<1·0 (n.d.)	<0·01 (<1*)	<0·5 (0*)	63·4 (36*)	3·2 (58*)

The concentrations of metal ions in recombinant factor VIII alone and plasma-derived factor VIII/VWF before and after treatment with iminodiacetate resin were measured as described in Materials and methods. Data represent the average values obtained from at least three separate experiments. The lowest detectable levels of Ca^{2+}, Cu^{2+} and Mn^{2+} were <0·5 mg/dl, <1·0 μg/dl and <0·01 mg/dl respectively. The concentrations of metal ions in the untreated sample were considered to be 100%. Asterisks indicate the values calculated from the concentration of metal ions eluted from the resins by HCl. n.d. not determined.

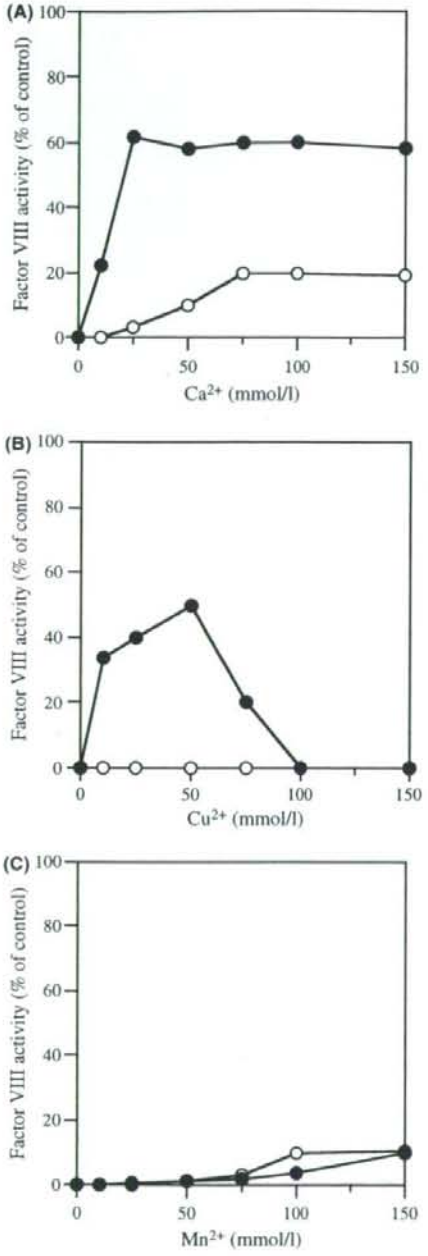

Fig 4. Inhibitory effect of metal ions on resin-induced inactivation of factor VIII in the absence or presence of von Willebrand factor (VWF). Recombinant factors VIII (*open circles*) and plasma-derived factor VIII/VWF (*closed circles*) were mixed with varying amounts of Ca^{2+} (A), Cu^{2+} (B) and Mn^{2+} (C) and were treated with 10% (w/v) iminodiacetate resin, prior to measuring the activity of factor VIII. Factor VIII activity before and after resin treatment were considered to be 100% and 0% respectively. Factor VIII activity was plotted as a function of the concentration of metal ions.

mechanism of resin-treated inactivation of factor VIII. With plasma-derived factor VIII/VWF preparations, addition of Ca^{2+} inhibited the inactivation of factor VIII by *c.* 60%, saturating at 25 mM (Fig 4A). In addition, Cu^{2+} appeared to inhibit inactivation by *c.* 50% at 50 mM, although the activity could not be measured reliably as precipitation was evident in samples at Cu^{2+} concentrations exceeding 50 mM (Fig 4B). However, the addition of Mn^{2+} inhibited by only <10% even at the highest concentrations used (150 mM, Fig 4C). Taken together, these findings indicate that the presence of VWF helps to prevent loss of metal ions from factor VIII, and that Ca^{2+} plays a more important role than Mn^{2+} or Cu^{2+} in conserving the functional structure of factor VIII, thereby supporting our findings.

Comparison of iminodiacetate resin-treated and immunodepleted plasma with factor VIII-deficient plasma

To examine whether iminodiacetate resin-treated plasma was functionally similar to immunodepleted plasma used as factor VIII- or V-deficient plasma, the levels of factor VIII and V activity in 50 samples with varying levels of activity were measured and compared using iminodiacetate resin-treated and immunodepleted plasma as factor VIII- or V-deficient plasma, respectively, in the APTT- or PT-based one-stage clotting assay. As factor V activity completely disappeared in the resin-treated plasma, resin-treated plasma was adjusted by adding bovine-derived factor V up to 100%, prior to measuring the factor VIII activity in APTT assay. We observed an excellent correlation ($r = 0{\cdot}997$) between the values of factor VIII activity obtained in iminodiacetate resin-treated and immunodepleted plasma (Fig 5A). The limited low level of factor VIII activity measured in iminodiacetate resin-treated plasma was 0·2%. Similarly, an excellent correlation ($r = 0{\cdot}997$) was apparent between the values of factor V activity obtained in resin-treated and immunodepleted plasma in PT assay (Fig 5B). Similarly, the limited low level of factor V activity obtained in resin-treated plasma was 0·2%, supporting that iminodiacetate resin-treated and immunodepleted plasma were functionally similar. We further measured the factor VIII activity using resin-treated plasma in a two-stage clotting assay. The levels of factor VIII activity obtained in this assay showed an excellent correlation with those obtained in a one-stage clotting assay ($r = 0{\cdot}995$, data not shown). These results indicate that iminodiacetate resin-treated plasma would be useful as a new laboratory reagent for factor VIII- and V-deficient plasma.

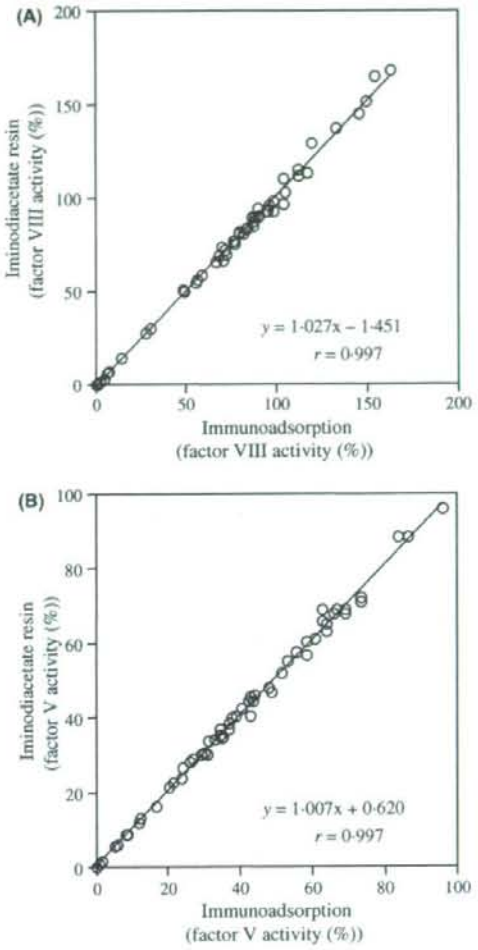

Fig 5. Correlation between iminodiacetate resin-treated and immunodepleted plasma for measurement of factor VIII and V activity. Factor VIII (A) or V (B) activity in plasma with varying levels of factor VIII or V activity, respectively, was measured using plasma samples treated with iminodiacetate resin or immunodepletion in a one-stage clotting assay. The activity level of factor VIII or V obtained using iminodiacetate resin-treated plasma was plotted against that using immunodepleted plasma. All the values obtained were used in the correlation analysis.

Discussion

Cation-exchange resins are classified based on their functional groups. In the present study, we focused on their functional disparities and compared the effects of treatment with resins on the activity of factors VIII and V using three representative groups of resins: sulfonate (SO_3^-), carboxylate (COO^-) and iminodiacetate ($N = (CH_2COO)_2^-$). The procoagulant activity of both factor VIII in plasma and recombinant factor VIII, were completely lost by incubation with the iminodiacetate resins. Similarly, factor V activity in resin-treated plasma was also undetectable, whilst that of the other coagulant factors was not affected. Resin-induced selective inactivation of factors VIII and V was not due to direct adsorption to the resins, but deprivation of the metal ions (predominantly Ca^{2+}) from their cofactor molecules. This is strongly supported by earlier studies, which state that factor VIII possesses a structural homology similar to that of factor V (Kane & Majerus, 1981; Vehar *et al*, 1984; Pemberton *et al*, 1997) and that both cofactors possess divalent metal ion-binding motifs. Therefore, we demonstrated that the resin-induced inactivation mechanism is closely associated with the presence of metal ions.

Ethylenediaminetetraacetic acid also inactivate factors VIII and V by chelating metal ions and promoting dissociation of the heavy and light chains (Fay, 1988), and this property has been used to purify the isolated heavy and light chains of these factors. Reactivity of the EDTA-separated chains with factor VIII or V alloantibodies is retained to some extent, and factor VIII or V activity can be reconstituted from the isolated heavy and light chains by adding metal ions. Unexpectedly, in the present study, the reactivity of the resin-treated factor VIII with anti-C2 alloantibodies was abolished, which indicated that severe disturbances in the conformational structure result in the loss of antigenicity of the C2 domain. Therefore, it appears that the mechanism of iminodiacetate resin-induced inactivation of both factors VIII and V is different from that of EDTA-induced inactivation. The reason for this difference is not clear, but it may be that the loss of metal ions by resin-induced adsorption is much greater compared with EDTA, and consequently the resin might severely deplete the critical molecular metal ions necessary to conserve the conformational structure.

Metal ions can reconstitute factors VIII(a) and V(a) from the heavy and light chains (Fay, 1988; Adams *et al*, 2004). The roles of metal ions to reconstitute factor VIII(a) are entirely distinguishable. Cu^{2+} is predominantly required for the increase in the inter-chain affinity in factor VIII (Wakabayashi *et al*, 2001), whilst the increase in its specific activity requires Ca^{2+} (and Mn^{2+}) to modulate conformation on the anionic membrane (Fay, 1988; Wakabayashi *et al*, 2005). Ca^{2+} is also necessary for stable association of the heavy and light chains in factor V(a) (Sorensen *et al*, 2004), although the functional importance of Cu^{2+} in factor V is still unknown. Iminodiacetate resins removed the metal ions from factor VIII, which was then completely inactivated. Addition of exogenous Ca^{2+} blocked (by *c.* 20%) the resin-induced inactivation of factor VIII, whilst addition of Cu^{2+} or Mn^{2+} had any or a little effect (by <5% or <10% respectively). This suggests that Ca^{2+} appears to be more critical than Mn^{2+} or Cu^{2+} for retaining the suitable conformation required to regenerate factor VIII specific activity, consistent with earlier reports (Fay, 1988; Wakabayashi *et al*, 2001).

The present study showed that the presence of VWF prevents the decrease of factor VIII antigen by the action of iminodiacetate resins. Factor V antigen in resin-treated plasma was significantly decreased compared to factor VIII antigen. This discrepancy is probably because of the presence of VWF, supported by our present observation that factor VIII antigen in resin-treated recombinant factor VIII alone was not detected. VWF stabilizes the cofactor activity of factor VIII and the heavy chain–light chain association (Weiss *et al*, 1977; Fay, 1988; Kaufman *et al*, 1989), but it could not inhibit the decrease of factor VIII activity by the resin. This might indicate that the Ca^{2+}-binding responsible for the specific activity of factor VIII is not protected by VWF, but that for the antigenic structure of factor VIII is protected by VWF. Two major VWF-interactive sites in factor VIII have been located in the acidic region of the A3 and C2 domains (Lollar *et al*, 1988; Saenko *et al*, 1994). Therefore, the Ca^{2+}-binding site(s) responsible for the antigenic structure of factor VIII may overlap or be located close to the VWF-binding site(s) on the factor VIII light chain. More recently, a Ca^{2+}-binding site with high affinity (K_d, *c.* 10 μM) in factor VIII has been identified in the A1 domain of the heavy chain using alanine-scanning, site-directed mutagenesis (Wakabayashi *et al*, 2004). The presence of multiple Ca^{2+}-binding sites with low affinity (K_d, *c.* 4 mM) in the light chain has also been speculated. However, the Ca^{2+}-binding site(s) in the factor VIII light chain has still not been identified.

The immunoadsorption approach is a well-known technique for the preparation of deficient plasma samples used as specific substrates for clotting assays. Attempts to specifically deplete factor VIII or V have been reported (Ofosu *et al*, 1980). Both the antigen and activity of factors VIII and V can be specifically removed by using the property of antibodies; however, several problems may arise while implementing this approach. The presence of residual factor VIII or V is observed by the limitation of binding capacity to antibody. Furthermore, residual factor VIII is related to its linkage to VWF, leading to high-molecular weight complexes that cannot completely react with specific antibodies because of steric hindrance. VWF may be removed from plasma in the form of factor VIII/VWF complex. In addition, monoclonal antibodies are expensive, and the column coupled with antibody has a limited period of use. On the other hand, the approach presented here can lead to the complete inactivation (<0·1%) of factor VIII or V in a short time, independent of the presence of VWF, although this resin inactivates both factors at the same time. In comparison with immunodepletion, this process costs less and has higher mass productivity. Furthermore, regeneration of the iminodiacetate resin enables its repeated use. In fact, iminodiacetate resin-treated plasma is functionally equivalent to immunodepleted plasma, as indicated by measuring factor VIII or V activity in 50 samples as substrates for factor VIII- or V-deficient plasma respectively. Furthermore, the factor VIII activity measured using resin-treated plasma was not significantly different between the one-stage and two-stage clotting assays. Finally, we propose that iminodiacetate resin-treated plasma is very useful as a new laboratory reagent for factor VIII- and V- deficient plasma.

Acknowledgements

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多彩な解離性障害を呈した重症型血友病Aの1例

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要　旨

症例は14歳の男子．生後9か月時に血友病Aの診断を受け，現在は第VIII因子製剤を定期的に補充する在宅自己注射療法を行っている．生来まじめでおとなしい性格であり，厳しい父に対して反抗したこともなく，学校の成績も優秀であった．X年8月末（14歳5か月時）に，持続する腹痛を主訴に当科を受診したが，外科にて慢性虫垂炎と診断され虫垂切除術を施行した．術後の経過は良好であったが，その後，左下肢の知覚消失を訴え，さらに上腹部痛も出現した．自宅で患児の腹痛について父と激しい口論中に，突然過呼吸状態となり，意識消失とともに四肢の強直が出現した．その発作以降，父に関する記憶が消失し，また，同様の発作が頻回に出現するようになったため，X年10月に解離性障害の疑いにて当科に入院した．入院による精神療法と家族（支援）療法を開始し，その際両親の面会制限を行った．入院中の対応で葛藤が軽減し高校に合格したことで，退院可能となった．本症例の解離性障害の発症には血友病というX連鎖劣性遺伝性疾患特有の母・息子関係と父・息子関係とが深く関与していると思われた．家族関係の精神的負担の大きい血友病症例と長期に関わる場合は，身体症状のみならず，疾患の遺伝様式からもたらされうる特殊な親子関係を理解した上で，両親の児に対する関わりや父母の連合といった家庭環境，さらに児自身の精神的心理的側面をも同時に注意深く対応する必要性が示唆された．

キーワード：解離性障害，血友病，家族関係，X連鎖性劣性遺伝

はじめに

小児は，家庭環境を含めた周囲の環境変化に影響を受けやすく，また，心的ストレスを自分で理解し解決する能力が未熟であり，その心的ストレスを身体症状に置き換えて発散することが多い[1]．その背景には，周囲の環境やストレスに対する児自身の適応能力の未熟性はもちろんのこと，母子家庭や父子家庭といった家庭環境の偏り，家族内のコミュニケーション不足・不全，養育者自身の未熟性など，システムとしての家族の問題が大きく関連していると思われる[2]．

解離という概念はJanetにより提唱されたもので，感情，感覚，運動および思考の統合が障害された状態を表す[3]．解離性障害は，アメリカ精神医学会の診断分類では1980年のDSM-III，国際的な診断分類では1992年のICD-10ではじめて登場した概念である．患者自身は気づいていない動機（心因）によって，意識，記憶，同一性あるいは環境についての知覚といった，個人にとって通常は統合されているはずの機能が破綻することによって生じるさまざまな障害のことをいう．解離性健忘，解離性遁走，解離性混迷，トランスおよび憑依障害，解離性運動障害や解離性けいれんなどに分類される[4]．あくまでも症状の原因となる身体的基礎疾患が否定された場合に診断される．

今回，血友病というX連鎖劣性遺伝性疾患特有の家族関係を背景として発症し，環境調整を中心とした入院治療で症状が軽快した解離性障害の1例を経験した．

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症　例

14歳（中学3年生）の男子．

主訴：痙攣発作と健忘

家族歴：両親と妹の4人家族．母方祖母の兄弟3人に出血傾向あり．母は血友病保因者と診断されていた．

既往歴：出生と発達発育歴に異常なし．生後9か月時に頭蓋内出血を発症し血腫除去術を受け，その際，重症血友病Aと診断された．その後，第VIII因子同種中和抗体（インヒビター）が出現したため，2歳時に当科を紹介された．インヒビター力価は3歳時に75BU/mLまで上昇し，出血時にはバイパス製剤を使用していた．6歳時に免疫寛容療法（当初は第VIII因子製剤80単位/kg×2回/週）を開始したところ，1年以内にインヒビターは消失した．現在は週3回第VIII因子製剤の定期的在宅自己注射を行っており，重篤な出血はほ

とんどない.

病前性格：まじめでおとなしく心優しい性格．学校では中心的存在ではなかった．学力は高く学校での問題行動もなかったが，頑固で，自分がまじめだと思われることを極端に嫌がる面があった．家庭では自ら意見を強く主張したり，わがままを言うことはまれであった．父は患児に厳しかったが，患児は母に愚痴を言うことはあっても，父に反抗したことはなかった．常に母のことを気にかけ，自分が良い成績をとらなければ母が父に怒られると気遣い，勉強を頑張っていた．中学3年生になり高校受験を控え，不安とあせりを感じている様子がうかがえた．

家族背景・交友関係：父は，患児が中学に入り塾へ通い始めた頃から勉強に関してさらに患児に厳しくなった．父としては，患児が好きであり成功してほしいからこそ厳しくしていたという．一方，母は，患児が勉強はできなくてもいいから健康ですくすくと育ってほしいと思っていたが，父に対しては自分の考えをはっきりとは言えなかった．妹は，患児の5歳年下で兄妹の関係は良好であった．兄を見て育っているので父に怒られないように要領よくやっていた．患児の友人関係は幅広くはないが，学校での友人とのトラブルはなく，仲の良い同級生の友人も3人いた．

現病歴：X年8月末（14歳5か月時）から右下腹部痛を訴えた．出血は否定的であり，外科に紹介し9月に慢性虫垂炎の診断で第VIII因子補充療法下に虫垂切除術をうけた．術中・術後と退院後の経過は良好であったが，9月末には運動会の練習後，膝関節痛が出現した．膝関節出血と考え，患児の判断で第VIII因子製剤の在宅自己注射を連日行い，関節痛はいったん改善した．その後，左下肢の知覚の消失を訴えたため当院整形外科を受診したが，運動機能に異常なく経過観察中であった．

10月2日から上腹部痛が出現した．10月5日夜，腹痛の原因をめぐって父と話し合っているうちに，初めて患児が父に反抗し，激しい口論となった．その際，突然過換気状態となり，意識が消失するとともに四肢の強直が出現した．しばらくして四肢強直は回復したが，その直後から父に関する事柄を中心とした記憶が消失し，その後も同様の発作を繰り返したため，10月6日に当科に入院した．

入院時現症：体温37.1℃，脈拍76/分，呼吸数20/分，血圧128/68mmHg，心音整，肺ラ音聴取せず．腹部平坦軟で上腹部に圧痛あり，肝・脾触知せず．項部硬直なし．

入院時検査所見：血液検査，頭部MRI検査，脳波検査および胃透視検査のいずれも異常を認めず．

心理検査所見：当院精神科にて心理検査ならびに知能検査を行い，以下のような結果を得た．WISC-IIIで言語性IQ＝108，動作性IQ＝78，全検査IQ＝93と全体的には平均域に入っていたが，言語性検査の成績に比べて動作性検査が有意に低かった．群指数や下位項目，検査時の様子を総合的に評価し，教科書的な知識や問題解決の戦略に関する知識は優れているものの，人との付き合いや生活適応において必要な力が未熟であるため，小さな挫折や対人関係のもつれにもつまずく可能性が高いと思われた．また，動作性IQの低値は学業の伸び悩みと関連していた可能性がある．ロールシャッハテストでは，思考過程そのものに病的な歪みは見られないものの，目にした情報を捉える時点で自分にとって都合のいいように歪めてしまう傾向が見られた．すなわち，自分にとって不利な問題や自らの価値が脅かされるような事実に直面すると，恐怖や不安を感じないように自分に都合のいい解釈をすることで精神の安定を図ろうとするという傾向が強いと考えられた．困難への対処力や欲求不満に耐える力などが未熟である中で，現実的なストレスが重なり，患児の処理能力を超えたため，症状への転換が起こったと考えられた．

診断：頭部画像検査，脳波検査および胃透視検査などの検査により中枢神経系あるいは消化器系の器質的疾患は否定され，また心理検査により統合失調症も否定的であった．言語性IQと動作性IQに差が認められたが，それまで発達の異常を指摘されたことはなく，また対人性，コミュニケーションの障害などの発達障害を示唆する所見はなかった．一方，転倒による打撲や尿失禁などは一切なかったこと，医療者の面前での症状が特に強かったこと，発作中でも医療者の指示に従う動作を見せ，また坐薬を入れるとの声かけにより発作が消失したことなどから，てんかんとは診断できず，解離性障害による偽発作と考えた．患児にとって父との葛藤，高校受験など強いストレスの存在下で，知覚障害，偽発作や健忘という多彩な症状が出現していることから，ICD-10に基づき，解離性健忘と解離性知覚障害，解離性けいれんが混合した混合性解離性（転換性）障害と診断した．

入院後経過：

1．第一期（解離症状が頻繁にみられた時期）：中学3年の10～11月

入院後は嘔気と胃痛とを訴え，その後に過呼吸状態となり意識を消失するという一連の発作が毎晩のようにみられた．徐々に発作回数および持続時間は増加・延長し，その症状も増悪してきた．我々は，父母との面接を頻繁に行う一方，児については父のみならず母との面会も最小限に制限して母子分離を図りながら，両親の連合の強化を目指した．これにより，発作の回

数は1〜2日に1回とやや減少し持続時間も短くなった．しかし，父に関する記憶は回復しなかった．また，同室の年下の患児に対し心優しい面をみせる一方で，医療者に対してはわがままや文句を言うことが多くなり，攻撃的な部分が見られるようになってきた．

父は，医師に対しては自らに非があると言っていたが，母には自分の行ってきたことは間違っていないと主張していたという．我々の話を聞こうとする姿勢も希薄であり，父の協力を得ることは困難と思われた．

母は患児の入院に対し非常な不安を抱いていた．患児に対する教育方針の不一致から，いずれ父とは離婚したいと考えていた．父と向き合うようにしてほしいという我々の願いにも，当初は難しいと言っていたが，徐々に父と対話をするよう努力している様子がうかがえた．

3人の友人については，会えば誰かはわかるが，記憶はあいまいであった．彼らは患児のその状態を理解し受け入れていた．10月末には患児が外出して友人と遊びたいと訴え，母親同伴で何度か外出したが発作を起こすことなく無事帰院できた．この友人との外出が非常に楽しくストレスの解消になるようであった．

学習面に関しては，入院したこともあり勉強がはかどらず，入院中に受けた中間試験の成績は学年平均以下であったが，その結果を受容しがたい様子であった．テスト後の懇談でも希望の高校は厳しいと言われ，非常にショックを受けていた．入院当初から当院院内学級に通級していたが，学級でも病室でも患児が自分のペースで学習できるよう指導した．また，院内学級担任および児童精神科医と，患児の病状や言動，治療方針などに関して頻繁に情報交換を行うことにより連携を密にし，一貫した対応を行うよう心がけた．

2．第二期（攻撃性が前面に出ていた時期）：中学3年の12月

発作の回数は週1回程度まで著減してきた．記憶に関しては，本人は戻ってないと言う一方で，実際には誰も患児には伝えていない過去の事柄を「俺は…だったらしい」という言い方で表現するようになり，徐々に記憶が回復しつつあるようであった．

しかし，父に関しては，抑圧されていた記憶が意識の表層へ再び顕在化するにつれて，強い憎しみを感じるようになってきており，「父親を許すつもりはない」，「父親のエゴのせいでこの2年間はめちゃくちゃになった．父親が母親を叱るから自分は一生懸命勉強したのに希望校にも行けなくなった．しかも父親は自分に直接ぶつかって来なかった．父親の態度が変わっても家に帰るつもりはない」と言い，さらに，「血友病の自分が生まれてきたから家族がめちゃくちゃになった．自分がいなかったらもめることはなかった．3人で仲良く暮らして欲しい」と話すようになった．この頃から，憎しみや怒りだけでなく，不安・焦燥などの自らの感情を，院内学級での日記に文章で表出することが多くなってきた．適宜面接を行い，患児の話を傾聴し，支持・受容するようにした．

3．第三期（改善傾向となった時期）：中学3年の1〜3月

このように父親に対する感情や自らの疾患についての思い，受験への不安などを吐露できるようになるに従い，精神的に落ち着きをみせるようになり発作もほぼ消失した．ランクを下げた高校を受験してもよいと言い出し，前向きに勉強に励むようになった．1月中旬から母に，「父親と会いたい．会って記憶を取り戻し，家に帰って普通の生活に戻りたい」と言うようになり，心理面での変容が生じているものと思われた．1月末に試験的に父と対面したが，すぐに入院当初と同様の発作が起こった．しかしその後も父に会いたがったため，週1回程度短時間の面会を行うこととした．その後は発作を起こすことなく面会できた．

2月初め突然，「父のことを思い出した」と言い出し，初めて父と5分ほど会話をすることができた．その後も父に頻繁に会いたがり，勉強は手につかない様子であった．会った後は，憎しみの気持ちを口にすることが多かった．2月中旬の入試当日は病棟から試験会場に向かい，発作もなく試験を終えた．無事合格し，その後はますます家に帰りたがり，退院後の生活を話すようになり，表情も穏やかになった．2月末から父との対面を増やし，自宅への試験外泊も何度か行ったが，発作はみられなかった．

この間，父は徐々に我々に本音を打ち明けるようになった．「自分の思うようにならない息子に対し，何を話せばいいか分からない」と話しつつも，「自分と対面することで息子の記憶が戻るきっかけとなればいいと思う」と患児を思いやる気持ちも口にするようになった．対面時はぎこちないながらもやさしく声をかけようと試みていた．

この時期も，当初は依然として父母の間に会話は少なく，父が母に対して攻撃的な発言をおこなうだけであったため，母も「もう限界だ」と漏らしていた．しかし，患児と父との対面の話が出てからは，主に連絡事項とはいえ，夫婦間で会話をする機会が増え，母にも少し明るい表情がみられるようになった．患児の心の変容，高校合格という目に見える成功体験を契機に両親の連合も家庭の安定に向けて強化されているように思われた．3月11日に退院したが，退院後，発作はほとんどみられない．第VIII因子製剤の定期補充療法継続のために小児科に通院しているが，両親の付き添いはなく，本人のみで受診することが多い．

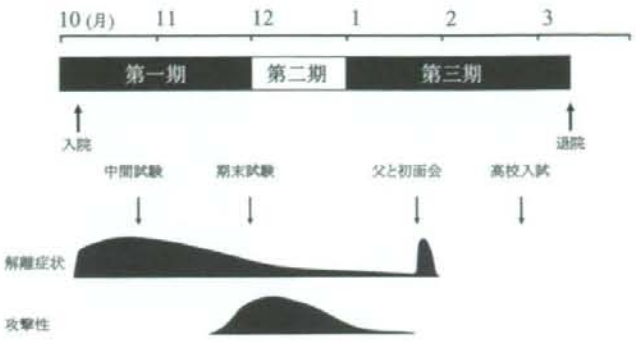

図1　経過表

考　案

本症例は上腹部痛，知覚障害，意識消失，偽発作，および健忘という多彩な症状で解離性障害を発症した．血友病Aという基礎疾患が存在したため，出血を含めた器質的疾患の否定が重要であったが，入院後の精査により否定された．高校受験を控えていた時期に虫垂炎にて入院し，勉強ははかどらず，患児自身も成績に伸び悩みを感じていた．厳しい父からの勉強のプレッシャーはさらに強くなったが，第二反抗期を迎えていた患児は，生まれて初めて父と激しく口論するという強い心的ストレスを体験した．これらのストレスが重なった際に知覚障害や偽発作，健忘などの解離症状が出現しており，ICD-10に基づき混合性解離性（転換性）障害と診断した．

診断には器質的疾患の除外の他に，統合失調症や身体表現性障害など他の精神疾患との鑑別も重要である．ICD-10，DSM-IV両者において解離性障害と身体化障害は別のカテゴリーに分類されるが，症状の合併が高率に見られることから，臨床上明確に区分することは容易ではない[8]．本症例は，現在は身体化障害の診断には至っていないが，その前駆状態であった可能性もあり，継続観察が必要である．なお，本症例では，対症療法として腹痛に対し胃粘膜保護剤を，過換気に対し抗不安薬を処方したが，効果はほとんど認めなかった．しかしながら，薬剤の効果が小さいことを患児とその家族に認識させるためにも対症療法は用いてよいのではないかと考える．

本症例における発症背景の一つには患児の適応能力の未熟性がある．困難への対処能力，欲求不満に耐える能力などが未熟であるところに心的ストレスが重なり，ストレスからの逃避あるいは回避のための自己保全機序として解離症状が出現したと思われる[6]．また，患児が血友病であるという背景は重要であろう．血友病Aは凝固第VIII因子活性の低下ないしは欠損により出血傾向を来たすX連鎖劣性遺伝性疾患である[7]．母方のX染色体上の第VIII因子の遺伝子異常が，男児の1/2に受け継がれて発症する．遺伝子の突然変異により発症する場合も約3割ある[8]ものの，多くの場合は母が保因者である．このため，一般に血友病患児をもつ母は自分からの遺伝のために息子がこのような病気になったという思いがある．また，幼児～学童期には転倒や打撲により皮下出血や関節内・筋肉内出血を頻繁に引き起こすため，過干渉になりがちで母子分離が困難になりやすい．一方，父には，自分ではなく母からの遺伝により息子が血友病になったとの思いがある上，遺伝を通じた強固な母子関係に自分の入り込む余地がなく，母に息子をとられたという思いのある場合が多い[9][10]．本症例でも，確実保因者の母から重症型血友病Aの患児が生まれており，母は患児のことを非常に心配し「勉強はできなくてもいいからとにかく元気にすくすく育ってほしい」と言い，父は「血友病のことがあり母と息子のつながりは非常に深い．それゆえに息子を取り戻したいと思っていた」と話していた．このような長年にわたる母子関係，父子関係を中心とする特異な家族関係が，高校受験を契機として患児の解離性障害の発症に大きく関わったと思われた．

重症型血友病Aは，1960年代以前には，生命予後の不良な疾患であったが，補充療法剤としてクリオプレシピテートが開発され，平均寿命は20歳を越えるようになった．さらに高濃縮凝固因子製剤が登場し，ウイルス不活化により安全性も確保されるようになった．現在では，モノクローナル抗体や遺伝子組み換え技術による高純度な製剤による定期補充療法により，患児のQOLは向上し，健常児と変わらぬ生活を送ることも可能となっている．医療者から見た「血友病のコン

トロール」は，出血の回数や程度を指標とし，四肢関節や筋肉の機能状態による評価が主体となりがちである．その観点からは，血友病Aは今日充分コントロール可能な疾患となった．一方，血友病患者を抱える家族の親子関係，あるいは血友病患者自身の精神的心理的側面についてはこれまでにも検討されてきたが[9)~11)]，血友病患者が解離性障害を発症するまでに至った報告は稀である．しかしながら，血友病患児を診療するなかで精神的心理的問題を抱える患児に遭遇することは必ずしも稀ではなく，すべての血友病患児および家族にとって起こりうる問題であると思われる．血友病の疾患のコントロールが出血の管理のみに留まるものでないことは言うまでもなく，ここに血友病の包括医療の確立が求められる．

我々は，血友病患者の平均寿命の延長とQOLの向上をめざしつつ，血友病患児を全人的に診察する重要性と必要性に迫られているといえよう．また，このことは血友病だけに留まるものではなく，X連鎖劣性遺伝疾患の患児を診療する場合には，遺伝形式に依存した疾患特有の家族関係の問題を内包しており，診断当初から家族のシステムが機能不全に陥る可能性があることを念頭におきながら，患児のみならず家族全体と関わり，支援していくことが重要であろう．

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Dissociative Disorder with Diverse Symptoms in a School Boy with Severe Hemophilia A

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We reported a case of dissociative disorder with severe hemophilia A. A 14-year-old hemophiliac boy was admitted to our hospital with dissociative disorder in October. He had a warm personality and had done well at school. One month before hospitalization, he underwent an appendectomy. Although postoperative recovery was uneventful, sensory disorder of the lower extremities developed, following epigastric pain. During a ferocious argument with his father over the cause for his abdominal pain, his whole body stiffened and he lost consciousness. Immediately thereafter, he lost memory of his father, and similar paroxysms occurred frequently. After admission, environmental intervention by limiting hospital visitation of parents and including family psychotherapy gradually resolved the intrafamilial conflict, and the symptoms improved. He passed the high school entrance exam and was discharged after a five-month hospital stay. The unique mother/father-son relationships of hemophilia A patients, that is an X-linked recessive inherited disorder, would cause the development of dissociative disorder. It would not be enough to evaluate somatic symptoms only, but we should also take familial relationships such as parental union and the active involvement of parents in mental growth of a diagnosed child. The psychological and psychiatric aspect should be carefully observed as well.

Letters to the Editor

Elevation of B cell-activating factor belonging to the tumour necrosis factor family (BAFF) in haemophilia A patients with inhibitor

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Dear Sir,

The development of factor VIII (FVIII) neutralizing antibodies (inhibitors) is one of the serious complications in the clinical management of haemophilia A. As replacement therapy with FVIII concentrates is ineffective or markedly impaired in this setting, the management of inhibitor patients is full of difficulty. Although FVIII inhibitor will develop by both B cell- and T cell-dependent immune system (1, 2), the precise immune regulatory mechanism of inhibitors has not been fully addressed.

Recently, new ligands, BAFF (B cell-activating factor belonging to the tumour necrosis factor [TNF] family, also known as BlyS) and APRIL (a proliferation inducing ligand), have been found to regulate immune system. BAFF is a member of the TNF superfamily of ligands and is involved in the survival and maturation of B cells (3). Another member of the superfamily, APRIL, also stimulates B- and T-cell proliferation and triggers humoral immune responses (4). BAFF binds to the TNF-related receptors such as B-cell maturation antigen (BCMA), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and BAFF receptor (BAFFR), whereas APRIL binds to TACI and BCMA and to heparan-sulfate proteoglycans (5). Such BAFF/APRIL ligand-TNF related receptor interaction comprises a complex network that is critically involved in the induction and regulation of humoral immunity. We hypothesized that BAFF and/or APRIL would participate in the development and preservation of inhibitors. To test the hypothesis, we measured BAFF and APRIL levels in haemophilia A patients with and without inhibitors.

Citrated plasma samples were obtained from 25 healthy individuals and 21 haemophilia A patients without inhibitors. Sixteen samples were from eight haemophilia A patients with inhibitors at different times. Mean (± standard deviation [SD]) historical peak inhibitor titers were 420.2 ± 877.8 BU/ml (range 8.2–2586). Inhibitors had been present at least for nine months when sample plasma was taken. All haemophilia A patients with and without inhibitors were diagnosed in our laboratory as severe or moderate type haemophilia A. Since BAFF level is significantly increased in patients with chronic hepatitis C virus (HCV) (6), haemophilia A patients with HCV infection were not enrolled in this study.

Plasma BAFF and APRIL levels were measured using specific ELISA kits (R&D Systems, Minneapolis, MN, USA and Bender MedSystems, Burlingame, CA, USA, respectively). Each sample was tested in duplicate. Total plasma IgG was also measured with a standard immunoturbidimetric test. Statistical analysis was performed using unpaired t-tests between groups.

Mean (± SD) inhibitor titer in haemophilia A patients with inhibitors was 104.0 ± 191.9 BU/ml (range 1.2–742). Mean (± SD) age of healthy individuals was 16.7 ± 14.0 years (range 1–42), that of haemophilia A patients without inhibitors was 15.0 ± 6.1 years (range 4–26), and that of haemophilia A patients with inhibitors was 9.4 ± 10.6 years (range 3–44). There was no significant difference in age among these groups.

In non-haemophiliac individuals (n=19), the plasma levels of BAFF and APRIL correlated well with those of serum ($p<0.001$, $R^2=0.92$; $p<0.001$, $R^2=0.94$, respectively), confirming the validation of these assays using citrated plasma samples.

Mean plasma BAFF levels (± SD) were significantly higher in haemophilia A patients with inhibitors (896 ± 191.4 pg/ml, range 594–1,399) than in healthy controls (746 ± 220 pg/ml, range 375–1,269) ($p<0.05$) and in haemophilia A without inhibitors (751 ± 236 pg/ml, range 352–1,056) ($p<0.05$) (Fig. 1, left). There was no significance between BAFF levels in healthy controls and in haemophilia A patients without inhibitors ($p=0.938$). In APRIL levels, no significance was observed between in healthy controls (10.0 ± 7.5 ng/ml, range 0.4–24.0) and in haemophilia A patients without inhibitors (8.2 ± 6.1 ng/ml, range 0.0–17.4) ($p=0.415$), between in healthy controls and in haemophilia A patients with inhibitors (15.1 ± 15.6 ng/ml, range 0.1–51.3) ($p=0.169$), and between in haemophilia A patients without inhibitors and those with inhibitors ($p=0.101$). These results suggest that BAFF is involved in the regulation of immunological response toward preservation of inhibitor. Meanwhile, significant differences of BAFF/APRIL levels were not observed between severe type and moderate type of haemophilia A (data not shown). No evident correlation between BAFF/APRIL

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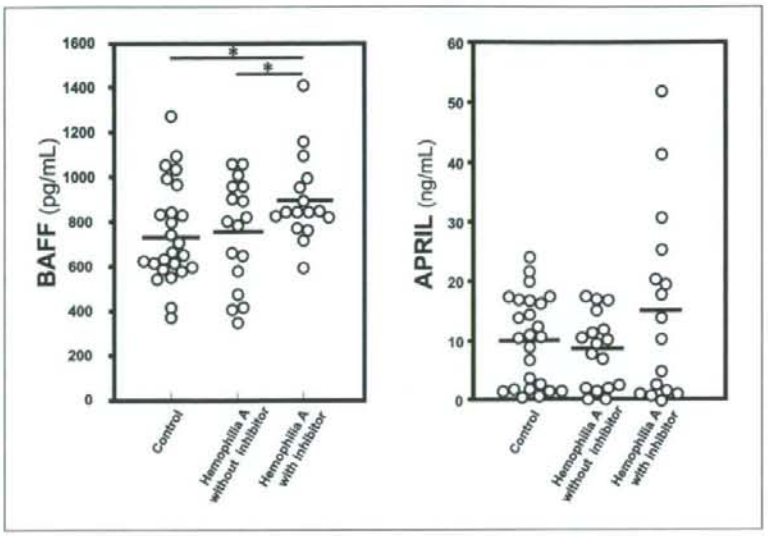

Figure 1: Plasma BAFF and APRIL levels in healthy controls, haemophilia A patients without inhibitors, and haemophilia A patients with inhibitors. Bars represent mean. Left: Plasma BAFF levels. Right: Plasma APRIL levels. *: $p<0.05$.

level and inhibitor titer, historical peak, and duration of inhibitor was confirmed (data not shown). Total IgG levels were within normal range consistent with age in all haemophilia A patients (data not shown). No correlation was observed between BAFF and IgG levels in haemophilia A patients with and without inhibitors (data not shown), suggesting that elevated BAFF levels in haemophilia A patients with inhibitors were insufficient for aberrant elevation of total IgG. Further IgG subclass analysis will be helpful to investigate the role of BAFF in inhibitor preservation as BAFF might induce IgG subclass switch.

Previous mouse studies demonstrated that constitutive BAFF overexpression results in the survival of autoreactive B cells (3, 7, 8), leading to a breakdown of peripheral tolerance. In this setting, autoimmune disorders develop in mice through aberrant activation of B cells, spontaneous production of multiple autoantibodies and polyclonal hypergammaglobulinaemia. In humans, elevated BAFF levels are correlated with hypergammaglobulinaemia and several B cell-mediated autoimmune diseases (9–11). Previous studies showed that anti-FVIII antibodies in the circulation and anti-FVIII antibody-secreting cells in the spleen and bone marrow persist for a long time even after termination of FVIII treatment (12). In haemophilia A patients with inhibitors, elevated BAFF levels would allow anti-FVIII antibody-secreting plasma cells to survive and produce inhibitors.

On the other hand, APRIL levels showed little difference among healthy controls, haemophilia A patients without inhibitors, and those with inhibitors. Although APRIL promotes IgA and IgG1 class switching in mouse IgM^+IgD^+ B cells (13), elevated APRIL levels in some haemophilia A patients with inhibitors remains of unknown significance. Further investigations will be required to address the role of APRIL in the haemophilia A patients with inhibitors.

Hitherto, congenital haemophilia A patients with inhibitors have received fewer B cell-targeted therapies such as rituximab therapy (14, 15) than acquired haemophilia (16). Treatment with a BAFF antagonist such as belimumab, a fully human monoclonal antibody that specifically binds to and neutralizes BAFF, was started in rheumatoid arthritis patients and demonstrated safety and efficacy (17). Targeting BAFF may represent a new therapeutic strategy in a subset of haemophilia A patients with refractory inhibitors presenting elevated BAFF levels.

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The Factor VIIIa C2 Domain (Residues 2228–2240) Interacts with the Factor IXa Gla Domain in the Factor Xase Complex*

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Factor VIIIa functions as a cofactor for factor IXa in the phospholipid surface-dependent activation of factor X. Both the C2 domain of factor VIIIa and the Gla domain of factor IXa are involved in phospholipid binding and are required for the activation of factor X. In this study, we have examined the close relationship between these domains in the factor Xase complex. Enzyme-linked immunosorbent assay-based and surface plasmon resonance-based assays in the absence of phospholipid showed that Glu-Gly-Arg active site-modified factor IXa bound to immobilized recombinant C2 domain (rC2) dose-dependently (K_d = 108 nM). This binding ability was optimal under physiological conditions. A monoclonal antibody against the Gla domain of factor IXa inhibited binding by ~95%, and Gla domainless factor IXa failed to bind to rC2. The addition of monoclonal antibody or rC2 with factor VIIIa inhibited factor IXa-catalyzed factor X activation in the absence of phospholipid. Inhibition was not evident, however, in similar experiments in the absence of factor VIIIa, indicating that the C2 domain interacted with the Gla domain of factor IXa. A fragment designated C2-(2182–2259), derived from V8 protease-cleaved rC2, bound to Glu-Gly-Arg active site-modified factor IXa. Competitive assays, using overlapping synthetic peptides encompassing residues 2182–2259, demonstrated that peptide 2228–2240 significantly inhibited both this binding and factor Xa generation, independently of phospholipid. Our results indicated that residues 2228–2240 in the factor VIIIa C2 domain constitutes an interactive site for the Gla domain of factor IXa. The findings provide the first evidence for an essential role for this interaction in factor Xase assembly.

Factor VIII, a plasma protein that participates in the blood coagulation cascade, is deficient or defective in individuals with hemophilia A. Factor VIII circulates in plasma as a noncovalent complex with VWF,[2] which stabilizes the synthesis and activity of the cofactor. Mature factor VIII is synthesized as a single chain polypeptide of ~300 kDa consisting of 2,332 amino acid residues (1, 2). Based on internal homologies of the amino acid sequence, factor VIII has three types of domains arranged in the order of A1-A2-B-A3-C1-C2 (3). Factor VIII circulates in the plasma as a heterodimer of a heavy chain, consisting of the A1, A2, and heterogeneous fragments of partially proteolyzed B domains, together with a light chain consisting of the A3, C1, and C2 domains (1, 3).

The carboxyl-terminal 159 amino acids of factor VIII comprise the C2 domain, which is involved in binding to both VWF (4–6) and phospholipid membrane surfaces (6, 7). Binding in this domain appears to be competitive and mutually exclusive (4, 5, 8, 9). The C2 domain has also been shown to participate in binding to factor Xa and thrombin (10, 11). Additionally, a major epitope for allo- and autoantibodies and for monoclonal antibodies has been located within the C2 domain (4, 6, 12), indicating that this region could be an antigenic "hot spot." Consequently, important aspects of the expression and regulation of factor VIII appear to be governed by the structure and function of the C2 domain.

Factor VIIIa functions as a cofactor for factor IXa in the anionic, phospholipid surface-dependent conversion of factor X to Xa. In intrinsic factor Xase, factor VIIIa binds to factor IXa and increases the k_{cat} for factor Xa formation by several orders of magnitude compared with factor IXa alone (13). The A2 domain of factor VIIIa interacts with the catalytic domain of factor IXa, and the A3 domain interacts with the first epidermal growth factor domain (14). Although the affinity of isolated A2 for factor IXa is low (K_d ~ 300 nM), it amplifies the enzyme activity of factor IXa by modulating an active site in the catalytic domain, and this interaction defines the cofactor activity of factor VIIIa (15). Factor IXa-interactive sites in the A2 domain are located in at least three regions, within residues 484–509 (16), 558–565 (17), and 708–717 (18), respectively.

In contrast, the high affinity (K_d ~ 15 nM) of the isolated factor VIIIa light chain for factor IXa provides the majority of the binding energy for this interaction. To date, one region within the A3 domain, residues 1804–1818, has been identified

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[2] The abbreviations used are: VWF, von Willebrand factor; Gla, γ-carboxyglutamic acid; rC2, recombinant C2 domain; HRP, horseradish peroxidase; GDless, Gla domainless; EGR-ck, Glu-Gly-Arg-chloromethylketone; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography coupled to mass spectrometry; EGR-factor IXa, Glu-Gly-Arg active site-modified factor IXa; EGR-GDless factor IXa, Glu-Gly-Arg active site-modified Gla domainless factor IXa; BSA, bovine serum albumin; SPR, surface plasmon resonance; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody.

as a factor IXa-interactive site (19, 20). Recently, Blostein *et al.* (21) demonstrated that the light chain of factor VIIIa interacts with the Gla domain of factor IXa, which contains 12 post-translationally modified glutamic acid residues (γ-carboxyglutamic acid) and functions in calcium-dependent phospholipid binding (22). However, the site in the light chain of factor VIIIa responsible for interaction with the Gla domain of factor IXa remains to be identified. The C2 domain of factor VIIIa and the Gla domain of factor IXa are involved in phospholipid binding, and both bound sequences could be aligned structurally close. We speculated, therefore, that the C2 domain of factor VIIIa might interact with the Gla domain of factor IXa in the factor Xase complex.

In the present study, we have examined the interaction between the C2 domain of factor VIIIa and the Gla domain of factor IXa in the factor Xase complex, using a combination of functional and binding assays employing recombinant C2 domain (rC2), V8 protease-digested C2 fragments, synthetic peptides, and monoclonal antibodies. Our results indicated that residues 2228–2240 in the C2 domain contain an interactive site for the Gla domain of factor IXa. The findings provide the first evidence for an essential role of this interaction in factor Xase assembly.

MATERIALS AND METHODS

Reagents—Purified recombinant factor VIII was a generous gift from Bayer Corp. (Osaka, Japan). Two monoclonal antibodies, mAb IXa-GD, against the Gla domain of factor IXa and specific for calcium-dependent conformation, and mAb 3A6 against the heavy chain of factor IXa, were prepared (23, 24). A monoclonal antibody, ESH8, against the C2 domain of factor VIII and recognizing residues 2248–2285 (4, 5) was purchased from American Diagnostica Inc. (Stamford, CT). A horseradish peroxidase (HRP)-labeled monoclonal antibody was prepared using Peroxidase Labeling Kit-NH_2 (Dojindo Molecular Technologies Inc., Kumamoto, Japan). Human factor IXa, factor X, and EGR-ck (Hematologic Technologies, Inc., Essex Junction, VT), factor Xa (Enzyme Research Laboratories, Inc., South Bend, IN), thrombin (Sigma), recombinant hirudin (Calbiochem), and chromogenic Xa substrate S-2222 (Chromogenix, Milano, Italy) were purchased commercially. Gla domainless (GDless) factor IXa was prepared from factor IXa by limited chymotryptic digestion (25). Briefly, α-chymotrypsin was incubated with factor IXa in a 1:24 ratio (w/w) at 4 °C. The reaction was then quenched using 5 mM diisopropyl fluorophosphate, and the GDless factor IXa was separated from undigested factor IXa and the Gla domain-containing peptide (residues 1–42) using ion exchange chromatography. Molecular mass was estimated by SDS-PAGE. The amidolytic activity of GDless factor IXa was less than 0.2%. Phospholipid vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, and 30% phosphatidylethanolamine (Sigma) were prepared using *N*-octyl glucoside (26). Synthetic peptides consisting of overlapping sequences of 13 residues encompassing 2182–2249 within the factor VIII C2 domain were prepared by Biosynthesis (Lewisville, TX). They were purified by reverse phase HPLC (purity >95%) and were confirmed by mass spectrometry analysis.

Construction, Expression, and Purification of Factor VIII rC2—The rC2 was prepared using the protocol described by Takeshima *et al.* (27). Briefly, cDNA encoding the C2 domain of human factor VIII with a 4-amino acid NH_2-terminal extension (Val^{2169}–Tyr^{2332}) was constructed, transformed, and expressed in *Pichia pastoris* cells. The product was purified using ammonium sulfate fractionation and cation exchange HPLC (TSK-GEL CM-3SW; TOSOH Corp., Tokyo, Japan). SDS-PAGE analysis demonstrated >95% purity. The rC2 protein was identified as a single peak by a gel filtration and had a mass 18,626.6 on LC/MS analysis, closely matching the expected mass of 18,627.3.

Preparation of EGR-GDless Factor IXa—Factor IXa (10 μM) or GDless factor IXa (2.3 μM) was inactivated overnight at 4 °C by the addition of a 20-fold molar excess of EGR-ck in 20 mM HEPES, pH 7.2, 150 mM NaCl, and 0.01% Tween 20 (HBS buffer). Unbound EGR-ck was removed by extensive dialysis at 4 °C in the same buffer. Chromogenic assays demonstrated less than 0.2% residual activity of factor IXa or GDless factor IXa, respectively.

Preparation of rC2 Proteolytic Fragments—The rC2 (16.7 μM) was digested for 96 h at 37 °C with *Staphylococcus aureus* V8 protease (5.4 μM; Wako Pure Chemical Industries Ltd., Osaka, Japan) in 100 mM Tris-Tricine, pH 8.4, 150 mM NaCl, and 0.1% SDS. The digest was treated with the SDS-Out™ precipitation kit (Pierce) to remove the SDS and was fractionated by reverse phase HPLC using TSKgel ODS-100Z (5 μm; Tosoh Corp.). The reaction mixture was loaded onto a column equilibrated with 90% distilled H_2O, 10% acetonitrile in 0.1% trifluoroacetic acid and eluted with a linear gradient of 10–50% acetonitrile over 60 min. Fragments were detected at 216 nm and automatically collected in 500-μl aliquots and lyophilized. The fragments exhibited excellent solubility following their resuspension in HBS buffer. Protein concentrations were determined by the method of Bradford (28). Electrophoresis of the purified fragments followed by staining with GelCode Blue Stain Reagent (Pierce) showed >95% purity.

ELISA-based Binding Assay—Microtiter wells were coated with 200 nM rC2 (100 μl) in 100 mM sodium bicarbonate, pH 9.6, overnight at 4 °C. The wells were washed with HBS buffer and were blocked with the same buffer containing 5% BSA for 2 h at 37 °C. EGR-factor IXa or EGR-GDless factor IXa was then added and incubated in HBS buffer containing 1 mM $CaCl_2$ and 5% BSA for 2 h at 37 °C. Bound EGR-factor IXa was quantified by the addition of HRP-labeled anti-factor IXa mAb 3A6 and *o*-phenylenediamine dihydrochloride substrate. Reactions were quenched by the addition of 2 M H_2SO_4, and absorbances were measured at 492 nm using a Labsystems Multiskan Multisoft microplate reader (Labsystems, Helsinki, Finland). Control experiments demonstrated that mAb 3A6 did not affect the reaction between factor VIII and factor IXa (data not shown). The amount of nonspecific binding of HRP-labeled IgG in the absence of factor VIII was <5% of the total signal. Specific binding was recorded after subtracting the nonspecific binding. In competitive inhibition assays, the competitor proteins were incubated with 100 nM EGR-factor IXa for 2 h at 37 °C prior to the addition to immobilized rC2. The percentage of inhibition was calculated using the equation, (bound absorbance − non-

specific absorbance)/(maximum − nonspecific) × 100 (%). Absorbance in the absence of competitive protein or immobilized rC2 was regarded as maximum or nonspecific, respectively.

SPR-based Binding Assay—The kinetics of rC2 and EGR-factor IXa interaction were determined in SPR-based assays using a Biacore X instrument (BIAcore AB, Uppsala, Sweden). EGR-factor IXa was covalently coupled to the surface of a CM5 chip at a coupling density of ~7 ng/mm^2. Binding (association) of the ligand was monitored in 10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, and 0.005% surfactant P20, at a flow rate of 20 μl/min for 2 min at 37 °C. The dissociation of bound ligand was monitored for a 2-min period by replacing the ligand-containing buffer with buffer alone. The level of nonspecific binding corresponding to ligand binding to the uncoated chip was subtracted from the signal. The rate constants for association (k_a) and dissociation (k_d) were determined by nonlinear regression analysis (29, 30) using the evaluation software provided by Biacore AB. Equilibrium dissociation constants (K_d) were calculated as k_d/k_a.

Factor Xa Generation Assays—The rate of conversion of factor X to factor Xa was monitored in a purified system (18, 31). Factor Xa was generated at 22 °C in HBS buffer containing 1 mM $CaCl_2$ and 0.1% BSA. For assays in the absence of phospholipid, 200 nM factor VIII was activated by 10 nM thrombin. Thrombin activity was inhibited after 1 min by the addition of 2.5 units/ml hirudin, and factor Xa generation was initiated by the addition of 5 nM factor IXa and 1 μM factor X. Experiments in the absence of factor VIIIa were performed under the same conditions except for 20 nM factor IXa. For assays in the presence of phospholipid, 30 nM factor VIII was activated by 10 nM thrombin in the presence of 20 μM phospholipid. Thrombin activity was inhibited after 1 min by hirudin, and factor Xa generation was initiated by the addition of 0.5 nM factor IXa and the indicated amounts of factor X. Aliquots were removed at appropriate times to assess initial rates of product formation and added to tubes containing EDTA (100 mM final concentration) to stop the reaction. Rates of factor Xa generation were determined at 405 nm using a microtiter plate reader after the addition of chromogenic substrate, S-2222 (0.46 mM final concentration). Factor Xa generation was quantified by extrapolation from a standard curve prepared using known amounts of factor Xa.

ELISA for Factor VIII or EGR-factor IXa Binding to Phosphatidylserine—ELISA were performed using a minor modification of a previously reported method (7). Briefly, 50 μM 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (Sigma) in methanol was immobilized onto each well of a microtiter plate and allowed to air dry at 4 °C overnight. After washing with HBS buffer, the wells were blocked for 2 h at 37 °C with HBS buffer containing 5% BSA. Factor VIII or EGR-factor IXa was added to each well in HBS buffer containing 1 mM $CaCl_2$ and 5% BSA and incubated for 2 h at 37 °C. Bound factor VIII was detected using anti-factor VIII mAb ESH8, followed by HRP-labeled anti-mouse second antibody. Bound EGR-factor IXa was detected using HRP-labeled mAb 3A6.

NH_2-terminal Sequence Analysis—The C2 fragments were blotted onto polyvinylidene difluoride membranes, stained with Gelcode Blue, and excised. NH_2-terminal sequence analyses of the purified fragments were performed using an Applied Biosystems model 491 sequencer (Foster City, CA). Samples were subjected to 5 or 7 cycles of automated sequencing.

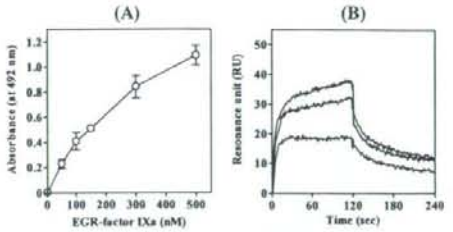

FIGURE 1. **Direct binding of EGR-factor IXa to rC2.** *A*, ELISA-based assay. Various concentrations of EGR-factor IXa were reacted with rC2 (200 nM) that had been immobilized onto microtiter wells for 2 h at 37 °C, as described under "Materials and Methods." Bound EGR-factor IXa was detected using HRP-labeled anti-factor IXa mAb 3A6. Absorbance values were plotted as a function of the concentration of EGR-factor IXa. Experiments were performed at least three separate times, and average ± S.D. values are shown. *B*, SPR-based assay. Various concentrations of rC2 were injected onto the EGR-factor IXa (~7 ng/mm^2) immobilized onto the sensor chip at a flow rate of 20 μl/min for 2 min, followed by a change of running buffer for 2 min as described under "Materials and Methods." The *three lines* illustrate representative response curves for the different concentrations of rC2 (100, 300, and 500 nM, respectively). Experiments were performed at least three separate times.

Solvent-accessible Surface Area Analysis—The solvent accessibilities at the interface for the residues 2182–2259 of the C2 domain were calculated from the atomic coordinates using Marc Gerstein's calc-surface program (32) available from the Helix Systems Web site. The atomic coordinates of human factor VIII and C2 domain were retrieved from the Protein Data Bank (code 2R7E and 1D7P, respectively). Values that are more positive represent a greater probability of surface exposure.

RESULTS

Binding of EGR-factor IXa to the C2 Domain—Blostein *et al.* (21) have recently reported that the Gla domain of factor IXa interacts with the light chain of factor VIIIa. The C2 domain of factor VIIIa and the Gla domain of factor IXa are involved in phospholipid-binding, and we surmised, therefore, that they could be juxtaposed in the factor Xase complex and that the C2 domain might associate directly with the Gla domain of factor IXa. To investigate this hypothesis, we initially examined the direct binding of factor IXa to immobilized rC2 using microtiter-based, solid phase binding assays. An active site-modified EGR-factor IXa preparation was used in these experiments to eliminate difficulties of interpretation in the presence of enzymatically active factor IXa. Various concentrations of EGR-factor IXa were incubated with immobilized rC2 (200 nM). Bound EGR-factor IXa was detected using anti-factor IXa mAb 3A6, recognizing the heavy chain of its protease. EGR-factor IXa bound to immobilized rC2 in a dose-dependent manner (Fig. 1*A*). Control experiments using an anti-C2 mAb demonstrated that immobilized rC2 was not affected by the ionic strength of the wash buffer or the duration of the wash and incubation steps subsequent to C2 binding (data not shown). To confirm the specificity of this binding, various concentrations of factor VIII or rC2 were preincubated with EGR-factor IXa (100 nM) in

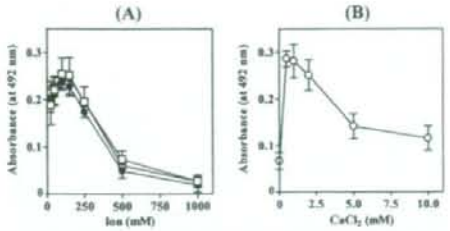

FIGURE 2. **Effect of ionic strength on EGR-factor IXa and rC2 interaction.** *A*, NaCl, KCl, and LiCl effect. EGR-factor IXa (100 nM) was incubated with immobilized rC2 (200 nM) in HBS buffer containing 1 mM $CaCl_2$, 5% BSA, and various amounts of NaCl (*open circles*), KCl (*closed circles*), and LiCl (*open squares*) in an ELISA-based assay. *B*, $CaCl_2$ effect. EGR-factor IXa was incubated with immobilized rC2 in HBS buffer containing 5% BSA and various amounts of $CaCl_2$. EGR-factor IXa bound in the absence of $CaCl_2$ refers to an experiment in the presence of 10 mM EDTA. Experiments were performed at least three separate times, and average ± S.D. values are shown.

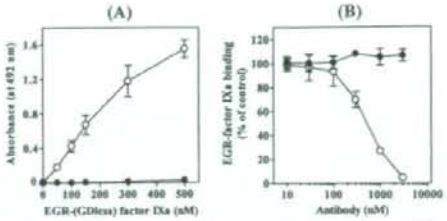

FIGURE 3. **Contribution of the Gla domain of EGR-factor IXa for rC2 interaction.** *A*, various concentrations of EGR-factor IXa (*open circles*) or EGR-GDless factor IXa (*closed circles*) were incubated with immobilized rC2 (200 nM) for 2 h at 37 °C in an ELISA-based assay. *B*, EGR-factor IXa (100 nM) was preincubated with various concentrations of mAb IXa-GD IgG (*open circles*) or normal murine IgG (*closed circles*) for 2 h at 37 °C, prior to incubation with immobilized rC2. Absorbance values for the EGR-factor IXa binding to rC2 in the absence of competitor represent the 100% level. The percentage of EGR-factor IXa binding was plotted as a function of antibody concentration. Experiments were performed at least three separate times, and average ± S.D. values are shown.

the fluid phase prior to addition to the immobilized rC2. Factor VIII and rC2 inhibited EGR-factor IXa binding to immobilized rC2 by ~90 and ~60%, respectively (data not shown), confirming specificity of the assay.

We further evaluated interactions by an alternative approach using real time SPR-based assays. This technique provides information on kinetic and equilibrium binding constants (29, 30). A range of concentrations of rC2 were added to EGR-factor IXa immobilized onto a sensor chip. Fig. 1*B* shows representative curves corresponding to the association/dissociation of rC2. The data could be comparatively well fitted by nonlinear regression using a 1:1 binding model with drifting base line. Kinetic constants showed that rC2 bound to EGR-factor IXa with mild affinity (K_d = 108 ± 27 nM, $k_d/k_a = 2.45 \times 10^{-2}$ $s^{-1}/2.36 \times 10^5$ M^{-1} s^{-1}). In ELISA-based assays, the apparent K_d value appeared to be higher (~400 nM), compared with that obtained by SPR-based assays. Since ELISA is not an equilibrium binding assay, the multiple steps of incubation and wash may affect the detection for lower concentrations of EGR-FIXa. The results indicated that the C2 domain of factor VIII interacted directly with factor IXa.

Characterization of the Interaction between the C2 Domain and EGR-factor IXa—The light chain of factor VIIIa interacts with factor IXa in electrostatic and calcium-dependent mechanisms (19). To further characterize this interaction, factor IXa (100 nM) was mixed with various amounts of NaCl and incubated with immobilized rC2. Control experiments showed that the amount of immobilized rC2 or the reactivity of antibody was not affected even at a higher concentration of NaCl (data not shown). Binding of EGR-factor IXa to rC2 was maximal at physiological concentrations of NaCl (~150 mM; Fig. 2*A*). Higher concentrations of NaCl incrementally weakened this interaction, however, and consequently binding was significantly decreased by ~95% at elevated ionic strengths, supporting the salt sensitivity of this interaction. The Na^+-bound factor IXa drastically enhances catalytic activity toward factor X and increases the affinity for factor VIIIa (33, 34). However, other monovalent cations, K^+ and Li^+, also inhibited this binding similarly, suggesting that this effect was not due to a specific interaction of Na^+-factor IXa.

Ca^{2+} is known to be required for the structural and functional integrity of factor IXa, and hence the effect of Ca^{2+} on factor IXa-rC2 interaction was also examined in the current experiments. Binding of factor IXa to immobilized rC2 was investigated in buffer containing various amounts of $CaCl_2$. EDTA (10 mM) was added to the reaction mixtures to assess binding in the absence of Ca^{2+}. The presence of Ca^{2+} up to ~1.0 mM markedly increased factor IXa binding by ~6-fold compared with that in the absence of Ca^{2+} (Fig. 2*B*). Optimal binding was observed at approximately physiological concentrations of free Ca^{2+} (~1.3 mM). Binding was significantly inhibited by increments of Ca^{2+} >1 mM. The data were consistent, therefore, with a role for Ca^{2+} in C2-factor IXa interaction, although it was not possible to distinguish between a direct or indirect role for Ca^{2+} in mediating this effect.

Binding of the Gla Domain of Factor IXa to the C2 Domain—To investigate whether the Gla domain of factor IXa participates in direct interactions with the C2 domain of factor VIII, EGR-GDless factor IXa was prepared by chymotrypsin digestion and EGR-ck labeling, as described under "Materials and Methods." Control experiments demonstrated that EGR-GDless factor IXa and EGR-factor IXa were similarly reactive with anti-factor IXa mAb 3A6 in the ELISA (data not shown). The binding of EGR-GDless factor IXa to immobilized rC2 was markedly lower than that of EGR-factor IXa even at the maximum concentration employed (500 nM; Fig. 3*A*). SPR-based assays also showed that rC2 failed to react with EGR-GDless factor IXa (data not shown). In addition, competitive experiments using an anti-factor IXa mAb, mAb IXa-GD, recognizing the Gla domain of factor IXa and dependent on the presence of Ca^{2+}, demonstrated that the monoclonal antibody blocked binding of EGR-factor IXa to rC2 (up to ~95%) in a dose-dependent manner (IC_{50}; 758 ± 93 nM) (Fig. 3*B*). These findings were in keeping with a significant role for the Gla domain of factor IXa in direct binding to the C2 domain of factor VIII.

To assess the functional role of the interaction between the C2 domain and Gla domain of factor IXa in the factor Xase complex, we examined the effect of rC2 or mAb IXa-GD on factor VIIIa/factor IXa-mediated activation of factor X in an

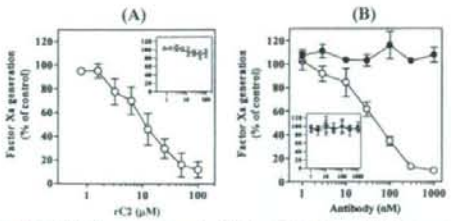

FIGURE 4. **Inhibition of rC2 and mAb IXa-GD in factor VIIIa/factor IXa-mediated factor X activation in the absence of phospholipid.** Various concentrations of rC2 (*open circles*) (*A*) or mAb IXa-GD (*open circles*) (*B*) or normal murine IgG (*closed circles*) were preincubated with 5 nM factor IXa for 2 h at 37 °C. Factor Xa generation was initiated by the addition of thrombin-activated factor VIIIa (200 nM) and 1 μM factor X under the conditions described under "Materials and Methods." The initial rate of factor Xa generated in the absence of competitor represents the 100% level and was 1.19 ± 0.14 nM/min. Initial rates of factor Xa generation were plotted as a function of rC2 or antibody concentration. The *inset* shows the experiments in the absence of factor VIIIa under similar conditions except for 20 nM factor IXa. The initial rate of factor Xa generated in the absence of competitor (100% level) was 0.0034 ± 0.0001 nM/min. Experiments were performed at least three separate times, and average ± S.D. values are shown.

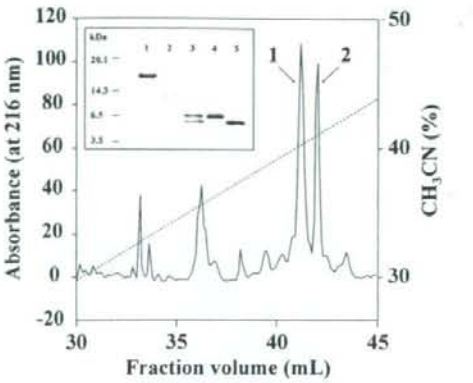

FIGURE 5. **Reverse phase HPLC and SDS-PAGE of V8 protease-cleaved rC2.** *S. aureus* V8 protease-cleaved C2 fragments were fractionated by reverse phase HPLC. The *broken line* represents acetonitrile concentration (10–50%), and fragments were detected at 216 nm (*solid line*). The *inset* shows that the proteins were analyzed by SDS-PAGE using 16.5% peptide gel under nonreducing conditions, followed by staining with GelCode Blue. *Lane 1*, 1.5 μg of rC2; *lane 2*, 0.75 μg of V8 protease; *lane 3*, 1.5 μg of rC2 digested with 0.75 μg of V8 protease; *lane 4*, 0.5 μg of fragment in peak fraction 1; *lane 5*, 0.5 μg of fragment in peak fraction 2. The positions of molecular mass markers in kDa are indicated to the *left*.

amidolytic assay. For this assay, 200 nM factor VIII was activated by thrombin and incubated with mixtures of 5 nM factor IXa and various concentrations of rC2 or mAb IXa-GD. Factor Xa generation was initiated by the addition of 1 μM factor X. The rC2 competes with factor VIII for binding to phospholipid membranes, and for this reason, the assays were performed in the absence of phospholipid. The addition of rC2 and mAb IXa-GD markedly decreased the rates of factor Xa generation (by >90%) in a dose-dependent manner, with the IC_{50} values of 10.9 ± 3.8 μM and 43.2 ± 15.3 nM, respectively (Fig. 4, *A* and *B*). To exclude the possibility that rC2 and mAb IXa-GD directly affected factor IXa-catalyzed activation of factor X, factor Xa generation was further examined in the absence of factor VIIIa. As expected, there was little inhibition of factor Xa generation in the presence of rC2 or mAb IXa-GD (Fig. 4, *inset*), confirming that the reactions were governed by factor VIIIa. The results indicated that association between the C2 domain of factor VIII and the Gla domain of factor IXa played a significant role in the assembly of the factor Xase complex and hence factor IXa-catalyzed activation of factor X in the presence of factor VIIIa.

Purification and Characterization of rC2-digested Fragments—To localize factor IXa-interactive regions within the C2 domain, limited *Staphylococcus aureus* V8 protease digests of rC2 were prepared. SDS-PAGE analysis demonstrated the presence of two large fragments of apparent mass 7.5- and 6.2-kDa that were significantly smaller than the initial rC2 (~16 kDa) (Fig. 5*B*). The digestion of rC2 by V8 protease required denaturing conditions with 0.1% SDS, and we confirmed that the binding of SDS-treated, uncleaved rC2 to EGR-factor IXa was similar to that of the non-SDS-treated product in the ELISA-based assay (data not shown). EGR-factor IXa bound to immobilized, unfractionated V8 protease-cleaved rC2 in a dose-dependent manner (data not shown), prompting us to further isolate and purify the cleaved fragments. The two fragments were not able to be separated by ion exchange HPLC using CM, Mono-Q, and Mono-S under any salt and pH conditions but were resolved by reverse phase HPLC (Fig. 5). SDS-PAGE confirmed that these fractions, designated as peak 1 and peak 2, represented the 7.5- and 6.2-kDa fragments, respectively (Fig. 5, *inset*). Some smaller peaks were observed, but the bands were poorly detectable in SDS-PAGE and appeared to represent further minor degradation of rC2. The peak 1 and peak 2 fractions were further characterized by size exclusion chromatography, and each was shown to elute as a single peak, indicating that the fragments were monomeric in solution (data not shown).

Automated NH_2-terminal sequence analysis identified that the sites of cleavage responsible for the generation of the two C2 fragments were located at residues Glu^{2181}-Ser^{2182} and Glu^{2259}-Phe^{2260} for the ~7.5- and ~6.2-kDa fragments, respectively (Table 1). In addition, LC/MS analysis indicated that the molecular mass of fragment 1 (7.5 kDa) and fragment 2 (6.2 kDa) was 8827.25 ± 5.70 and 7355.63 ± 0.68 Da, respectively. On the basis of the previous molecular masses determined by LC/MS, NH_2-terminal sequence analysis, and specificity of V8 protease cleavage site (-Glu-*X* and/or -Asp-*X*), the deduced protein sequences of the two C2 fragments matched Ser^{2182}–Glu^{2259} (expected mass, 8823.08 Da) and Phe^{2260}–Glu^{2322} (expected mass, 7354.39 Da). Hence, the ~7.5- and ~6.2-kDa C2 fragments were designated as C2-(2182–2259) and C2-(2260–2322), respectively.

Binding of the Isolated C2 Fragments to EGR-factor IXa and the Effects on Factor Xa Generation—To determine whether the C2-(2182–2259) and/or C2-(2260–2322) were able to bind to factor IXa, we investigated direct binding in ELISA-based assays, as described above. EGR-factor IXa bound directly to immobilized C2-(2182–2259) (600 nM) in a dose-dependent manner, although in this instance, the binding efficiency was weaker than that of the uncleaved rC2 (Fig. 6*A*). In contrast, very limited binding of EGR-factor IXa to immobilized