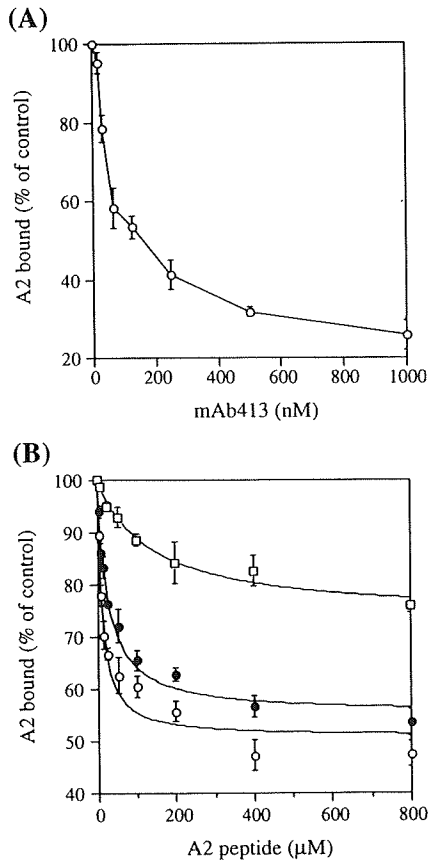

Fig. 5. Effect of anti-A2 mAb413 and A2 peptides on A2 subunit binding to Ah-plasmin — The A2 subunit (60 nM) was preincubated with varying amounts of anti-A2 mAb413 F(ab′)$_2$ for 1 h (A) or mixed with varying amounts of the A2 peptides (479–504 peptides; open circles, 484–509 peptides; closed circles, and 489–514 peptides; open squares) (B), prior to reaction with Ah-plasmin (100 nM) immobilized onto microtiter wells. Bound A2 was detected using biotinylated anti-A2 mAb (JR8). The absorbance values for the A2 subunit binding to Ah-plasmin in the absence of competitor were defined as 100%. The percentage of A2 binding was plotted as a function of antibody or A2 peptide, and the plotted data were fitted by nonlinear least squares regression to an Eq. (4) as described in Materials and methods.

factor VIIIa activity was ~50% of that obtained in the absence of A2. The activation rate constant (k_1) for factor VIII in the presence of A2 (200 nM) was ~4-fold lower compared than that observed in the absence of A2. Furthermore, the rate constant (k_2) for inactivation (and/or decay) was ~5-fold less than that observed in the absence of A2.

The activity of factor VIIIa at any time point likely represents contributions from non-activated molecules, activated molecules, and activated molecules that have decayed following factor VIII subunit dissociation. To precisely evaluate the effect of A2 subunit on plasmin-catalyzed inactivation of the cofactor, therefore, the experiments were repeated using factor VIIIa as a substrate. In these instances, the addition of exogenous A2 similarly inhibited factor VIIIa inactivation by plasmin in a dose-dependent manner (Fig. 1A, panel b). The inactivation rate constant (k) with factor VIIIa as substrate was similar to that (k_2) obtained using factor VIII. These results indicated that the A2 domain contributed to plasmin-catalyzed activation/inactivation of factor VIII(a).

3.2. EGR-factor IXa inhibition of factor VIIIa inactivation by plasmin

Factor IXa binds to factor VIIIa with high affinity (K_d: ~5 nM) via the A2 and A3 domains on an anionic membrane surface [29,30]. Since our above observation suggested that the A2 domain associates with plasmin during the activation and/or inactivation, we studied the effect of factor IXa on plasmin-catalyzed inactivation of factor VIIIa. Factor IXa and plasmin are serine proteases that inactivate factor VIIIa by proteolytic cleavage at Arg^{336} [11]. For this reason, an active-site modified EGR-factor IXa was used in place of factor IXa in these experiments. Factor VIIIa (25 nM) was mixed with saturating concentrations of EGR-factor IXa (40 nM) in the presence of phospholipid prior to the addition of plasmin (0.8 nM). In these circumstances, the inactivation rate constant in the presence of EGR-factor IXa was ~2.5-fold lower than that in its absence (0.115±0.010 min^{-1} and 0.264±0.030 min^{-1}, respectively) (Fig. 1B), supporting that factor IXa regulates plasmin-catalyzed factor VIIIa inactivation.

3.3. Effect of anti-A2 antibody on plasmin-catalyzed cleavage of factor VIII heavy chain

Up- and down-regulation of factor VIII activity is mediated by proteolytic cleavages at the A1-A2 domainal junction (Arg^{372}) and at both terminal regions in the A1 domain (Arg^{336} and Lys^{36}) of the heavy chain, respectively. A putative plasmin-interactive site in the A2 domain, which likely overlaps with the factor IXa-interactive sites, may be responsible for the cleavage of the factor VIII heavy chain. To examine this hypothesis, we assessed plasmin-catalyzed cleavage of the factor VIII heavy chain in the presence of an anti-A2 mAb413. This antibody

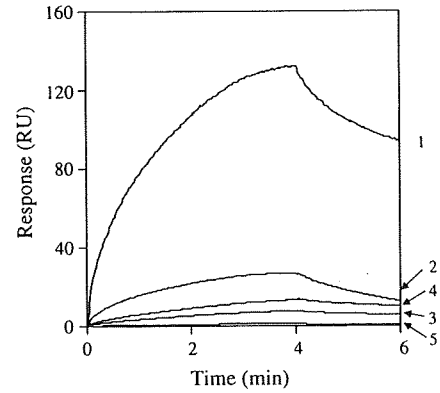

Fig. 6. Binding of recombinant A2 mutants to Ah-plasmin in an SPR-based assay — Recombinant wt-A2 or its mutants (120 nM) were incubated with Ah-plasmin immobilized on a sensor chip (~7 ng/mm^2) for 4 min, followed by a change of running buffer for over 2 min. Curves 1–5 show the association/dissociation curves of wt-A2, and its mutants, K377A, K466A, R471A, and R484A, respectively.

Table 3
Kinetic parameters of the interaction between recombinant A2 mutants and Ah-plasmin in a SPR-based assay

A2 mutant	k_{ass} $\times 10^4\ M^{-1}s^{-1}$	k_{diss} $\times 10^{-4}\ s^{-1}$	K_d^{app} nM
wt-A2	5.14±0.12	11.7±0.19	22.8
K376A	3.90±2.99	4.48±0.23	11.5
K377A	0.32±0.04	10.8±0.32	**338**
H378A	4.83±0.10	7.47±0.17	15.5
K380A	12.7±0.18	7.40±0.33	5.8
K466A	0.057±0.010	0.97±0.20	**170**
R471A	0.079±0.007	3.11±0.30	**394**
R484A	0.025±0.003	14.5±0.60	**5800**
Y487A	5.51±0.13	6.67±0.24	12.1
S488A	3.97±0.09	8.69±0.16	21.9
R489A	8.71±0.14	13.4±0.22	15.4
R490A	3.68±0.13	7.67±0.22	20.8
L491A	7.53±0.12	14.1±0.26	18.7
K493A	5.98±0.09	15.4±0.20	25.8
K496A	8.76±0.15	18.0±0.20	20.5
H497A	0.58±0.02	1.61±0.04	27.8
K510A	7.05±0.13	13.0±0.24	18.4
K512A	1.62±0.02	0.67±0.03	4.14

Reactions were performed as described under "Materials and methods". Parameter values were calculated by nonlinear regression analysis using the evaluation software provided by Biacore AB. The K_d values were calculated as k_{diss}/k_{asso}. A2 mutants, which had the greatest decrease in affinity for Ah-plasmin, and their K_d values are shown in bold. All experiments were performed at least three separate times, and average values and standard deviations are shown. K_d^{app}: apparent K_d value.

recognizes an epitope defined by residues 484–509, overlapping a factor IXa-interactive site, and blocks the interaction between the A2 subunit and factor IXa molecule [29]. Factor VIII (100 nM) was preincubated with the various concentrations of mAb413 IgG $F(ab')_2$ for 1 h and then treated with plasmin for 3 min. Fig. 2 shows the results from Western blotting of the cleavage reaction using biotinylated anti-A2 mABJR8 as the tracer. In the absence of mAb413, both $A1^{337-372}$–A2 (residues 337–740) and A2 (residues 373–740) fragments derived from the plasmin-cleaved factor VIII were observed (lane 2). In the presence of the antibody (lanes 3–8), the A2 fragment was depressed, whilst the density of the A1-A2 fragment (residues 1–740) was increased in a dose-dependent manner, demonstrating inhibition of cleavage at Arg^{372} and Arg^{336}, respectively. Intact A1–A2-B fragment was not detected in the presence of mAb413, indicating that this antibody did not affect cleavage by plasmin at Arg^{740}. The finding suggested that mAb413 blocked plasmin-catalyzed factor VIII heavy chain cleavage at Arg^{336} and Arg^{372}, but not at Arg^{740}. In contrast, this antibody did not affect plasmin-catalyzed cleavage of the light chain (data not shown). These findings indicated that a region within or close to residues 484–509 in the A2 subunit is critical for plasmin-catalyzed cleavage at Arg^{336} and Arg^{372} during activation and/or inactivation of factor VIII.

3.4. Binding of factor VIII to Ah-plasmin

To obtain direct evidence that the A2 domain contains a major plasmin-interactive site that contributes to enzyme docking and facilitates catalysis of cleavage of factor VIII heavy chain, a series of experiments were performed to assess plasmin binding to factor VIII subunits. In these experiments, an active-site modified plasmin, Ah-plasmin, lacking enzymatic activity, was utilized instead of plasmin. Interactions between the factor VIII subunits and Ah-plasmin were initially evaluated using a SPR-based assay. Several different amounts of factor VIII subunits were added to Ah-plasmin immobilized on a sensor chip. Fig. 3A shows representative curves corresponding to the association and dissociation of immobilized Ah-plasmin with factor VIII (panel a), intact heavy chain (panel b), intact light chain (panel c), A2 (panel d), or A1 subunit (panel e). The factor VIII fragments bound to Ah-plasmin in a dose-dependent manner. The data were assessed by nonlinear regression using a 1:1 Langmuir binding model and were shown to be not significantly different from those calculated using two-site model. Incomplete dissociation curves were obtained, however, because of mass transport issues. The K_d values obtained represent an apparent K_d for interaction, and its derivation may have been subjected to mass transport limitations. Kinetic constants indicated that factor VIII bound with higher affinity than the A2 subunit to Ah-plasmin (K_d: 3.1 nM and 22.6 nM, respectively) (Table 2). The intact heavy chain also bound with ~10-fold higher affinity than the light chain (K_d: 5.6 nM and 68.2 nM, respectively), similar to the calculated K_d value for factor VIII. However, the binding affinity determined for A1 was ~10-fold lower compared with that obtained for A2. The A2 domain readily dissociates from factor VIIIa, and the kinetics for the active form molecule could not be determined. These results indicated a more significant contribution of heavy chain compared with light chain, and a more prominent role for the A2 subunit compared with the A1 subunit.

We further evaluated factor VIII and plasmin interactions by an alternative approach using a solid-phase binding assay in which Ah-plasmin was immobilized onto microtiter wells. Factor VIII bound to Ah-plasmin was detected using biotinylated anti-A1 (58.12), anti-A2 (JR8), or anti-C2 (NMC-VIII/5) mAb IgG. These antibodies did not affect this binding reaction (data not shown). Results are presented in Fig. 3B and Table 2. Reactions of factor VIII subunits with Ah-plasmin yielded saturable binding curves. This method is not based on a true equilibrium binding assay, however, and the K_d values obtained represent an apparent K_d for the interactions. Nevertheless, the K_d value (6.7 nM) obtained for binding of factor VIII to Ah-plasmin in these experiments was similar to that calculated in the SPR-based assay. The binding affinity for the heavy chain was ~9-fold higher than that for the light chain. The A2 subunit also bound to Ah-plasmin with modest affinity (K_d: 40.7 nM), whilst the A1 failed to bind. Overall, the affinity values obtained by the ELISA-based assay were in a good agreement with those obtained in the SPR-based analysis, and were mutually supportive. The reasons for the reduced absorbance signal for factor VIII compared with those observed for the A2 subunits are not known, but may reflect fewer moles of factor VIII bound compared with the isolated subunits as a result of blocking accessible binding sites because of its larger size. Taken together, these results suggest that the A2 domain of heavy chain significantly contributes to factor VIII binding to plasmin.

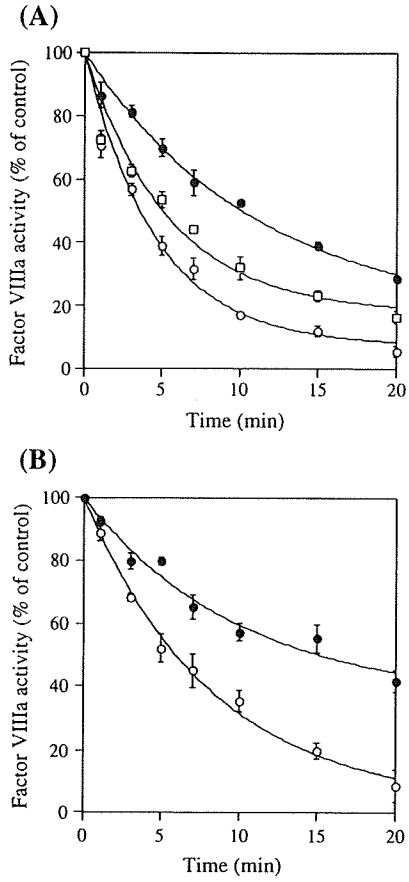

Fig. 7. Inhibitory effect of recombinant A2 mutant on factor VIIIa inactivation by plasmin — (A) Inhibition of R484A mutant on plasmin-catalyzed factor VIIIa inactivation: Isolated factor VIIIa (25 nM) was incubated with plasmin (0.7 nM) and phospholipid (10 μM) in the absence (open circles) or presence of recombinant A2 subunit (100 nM: wt-A2; closed circles, R484A mutant; open squares). Factor VIIIa activity was measured at the indicated times using a one-stage clotting assay. The initial activity of factor VIIIa (100% level) was ~50 U/ml. (B) Plasmin-catalyzed inactivation of factor VIIIa reconstituted with R484A mutant: Factor VIIIa reconstituted with the isolated A1/A3C1C2 dimer (200 nM) and recombinant A2 subunit (200 nM: wt-A2; open circles, R484A mutant; closed circles) was incubated with plasmin (1 nM). Factor VIIIa activity was measured at the indicated times using a one-stage clotting assay. The initial activity of reconstituted factor VIIIa with wt-A2 or R484A was ~35 U/ml (100% level). The value of factor VIIIa activity was plotted as a function of incubation time and the data were fitted to an Eq. (1) as described in Materials and methods.

3.5. Effect of 6-AHA on the binding of A2 subunit to Ah-plasmin

It is well-documented that plasmin interacts with substrates predominantly via exposed lysine-binding sites of the protease [15]. To examine the role of this lysine-binding in the current circumstances, the effects of 6-AHA, a specific competitor of lysine-binding, were examined in the ELISA-based assay. Fixed concentrations of A2 (60 nM) or light chain (120 nM) were incubated with immobilized Ah-plasmin in the presence of varying concentrations of 6-AHA. The 6-AHA blocked the interactions between light chain and plasmin in a dose-dependent manner by >90% (Fig. 4). The apparent K_i value was 6.8± 1.0 μM. In contrast, 6-AHA blocked A2 subunit and plasmin interaction weakly (~30%) with a K_i value of 11.3±2.3 μM. These K_i values (~10 μM) were similar to earlier reports [31] on the effects of 6-AHA on the binding affinity of plasmin, and supported the validity of this competition assay. The observation that 6-AHA markedly inhibited (>90%) light chain-plasmin binding suggests that this interaction is mainly dependent upon lysine-binding sites of the protease. In contrast, the observation that 6-AHA incompletely (~30%) inhibited binding to A2 suggests that plasmin interacts with this subunit predominantly through mechanisms independent of lysine-binding sites. Furthermore, the inhibitory effect of 6-AHA on the interaction between the heavy chain or factor VIII and Ah-plasmin was similar to that for the A2 interaction, indicating a more significant contribution of A2 on factor VIII and plasmin interaction through mechanisms independent of lysine-binding sites.

3.6. Localization of a plasmin-binding site within the A2 domain

Our Western blotting analyses showed that mAb413, recognizing residues 484–509 in the A2 domain, blocked plasmin cleavage of the heavy chain. To confirm that the inhibitory effect of the antibody resulted from blocking of A2 binding to plasmin, we performed a series of competition experiments using an ELISA. The A2 subunit (60 nM) was preincubated with varying amounts of antibody $F(ab')_2$ prior to incubation with immobilized Ah-plasmin. This antibody inhibited the A2 binding to Ah-plasmin by ~80% in a dose-dependent manner, with an IC_{50} of 151±19 nM (Fig. 5A).

To exclude the possibility that this inhibition of A2-plasmin binding resulted from conformational steric hindrance, and to further localize plasmin-binding sites within or around residues 484–509 in the A2 domain, a similar competitive assay was developed using three overlapping synthetic peptides encompassing amino acid sequences within residues 484–509. Control experiments demonstrated that these A2 peptides had little effect on the reaction between A2 subunits and the mAbJR8 used for detection. Both the 479–504 and 484–509 peptides dose-dependently blocked A2 subunit binding to Ah-plasmin by ~50%, and the apparent K_i values were similar (5.7±1.3 μM and 10.3±2.6 μM, respectively) (Fig. 5B). A peptide with a scrambled sequence was used as control and showed little inhibition (data not shown). Peptides 489–514 weakly inhibited binding of the A2 subunit and plasmin with >80% residual binding observed at the highest concentration (800 μM) of peptide. The K_i value for this third peptide (62.8±16.4 μM) was at least 5–10-fold higher than those recorded for the other two peptides. The results suggested that the A2 region comprising residues 479–509, in particular the 479–488 region, represented a plasmin-interactive site.

3.7. Plasmin interaction with the A2 mutants in a SPR-based assay

To further identify the A2 residues responsible for interaction with plasmin, a series of recombinant mutant A2 molecules

were prepared using a baculovirus expression system [26]. We focused on exposed positively charged residues involving lysine, and hydrophobic or hydroxyl group residues within or conformationally close to the 484–509 region. Selected residues were converted to alanine, generating a panel of 17 single-point A2 mutants. The secretion levels and conformational stabilities of the A2 mutants, and specific activities of factor VIIIa obtained from reconstituted mutated A2 and A1/A3C1C2 dimers are described in a recent report [26]. Interactions between our A2 mutants and Ah-plasmin were evaluated using a SPR-based assay (Fig. 6 and Table 3). The data fitted well in a comparative nonlinear regression analysis using a 1:1 Langmuir binding model. The binding affinity of wild-type A2 (wt-A2) for Ah-plasmin was equivalent to that obtained for plasma-derived A2, indicating that the arrangement of the plasmin-interactive site was similar in both preparations. It was especially noteworthy that the binding affinity of the R484A mutant to Ah-plasmin (K_d: 5800 nM) was ~250-fold lower than that of wt-A2, indicating that Arg^{484} plays a major role in plasmin binding. These results were consistent with our earlier competitive binding assays that indicated the presence of a lysine-independent plasmin-binding site within the 479–488 A2 region. In addition, 3 other A2 mutants (K377A, K466A, and R471A) also exhibited reduced binding affinity (by 10–20-fold) compared with wt-A2, suggesting that these residues contributed somewhat to plasmin binding. The remaining A2 mutants bound to plasmin with affinities similar to that of wt-A2. Collectively, these results indicated that residues of Arg^{484}, Lys^{377}, Lys^{466}, and Arg^{471} were involved significantly in A2 binding to plasmin.

3.8. Effect of R484A A2 mutant on plasmin-catalyzed inactivation of factor VIIIa

To investigate the functional role of Arg^{484} residue in the A2 domain that contributed to plasmin binding, we examined the inhibitory effects of R484A mutant on factor VIIIa inactivation by plasmin. Factor VIIIa (25 nM) was mixed with the A2 (100 nM) and incubated with plasmin (0.7 nM). In control experiments, the addition of wt-A2 was associated with an ~3.5-fold decrease in inactivation rate compared with that in the absence of A2 (0.065± 0.011 min^{-1} and 0.220±0.029 min^{-1}, respectively) (Fig. 7A). These data again confirmed the functional similarity of recombinant and plasma-derived A2 subunits. For quantitation, the change in the inactivation rate constant obtained by the addition of wt-A2 was regarded as 100% inhibition. As expected, the addition of R484A mutant, possessing ~250-fold reduced affinity for plasmin, weakly inhibited factor VIIIa inactivation by ~20% (0.187±0.026 min^{-1}).

To further elucidate the direct role of Arg^{484} in A2 on plasmin-catalyzed inactivation, we examined the effect of inactivation by plasmin using factor VIIIa reconstituted with the A1/A3C1C2 dimer and R484A A2 mutant as substrate. Reconstituted factor VIIIa with the A1/A3C1C2 dimer (200 nM) and wt-A2 or R484A A2 (200 nM) was reacted with plasmin (1 nM) as described in Materials and methods. Maximum factor VIIIa activity reconstituted with R484A mutant in this condition was similar to that with wt-A2 as previously reported [26]. Reconstituted factor VIIIa with R484A mutant was inactivated by plasmin with an ~2.8-fold decrease in inactivation rate, compared to that with wt-A2 (0.051±0.009 min^{-1} and 0.141±0.011 min^{-1}, respectively) (Fig. 7B). Taken together, these results demonstrated that the A2 domain, in particular Arg^{484}, provides a unique plasmin-interactive site that promotes enzyme docking during plasmin-catalyzed cleavage of the factor VIII heavy chain and cofactor inactivation.

4. Discussion

We have recently suggested that cleavage at Arg^{336} in the factor VIII molecule, a core reaction that regulates factor VIII(a) inactivation by plasmin, may be selectively modulated by interaction of the protease with the A2 subunit [20]. In the current study we present several lines of the direct evidence that the A2 domain does indeed contain a plasmin-interactive site. The presence of this site involved in proteolysis of heavy chain, was demonstrated using two complementary approaches, a SPR-based assay and an ELISA-based assay. Data obtained in conventional factor VIII binding assays using active plasmin are extremely difficult to interpret because plasmin-catalyzed cleavage of the cofactor might deplete the physiological binding response. We developed, therefore, a direct binding assay using Ah-plasmin, a catalytically inactive derivative of plasmin. The advantages of this approach have been confirmed in several previous studies using a range of different serine proteases including Ah-factor Xa [25,32,33]. Direct immobilization of Ah-plasmin may cause conformational changes, however, and crucial regions related to interaction may be buried. We utilized two distinct solid-phase binding assays in this study, therefore, and the results were mutually supportive. The data indicated that plasmin binds to the factor VIII heavy chain with high affinity (K_d: ~5 nM) and to the light chain with modest affinity (K_d: ~70 nM). Our observations further indicated that plasmin binds to the A2 domain with high affinity (K_d: ~20 nM) and does not bind to the A1, highlighting the role of the A2 domain as a plasmin-interactive site.

The A2 domain is known to contain a functionally essential region spanning residues 484–509, a highly basic spacer region exposed on the surface. This region participates in at least three protein interactions; factor IXa [29], alloantibody inhibitors developed in multi-transfused hemophilia A patients [34], and low-density lipoprotein receptor-related protein (LRP) which mediates clearance of factor VIII from the circulation [35]. Plasmin cleaves the factor VIII heavy chain at Arg^{336}, Arg^{372}, and Arg^{740}, resulting in activation and inactivation of the cofactor. In the present study utilizing functional and competitive binding assays with isolated A2 subunits, synthetic peptides and an anti-A2 mAb recognizing residues 484–509, we demonstrated a major contribution of the A2 domain in plasmin docking, mediating proteolytic activity.

Of interest was the observation that EGR-factor IXa inhibited (by ~2-fold) plasmin-catalyzed inactivation of factor VIIIa in the presence of phospholipid. Factor X, however, which binds with lower affinity (K_d: 1–3 μM [14]) to the A1 acidic region consisting of residues 337–372, did not affect plasmin activity

(data not shown). Furthermore, our recent observations indicated that plasmin action against factor VIII was little affected by the presence of either von Willebrand factor or phospholipid [20]. We can speculate, therefore, that factor IXa could protect rapid down-regulation of factor VIIIa by plasmin within the assembled tenase complex in a manner similar to the mechanism whereby factor VIIIa is protected by factor IXa from APC-catalyzed inactivation [36].

To date the best known inactivators of factor VIII(a) are APC and factor Xa. The factor VIIIa interactive sites for APC- or factor Xa-catalyzed inactivation have been already identified. APC interacts with the A3 domain (residues 2009–2018) of light chain and cleaves at Arg^{336} within A1 [37]. Factor Xa interacts with the A1 domain (residues 337–372) of the heavy chain and with the C2 domain of the light chain to cleave at Lys^{36} and Arg^{336} [14,25]. In contrast, based on our present and previous observations [20], plasmin interacts with the A2 domain and light chain for specific cleavage at Arg^{336} and Lys^{36}. Furthermore, the presence of von Willebrand factor and phospholipid modulates inactivation of factor VIIIa by APC and factor Xa [38–40], but has little effect on plasmin-mediated reactions. This evidence suggests that down-regulation of factor VIIIa by these three proteases in the coagulation system is governed by distinct interactive and cleavage mechanisms.

The preparation of a series of single-point A2 mutants allowed us to determine the critical amino acid residues of the plasmin-interactive site within the A2 domain. Recently, Sarafanov et al. [26] identified the binding site for LRP using these A2 mutants. In our study, the mutant R484A markedly reduced the affinity of A2 binding to plasmin (~250-fold) compared with wt-A2. The mutants K377A, K466A, and R471A also mildly reduced this affinity. In addition, the R484A mutant was considerably much less effective than wt-A2 at moderating plasmin-catalyzed inactivation of factor VIIIa. This indicates that Arg^{484} provides a major plasmin-interactive site that promotes enzyme docking during plasmin-catalyzed cleavage of the heavy chain.

Mutations corresponding to R484A and K377A are not listed in the hemophilia A database, but the specific activities of these preparations were similar to that of wt-A2. The specific activities of K466A and R471A correlate with those of the natural mutations, K466T and R471G, seen in mild/moderate hemophilia A [41,42]. Significant conformational disturbances produced by these four mutations are unlikely, therefore, and our findings strongly suggest that residue Arg^{484} is the major functional determinant of the A2 binding site for plasmin. The competitive assays using synthetic peptides and mAb413 with an epitope involving this residue demonstrated that Lys^{377}, Lys^{466}, and Arg^{471} appeared to be principally involved in plasmin docking to A2.

On the basis of the factor VIII homology with ceruloplasmin [43], the currently identified four residues appear to be arranged in a spatially adjacent fashion and exposed on the A2 surface. This provides an extended surface for plasmin binding juxtaposed to the Arg^{336} cleavage site in the A1. Interestingly, comparisons of amino acid sequences among human, porcine, murine, and canine factor VIII molecules (http://europium.csc.mrc.ac.uk) indicate that Arg^{484}, the major contributor for plasmin binding in our study, has less inter-species homology (Arg, Ser, Ser, and Thr, respectively) than the other three residues that are well-conserved. It may be that the mechanism of plasmin action against factor VIII is species-dependent.

The five kringle domains of plasmin, containing homologous lysine-binding sites, play an important role in binding to numerous ligands. A lysine-binding site is essentially constructed in three parts; a hydrophobic core, a cationic center, and an anionic center [44]. The binding mechanism involves hydrogen-bond and/or ion-pair interaction with the cationic or anionic center and van der Waals electronic interaction with the hydrophobic core [45]. In our studies, the lysine analog, 6-AHA, that interacts tightly with lysine-binding sites, inhibited only ~30% A2 binding to Ah-plasmin. Since Arg^{484} in A2 contributed more significantly than the lysine residues to plasmin binding, we suggest that plasmin interacts with Arg^{484} independently of lysine-binding sites. The lysine residues Lys^{377} and Lys^{466} may play a relatively minor role in a lysine-binding sites-dependent mechanism.

In contrast, the binding of factor VIII light chain to Ah-plasmin with mild affinity was completely blocked by 6-AHA, indicating that this interaction is dependent on lysine-binding sites. Plasmin interaction with the light chain, therefore, appears to be quite different from that with the heavy chain (A2 domain). The lysine residues in factor VIII are clustered in both the N-terminal portion (residues 1690–1695) and the middle portion (residues 1804–1818) of the A3 domain of light chain [2,3]. Three-dimensional modeling indicates that these sequences are located over an extended surface in the A3 domain [43], and it is tempting to speculate that the plasmin-interactive site within the light chain may be found in this region. Further studies are required, however, to clarify the lysine-binding sites-dependent mechanisms involved in plasmin interaction with the light chain of factor VIII.

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Selective factor VIII and V inactivation by iminodiacetate ion exchange resin through metal ion adsorption*

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Summary

The procoagulant activity of factors VIII and V depends on the presence of metal ion(s). We examined the effect of cation-exchange resins with different functional groups on both factors, of which only reaction with iminodiacetate resin resulted in the complete loss of their activity levels in plasma. However, the antigen level of factor VIII was preserved by >95%. This resin reduced divalent cations content present in factor VIII preparations, indicating that it inactivated this factor by direct deprivation of predominant Ca^{2+} ($>Mn^{2+}>>Cu^{2+}$), rather than adsorption of the factor itself. The antigen level of recombinant factor VIII alone was decreased by >95% by reaction with resin, whilst that complexed with von Willebrand factor was preserved by >95%. Iminodiacetate resin-treated plasma was evaluated by measuring factor VIII and V activity in plasma with various levels of either activity. These were significantly correlated to the values obtained using factor VIII- or V-deficient plasma prepared commercially by immunodepletion. We demonstrated that iminodiacetate resin-induced factors VIII and V inactivation is because of direct deprivation of metal ions, predominantly Ca^{2+}, which is more essential for the functional structure of their molecules. Furthermore, iminodiacetate resin-treated plasma would be useful as a substrate for measuring the activity of these factors.

Keywords: factor VIII, factor V, iminodiacetate resin, inactivation, metal ion.

Factors VIII and V function as crucial cofactors in the tenase and prothrombinase complexes that are responsible for factor X activation in an activated factor IX (factor IXa)-dependent reaction and for prothrombin activation in an activated factor X (factor Xa)-dependent reaction on phospholipid surfaces respectively (Mann *et al*, 1988, 1990). Factor VIII deficiency causes a severe congenital bleeding disorder known as haemophilia A. Factors VIII and V are synthesized as multi-domain, single chain molecules (A1-A2-B-A3-C1-C2) with a molecular mass of approximately 300 kDa (Kane & Majerus, 1981; Vehar *et al*, 1984). Both cofactors, which display similar homologies with approximately 40% identical sequences (Vehar *et al*, 1984; Kane & Davie, 1986), are formed by a heavy chain consisting of the A1, A2 and B domains, together with a light chain consisting of the A3, C1 and C2 domains. Factor VIII circulates in plasma as a heterodimer formed by heavy and light chains, whilst factor V circulates as a single chain.

Factor VIII circulates as a non-covalent complex with von Willebrand factor (VWF), which regulates the synthesis and stabilizes the cofactor activity of factor VIII (Weiss *et al*, 1977; Kaufman *et al*, 1989). Quantitative or qualitative defects in VWF result in a decreased level of circulating factor VIII. Critical sites for VWF interaction in factor VIII have been localized to the A3 and C2 domains (Lollar *et al*, 1988; Saenko *et al*, 1994). The association of factor VIII with VWF in this complex results in its increased circulatory half-life (Saenko *et al*, 1999) and enhanced stability of heavy chain–light chain interactions (Fay, 1988). VWF protects factor VIII from proteolysis by several serine proteases including activated protein C (Fay *et al*, 1991; Nogami *et al*, 2002) and factor Xa (Koedam *et al*, 1990; Nogami *et al*, 1999).

Factor VIII possesses a metal-binding motif similar to factor V. Binding reactions of the heavy and light chains of factor VIII(a) and V(a) are non-covalent and require a metal

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ion-dependent linkage with the A1 and A3 domains (Fay, 1988; Adams *et al*, 2004). The high-affinity Ca^{2+} (and Mn^{2+})-binding sites have been identified in the A1 domain of factors VIII and V by site-directed mutagenesis analysis (Sorensen *et al*, 2004; Wakabayashi *et al*, 2004). Cu^{2+}-binding sites are also located in the A1 and/or A3 domains of both molecules (Tagliavacca *et al*, 1997; Adams *et al*, 2004). Factor VIII and V activity can be reconstituted from isolated heavy and light chains in the presence of Ca^{2+}, Cu^{2+} and Mn^{2+} metal ions (Fay, 1988). Occupancy of the high-affinity Ca^{2+} (and Mn^{2+})-binding site promotes the cofactor activity of factor VIIIa by modulating the heterodimer conformations on the anionic membrane surfaces (Fay, 1988; Wakabayashi *et al*, 2005), and is necessary for a stable association between the heavy and light chains of factor V(a) (Sorensen *et al*, 2004). On the other hand, Cu^{2+} enhances the inter-chain affinity by approximately 100-fold rather than contributing to the specific activity of factor VIII (Wakabayashi *et al*, 2001), whilst its functional role for factor V is not known yet. However, these findings indicate that these metal ions are essential in conserving the functional structure of factors VIII and V.

To further elucidate the metal cation(s)-dependent function of both factors VIII and V, we investigated whether cation-exchange resins affected these molecules. We demonstrated that the cation-exchange iminodiacetate resin inactivated factors VIII and V, not by direct adsorption to the resin, but probably by deprivation of metal ions, predominantly Ca^{2+}, from the molecules. Furthermore, the iminodiacetate resin-treated plasma prepared by this new strategy would be very useful as a substrate for measuring the activity of both factors VIII and V.

Materials and methods

Reagents

Purified recombinant factor VIII preparations (Kogenate FS®) and plasma-derived factor VIII/VWF concentrates (Confact F®) were generous gifts from Bayer Corp. Japan (Osaka, Japan) and Chemo-Sero-Therapeutic Research Inc. (Kumamoto, Japan) respectively. VWF was purified from the factor VIII/VWF concentrates using gel filtration on a Sepharose CL-4B column and immune beads coated with immobilized anti-factor VIII monoclonal antibody (Shima *et al*, 1992). Enzyme-linked immunosorbent assay (ELISA) for detection of factor VIII demonstrated >95% purity of VWF. The cation-exchange resins purchased commercially were as follows (Table I): (1) iminodiacetate group: Muromac A-1, Muromac B-1 (both from Muromachi Chemicals, Fukuoka, Japan) and Amberlite IRC748 (Organo Corp., Tokyo, Japan); (2) sulfonate group: Amberlite IR120B Na, Amberlite IR124 Na and Amberlite 200CT Na (Organo Corp.); and (3) carboxylate group: Amberlite IRC50 and Amberlite IRC76 (Organo Corp.) were stored according to the manufacturer's instructions. Coagtrol-N (Sysmex, Kobe, Japan) was used as normal pooled plasma. Factor VIII- or V-deficient plasma prepared by immunodepletion was purchased from Siemens Healthcare Diagnostics (Deerfield, IL, USA). The EVALU-VIII and SYSCOR Sets were purchased from George King Bio-Medical Inc. (Overland Park, KS, USA) and were used as test samples for evaluating the various activity levels of factors VIII and V.

Table I. Functional groups of cation-exchange resins.

Type of functional group	Name®
Sulfonate	Amberlite IR120B Na
	Amberlite IR124 Na
	Amberlite 200CT Na
Carboxylate	Amberlite IRC50
	Amberlite IRC76
Iminodiacetate	Amberlite IRC748
	Muromac A-1
	Muromac B-1

Preparation of resin-treated plasma

The cation-exchange resins were dialysed in 150 mM NaCl buffer with 0·1% ethylenediaminetetraacetic acid (EDTA) for 4 h at 4°C to remove free metal ions from the resins, followed by further dialysis in 150 mM NaCl buffer overnight prior to use. Plasma was mixed with 10% (w/v) resin in polystyrene tubes for 2 h at 22°C with stirring. After centrifuging at 2000 *g*, the supernatants were adjusted by adding 0·5 M HEPES buffer, pH 7·0 in a 50:1 volume ratio.

Measurement of activity and antigen levels of factor VIII and V

The activity levels of factors VIII and V were measured by activated partial thromboplastin time (APTT)-based and prothrombin time (PT)-based one-stage clotting assays respectively (Wolf, 1953; Hardisty & MacPherson, 1962). In some cases, factor VIII activity was also measured in a two-stage clotting assay using thromboplastin generation test (Biggs *et al*, 1955). VWF activity was measured by an assay for ristocetin cofactor of VWF (MacFarlane *et al*, 1975). The antigen levels of factor VIII and VWF were measured by a two-site sandwich ELISA using alloantibodies with C2 epitopes and rabbit anti-human VWF (Dako, Glostrup, Denmark), respectively, as described previously (Shima *et al*, 1995). The levels of factor V antigen were measured by an ELISA using a commercial kit (Matched-Pair Antibody Set for ELISA of human factor V antigen, Affinity Biologicals, Ontario, Canada).

Measurement of metal ions concentrations in factor VIII preparations

Samples were heated at 95°C for 2 min and centrifuged at 3500 *g* for 30 min. Metal ion (Ca^{2+}, Cu^{2+} and Mn^{2+})

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).

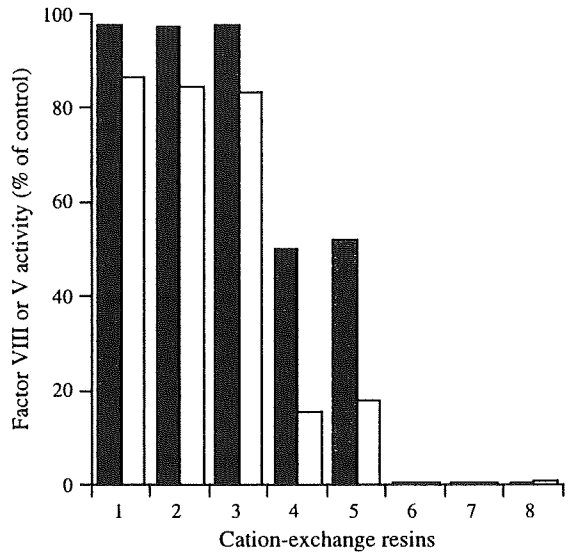

Fig 1. Activity of factor VIII and V in plasma incubated with cation-exchange resins. Normal plasma was mixed with 10% (w/v) cation-exchange resins at 22°C for 2 h. Resins 1–3 belong to the sulfonate group (Amberlite IR120B Na, Amberlite IR124 Na and Amberlite 200CT Na respectively), resins 4 and 5 belong 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.

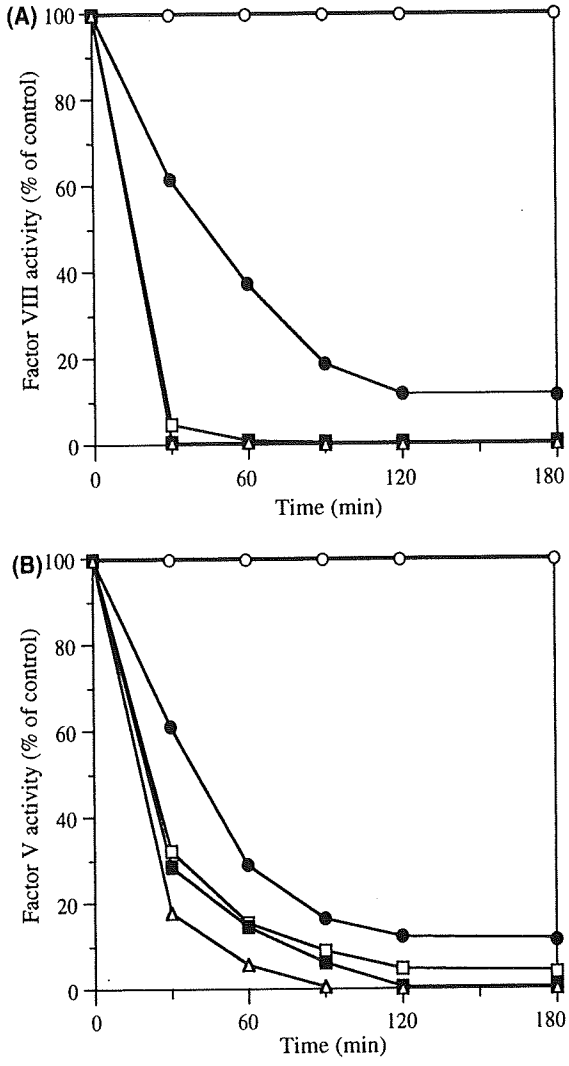

Fig 2. Inactivation of factors VIII and V in plasma by iminodiacetate resin. Varying amounts of iminodiacetate resin (%, w/v) were incubated with normal plasma at the indicated times prior to measuring the activity of factors VIII (A) and V (B) in a one-stage clotting assay. Symbols represent different 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.

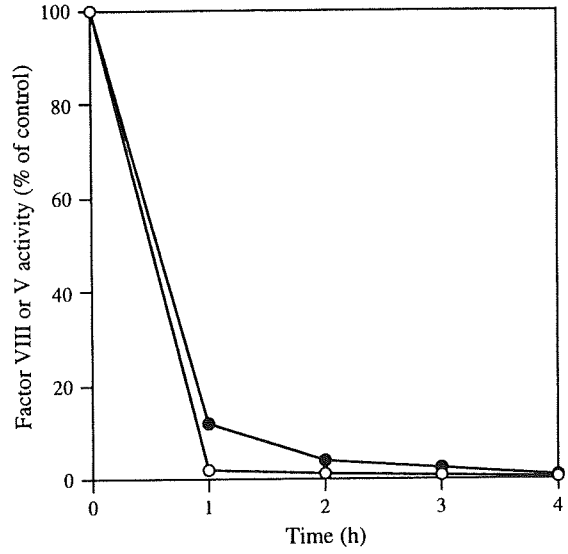

Fig 3. Inactivation of factor VIII in factor V-deficient plasma and factor V in factor VIII-deficient plasma by iminodiacetate resin. Iminodiacetate resin (10%, w/v) was mixed with factor V- and factor VIII-deficient plasma, following the measurement of the activity of factor VIII (*open circles*) or 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.

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 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.

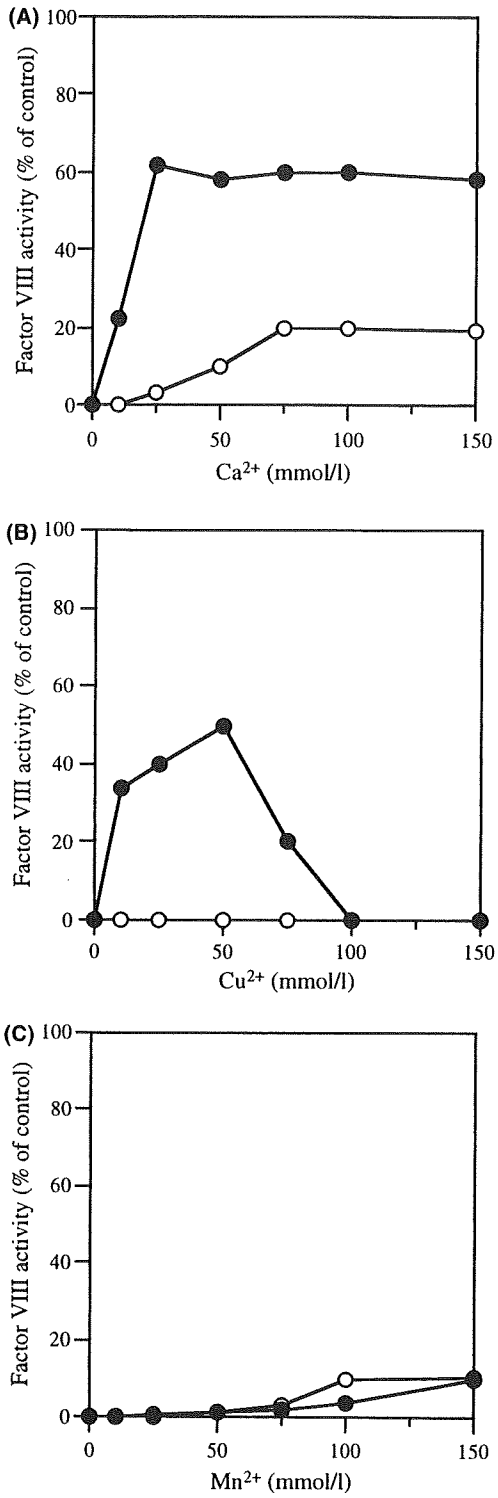

Fig 4. Inhibitory effect of metal ions on resin-induced inactivation of factor VIII in the absence or presence of von Willebrand factor (VWF). Recombinant factors VIII (*open circles*) and plasma-derived factor VIII/VWF (*closed circles*) were mixed with varying amounts of Ca^{2+} (A), Cu^{2+} (B) and 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 = 0·997) 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 = 0·997) 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 = 0·995, 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.

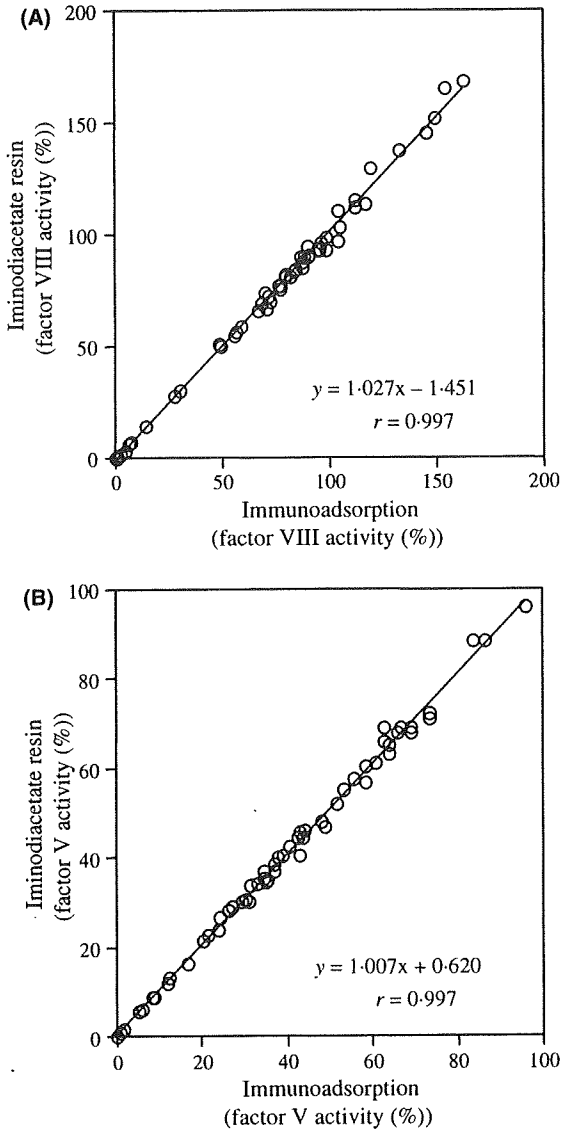

Fig 5. Correlation between iminodiacetate resin-treated and immunodepleted plasma for measurement of factor VIII and V activity. Factor VIII (A) or V (B) activity in plasma with varying levels of factor VIII or V activity, respectively, was measured using plasma samples treated with iminodiacetate resin 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．

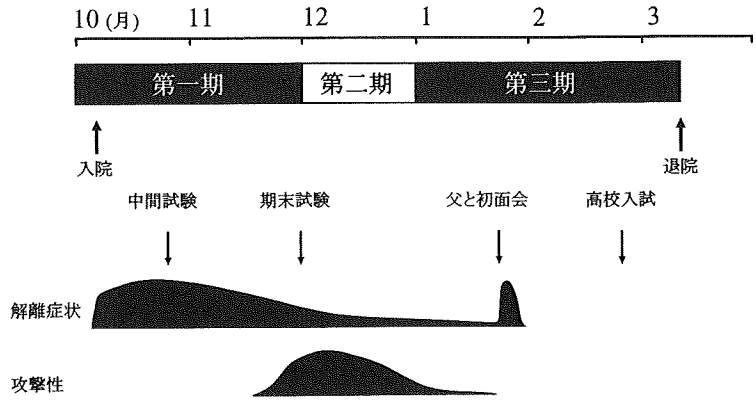

図1 経過表

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[5]．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.