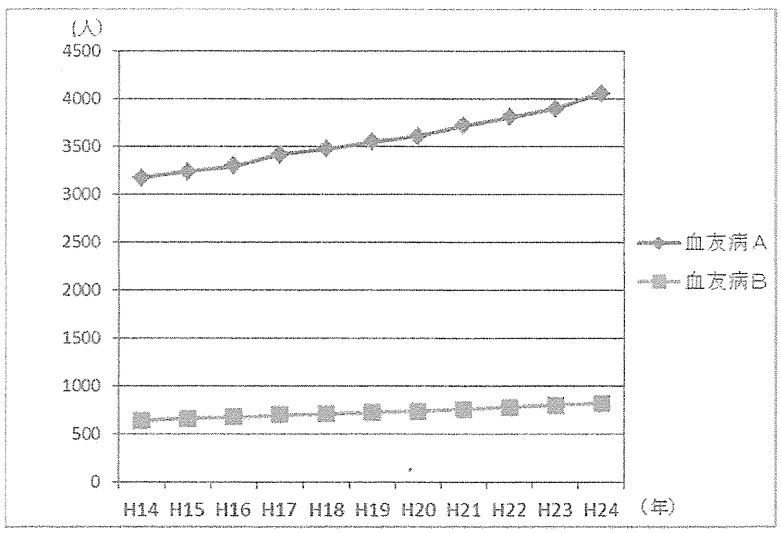
表 2　各国の血友病患者数と人口に対する比率

国	人口	患者数	人口に対する比率 （小数点以下切り上げ）
United Kingdom	62,641,000	6,575	9,527
Canada	34,482,779	3,380	10,202
Switzerland	7,907,000	701	11,280
France	65,436,552	5,735	11,410
Denmark	5,574,000	477	11,686
Germany	81,726,000	4,654	17,560
United States	311,591,917	17,485	17,821
Brazil	196,655,014	10,558	18,626
Argentina	40,764,561	2,133	19,111
Japan	127,817,277	5,446	23,470
Korea, Republic of	49,779,000	1,908	26,090
Russia	141,930,000	5,421	26,182
China	1,344,130,000	10,182	132,010

WFH 2011



瀧 正志. 厚生労働省委託事業 血液凝固異常症全国調査

図 1　わが国の血友病患者登録数

79%，中央的患者登録システムが完備されているのは57%であった。すべての患者が包括的ケアセンター（Comprehensive care centres: CCCs）あるいは血友病治療センター（Hemophilia treatment centre; HTC）でみられている国は64%であった。我が国では，血友病専門医のボードは，たとえば日本血栓止血学会標準化委員会血友病部会や小児血液がん学会止血血栓委員会が相当するが，患者の登録システムは確立されていない。英国ではHTCが40，CCCが29施設あり，また血友病専門医は United Kingdom Haemophilia Centre Doctors'

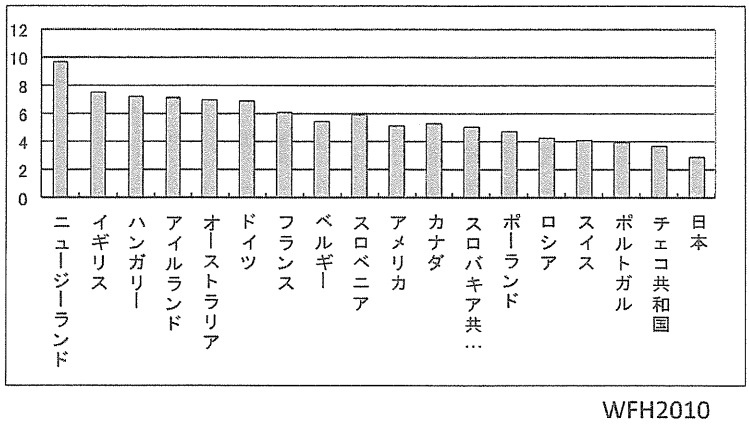


図２　国別一人当たりの製剤使用量

表３　包括的治療センターと血友病治療センターの定義[14]

包括的ケアセンター（CCC）	血友病治療センター（HTC）
・最低 40 人の重症血友病患者（FVIII/IX ＜1%）	・最低患者数の指定なし
・24 時間　専門的治療が可能	・24 時間 専門家がカバー
・24 時間　検査が可能	・検査対応可能（遅れあり）
・以下を含む，多くの専門分野に渡る包括的ケアチームによる治療を実施 フルタイムの血液学者，あるいは小児科医を１人は採用 熟練した看護士 経験豊富な理学療法士 ソーシャルワーカー データ管理	・以下を含む，多くの専門分野に渡る包括的ケアチームによる治療を実施 フルタイムの血液学者，あるいは小児科医を１人は採用 熟練した看護士 経験豊富な理学療法士 ソーシャルワーカー 十分な記録の保持
・在宅自己注射，予防処置，インヒビター治療，および ITI の実行	・CCC と協力して 在宅自己注射，予防処置，インヒビター治療，および ITI の実行
・産婦人科，整形外科，歯科，遺伝子学との連携	・CCC と協力して 産婦人科，整形外科，歯科，遺伝子学との連携
・診療監査の実行 (内部監査は必須，外部監査は実施するのが望ましい)	・診療監査の実行
・コンセンサスガイドラインを順守し， 医学教育も提供	・コンセンサスガイドラインを順守し， 医学教育も提供
・研究も実施する	

Organisation（UKHCDO）を中心に組織されている。米国では 141 の HTC があり，CDC（Centers for Disease Control and Prevention）や HRSA（Health Resources and Services Administration）よりサポートされている。我が国ではまだ CCCs の基準を満たす施設はない。人口 100 万人あたりの HTCs は欧州では中央値 0.84（0.62～1.11）である。欧州の基準から考えると我が国では 80～145 施設必要なことになる。

3. 診療連携

前述したように，我が国では諸外国のように血友病治療センターや血友病包括的ケアセンターが確立していない。したがって，血友病診療の質的差が生じることはいなめない。定期補充療法の普及により，患者の関節症の予後やQOLは改善しており特に1次的補充療法は血友病性関節症の発症や進行を抑制する。しかしながら，Manco Johnsonらが実施したオンデマンド治療法と1次定期補充療法群とのランダム化前向き調査では定期補充群でも多少関節スコアが悪化している[15]。さらに，思春期から若年成人年齢にかけてコンプライアンスが低下するために包括的な治療・ケア体制が必要である。したがって，血友病診療は小児科/血液内科/内科での止血治療のみならず，関節症の評価のための整形外科やリハビリテーション科，さらに口腔外科など他科との診療連携が必須である。また，日常の注射指導など看護部との連携や大手術の止血管理やインヒビター陽性例の治療あるいは救急対応などの専門施設との連携も重要である。

診療連携の第一歩は，院内連携システムの構築である。特に看護部，整形外科，内科/血液内科，医療相談課などとの連携を構築する。院内包括外来を実施するのが院内連携構築の第一歩になる（図3）。また，実際の生活，学校や職場などにおける様々な相談については患者会の紹介も重要である（全国ヘモフィリア友の会ネットワーク：http://hemophilia.web.fc2.com）。院内連携とともに，都道府県内あるいは地域の専門施設との連携体制も必要である。日本小児血液がん学会止血血栓委員会の小児血友病診療ネットワーク（小児血液がん学会止血血栓委員会，瀧　正志　委員長，事務局 m3asa@marianna-u.ac.jp）では全国の血友病診療施設321施設で計520名の医師が参加している（図4）。ブロック長及び都道府県代表が決められており，地域診療連携の基盤となる。また，インヒビターの手術などさらに高い専門性が必要な場合には，専門施設と患者在住の診療施設との連携で実施することも可能である。図5に奈良医大小児科と他施設との連携により整形外科手術の実施体制を示している。まずかかりつけの診療施設から血友病性関節症の手術に関するコンサルトを受け，あらかじめ凝血学的評価および整形外科的評価のために受診してもらい，小児科ではインヒビターの評価，バイパス製剤の効果判定を実施して整形外科では当該関節の評価と手術計画をたてる。手術時は小児科が止血管理を実施して，リハビリ科と連携して術後のリハビリを実施する。リハビリが安定したころに退院して，紹介先の診療施設でリハビリを継続する。専門施設と診療施設が連携することで高度の治療もよりスムーズに実施することができると思われる。

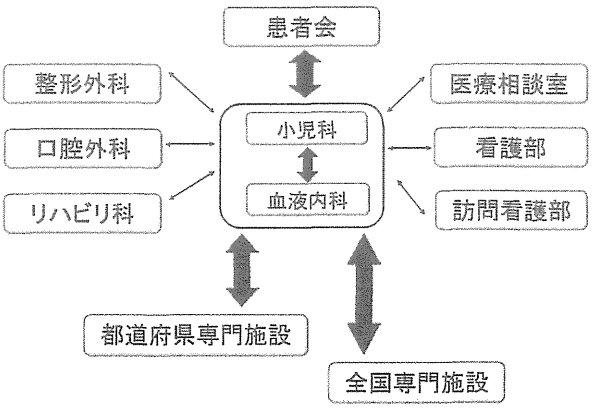


図3　院内外診療連携の構築

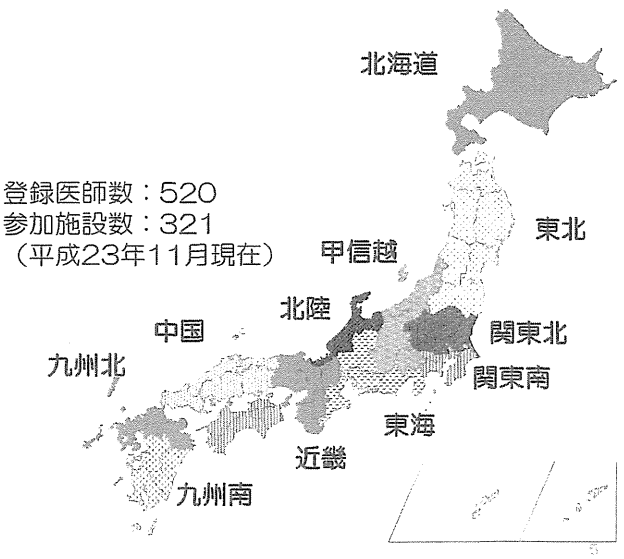


図4　小児血友病診療ネットワーク

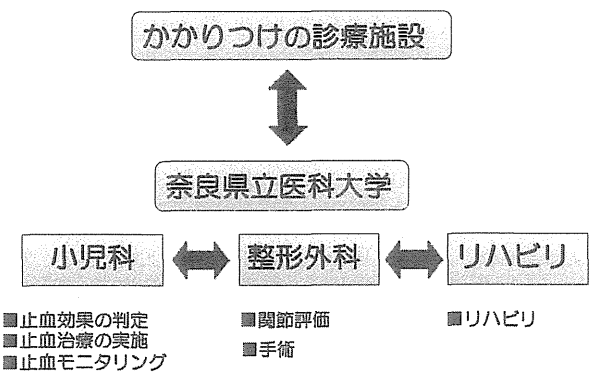


図5　血友病Aインヒビターの滑膜切除術の診療連携例

表4 UKHCDO Guideline リスト

Year	Reference	Title
2011	Haemophilia, 17, e877-883	UKHCDO guidelines on the management of HCV in patients with hereditary bleeding disorders 2011.
2011	Br J Haematol, 154, 208-215	Guideline on the management of haemophilia in the fetus and neonate
2010	CMGS Website	Practice Guidelines for the Molecular Diagnosis of Haemophilia A
2010	CMGS Website	Practice Guidelines for the Molecular Diagnosis of Haemophilia B
2010	Br J Haematol, 149, 498-507	A United Kingdom Haemophilia Centre Doctors' Organization guideline approved by the British Committee for Standards in Haematology: guideline on the use of prophylactic factor VIII concentrate in children and adults with severe haemophilia A
2009	UKHCDO Website	Emergency and out of hours care for patients with bleeding disorders–Standards of care for assessment and treatment
2008	Haemophilia, 14, 1099-1111	The molecular analysis of von Willebrand disease: a guideline from the UK Haemophilia Centre Doctors' Organisation Haemophilia Genetics Laboratory Network
2008	Haemophilia, 14, 671-684	Guideline on the selection and use of therapeutic products to treat haemophilia and other hereditary bleeding disorders. A United Kingdom Haemophilia Center Doctors' Organisation (UKHCDO) guideline approved by the British Committee for Standards in Haematology
2006	Haemophilia, 12, 301-336	The obstetric and gynaecological management of women with inherited bleeding disorders-review with guidelines produced by a taskforce of UK Haemophilia Centre Doctors' Organization
2006	Br J Haematol, 133, 591-605	The diagnosis and management of factor VIII and IX inhibitors: a guideline from the United Kingdom Haemophilia Centre Doctors Organisation
2006	Br J Haematol, 135, 603-633	A review of inherited platelet disorders with guidelines for their management on behalf of the UKHCDO
2005	Haemophilia, 11, 145-163	A framework for genetic service provision for haemophilia and other inherited bleeding disorders
2004	Haemophilia, 10, 218-231	Management of von Willebrand's disease: a guideline from the UK Haemophilia Centre Doctors' Organisation
2004	Haemophilia, 10, 199-217	The diagnosis of von Willebrand's disease: a guideline from the UK Haemophilia Centre Doctors' Organisation
2004	Haemophilia, 10, 593-628	The rare coagulation disorders–review with guidelines for management from the United Kingdom Haemophilia Centre Doctors' Organisation

4. 血友病の診療ガイドライン

血友病診療の標準化において診療ガイドラインは重要である。国際的には最近 WFH から非常に詳細なガイドラインが発表されている[16]。英国では血友病専門医による United Kingdom Haemophilia Centre Doctors' Organisation (UKHCDO) から血友病診療に関する様々なガイドラインが発表されている（表4)。わが国では，日本血栓止血学会標準化委員会血友病部会が中心となって，文献検索，実態調査および専門家の意見に基づきインヒビター非保有例[17]および保有例の診療ガイドラインが作成された[18]。現在，改訂版を製作中である。

5. 血友病の登録システム

わが国では国家規模の血友病患者の登録システムは存

在しない。欧州では仏，独，ギリシア，伊，オランダ，ノルウェー，スロバキア，スペイン，英国で各患者が登録されている。血友病患者の登録システムは我が国における血友病の疫学に重要であるが，血友病診療の国際比較や血友病診療の政策においても必須である。現在，厚労省の研究班で新規発生血友病患者に関する前向き調査が実施されている（厚生労働科学研究事業　血友病の治療とその合併症の克服に関する研究　分担研究「第VIII，第IX因子製剤のインヒビター発生要因に関する研究」：事務局　奈良県立医科大学小児科　pedlab@naramed-u.ac.jp）。

最後に

血友病診療の基礎と，小児期から成人への移行期のポイントと我が国の課題について解説した。移行期の治療は小児科と内科/血液内科との谷間にあるが，以後の患者のQOLを大きく作用する重要な時期である。止血治療のみならず様々な領域や職種と連携して診療を進める必要がある。今後，血友病の治療は長時間作用型製剤などの新たな製剤の開発のみならず遺伝子治療など次世代の治療も期待されている。国際的にみて我が国の診療レベルが遅れないためにも我が国における血友病診療の標準化と連携体制の構築が急務であると思われる。

著者のCOI（conflicts of interest）開示：嶋緑倫；講演料（ノボノルディスクファーマ株式会社，バクスター株式会社），研究費・助成金（中外製薬株式会社），寄付金（CSLベーリング株式会社，ノボ ノルディスクファーマ株式会社）

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bjh research paper

Different factor VIII neutralizing effects on anti-factor VIII inhibitor antibodies associated with epitope specificity and von Willebrand factor*

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Summary

Inhibitor neutralization therapy based on factor (F)VIII replacement is used for haemostatic treatment in haemophilia A patients with inhibitors on low responder, but effects appear to depend on various properties of inhibitors. We investigated this nature by evaluating the global coagulation function in timed-reactions after mixing FVIII (1 U/ml) with anti-FVIII alloantibodies containing distinct epitopes (2·5 Bethesda units/ml). Thrombin generation assays showed that peak thrombin and mean velocity to peak thrombin were depressed by anti-C2 type 1 inhibitors to significantly greater extents than by anti-A2 type 1 and anti-C2 type 2 (2- to 6-fold and 10- to 20-fold, respectively). In the presence of FVIII-von Willebrand Factor (VWF) complex, the anti-C2 type 1-mediated decreased thrombin generation was reduced by 20–40%, reflecting the protective function of VWF. However, the activities of anti-A2 type 1 were little affected, and that of anti-C2 type 2 was rather enhanced by *c.* 2·5-fold, relative to FVIII. Clot waveform analysis also showed similar patterns. Anti-FVIII monoclonal antibodies with well-defined characteristics demonstrated similar reactions to those with polyclonal inhibitors. In conclusion, the neutralizing effects of FVIII(-VWF) depending on epitopes could have significant therapeutic implications, and it could be important to determine inhibitor properties in order to predict the effects of infused FVIII in neutralization therapy.

Keywords: factorVIII, inhibitor, neutralization therapy, haemophilia A, epitopes.

Factor (F)VIII, a plasma protein deficient in individuals with the severe congenital bleeding disorder, haemophilia A (HA), functions as a cofactor in the tenase complex, responsible for phospholipid (PL)-dependent FIXa-mediated activation of FX (Mann *et al*, 1990). The FVIII molecule is arranged into three domains (A1-A2-B-A3-C1-C2) based on amino acid homology, and is processed into a series of heterodimers, generating a heavy chain (HCh) consisting of A1 and A2 domains together with heterogeneous fragments of proteolysed B domain linked to a light chain (LCh) consisting of A3, C1, and C2 domains. The catalytic efficiency of FVIII in the tenase complex is markedly enhanced by conversion into FVIIIa, through limited proteolysis by thrombin and FXa (Eaton *et al*, 1986). Both enzymes proteolyse at Arg^{372} and Arg^{740} in the HCh, resulting in the generation of 50-kDa A1 and 40-kDa A2 subunits. The 80-kDa LCh is proteolysed at Arg^{1689} producing a 70-kDa subunit. Proteolysis at Arg^{372} and Arg^{1689} is essential for generating FVIIIa cofactor activity (Fay, 2004). Cleavage at the latter site liberates FVIII from its carrier protein, von Willebrand factor (VWF; Lollar *et al*, 1988). FVIIIa activity is down-regulated by activated protein C, following cleavage at Arg^{336} (Eaton *et al*, 1986).

FVIII inhibitors develop as alloantibodies (alloAbs) in 20–30% of multi-transfused HA patients (Oldenburg *et al*, 2000). The reduction or disappearance of FVIII coagulant activity (FVIII:C) in the presence of anti-FVIII antibodies is associated with impairment of FVIII(a) cofactor function mediated by binding to functionally essential regions on FVIII. Anti-FVIII inhibitor antibodies either inhibit FVIII:C completely or incompletely at saturating concentrations, corresponding to a classification of type 1 or type 2, respectively (Gawryl & Hoyer, 1982). Major inhibitory epitopes have been localized to one or both of the A2 and C2 domains (Prescott *et al*, 1997). Anti-C2 antibodies sub-classified as

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type 1 prevent the binding of FVIII to PL and VWF (Shima *et al*, 1993), whilst those classified as type 2 prevent the association of FVIII with thrombin and FXa (Meeks *et al*, 2007; Matsumoto *et al*, 2012). Anti-A2 antibodies prevent the association of FVIIIa with FIXa (Fay & Scandella, 1999).

Clinical treatment protocols for HA patients with inhibitor are based on replacement therapy using FVIII concentrates ('neutralization therapy') and so called 'bypassing therapy' utilizing recombinant FVIIa (rFVIIa) and plasma-derived activated prothrombin complex concentrates (APCC). The former type of therapy is usually regarded as first choice for inhibitor patients classed as low responders, whilst the latter protocols are used for those classed as high responders. Klintman *et al* (2010) reported that mixtures of FVIII and bypassing agents (rFVIIa and APCC) significantly potentiated coagulation effects *in vitro* compared to bypassing agents alone in plasmas from HA patients with inhibitor. In addition, we have recently demonstrated that FVIII was activated by limit proteolysis by rFVIIa or APCC (Soeda *et al*, 2010; Yada *et al*, 2013), and that this activation was not impaired by the presence of anti-FVIII inhibitors. Furthermore, anti-C2 type 1 inhibitors depressed the inactivation phases in both rFVIIa-mediated and APCC-mediated reactions, resulting in relatively persistent, elevated levels of FVIII:C (Yada *et al*, 2011, 2013). These findings suggested further studies were warranted to determine if the coagulation effects of neutralization therapy in HA patients with inhibitor might be governed by various characteristics of the antibodies (epitope specificity, kinetics, mechanisms of inhibition of FVIII function, etc.). In the present study, we have examined different FVIII inhibitors in *in vitro* models of neutralization therapy using mixtures of FVIII and well-defined anti-FVIII antibodies.

Materials and methods

Reagents

Recombinant FVIII preparations (Kogenate FS®) and plasma-derived FVIII-VWF concentrates (Confact F®) were provided by Bayer Corp. Japan (Osaka, Japan) and Chemo-Sero-Therapeutic Research Inc. (Kumamoto, Japan), respectively. VWF was purified from FVIII-VWF concentrates by gel filtration using Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden) and immune-beads coated with immobilized anti-FVIII mAb (Shima *et al*, 1992). Enzyme-linked immunosorbent assays of FVIII demonstrated VWF purity of >95%. Recombinant lipidated tissue factor (TF; Innovin®; Dade Behring, Marburg, Germany), ellagic acid (Sysmex, Kobe, Japan), thrombin-specific fluorogenic substrate (Bachem, Bubendorf, Switzerland), thrombin calibrator (Thrombinoscope, Maastricht, Netherlands), and FVIII-deficient plasma (George King Biomedical, Overland Park, KS, USA) were purchased from the indicated vendors. Anti-C2 mAbs ESH4 (epitope 2303–2332) and ESH8 (epitope 2248–2285) were purchased from American Diagnostica Inc. (Greenwich, CT, USA). An anti-A2 mAb, JR8, was obtained from JR Scientific Inc. (Woodland, CA, USA). PL vesicles (phosphatidylserine/phosphatidylcholine/phosphatidylethanolamine; 10/60/30%; Sigma, St Louis, MO, USA) were prepared using *N*-octylglucoside (Mimms *et al*, 1981).

Anti-FVIII inhibitor alloAbs

Six anti-FVIII inhibitor alloAbs were obtained from Japanese patients with congenital severe HA. IgG fractions were prepared using protein A-Sepharose (Amersham Biosciences). The inhibitor titres of antibody IgGs were determined using Bethesda assays. The two kinetic patterns of FVIII inactivation by anti-FVIII antibodies (type 1 and type 2 behaviors) were determined in one-stage clotting assays. Epitopes of these antibodies were determined by sodium dodecyl sulfate polyacrylamide gel elctrophoresis and Western blotting using isolated FVIII fragments. The properties of these anti-FVIII antibodies used in the present study are summarized in Table I. All experiments were performed using blood samples obtained from patients enrolled in the Nara Medical University Haemophilia Programme after informed consent following local ethical guidelines.

In vitro *model of neutralization therapy*

Purified inhibitor IgG at a final concentration (f.c.) of 2·5 Bethesda units (BU)/ml was added to FVIII-deficient plasma. The reconstituted HA inhibitor plasma samples were then incubated with FVIII or FVIII-VWF (f.c. 1 U/ml) at 37°C. FVIII-VWF was prepared by a 1 h-incubation of equivalent amount of FVIII and VWF at 37°C. Aliquots were obtained at the indicated times and coagulation function assessed using global coagulation assays.

Thrombin generation assays

Calibrated automated thrombin generation assays were performed as previously described (Matsumoto *et al*, 2009).

Table I. Properties of anti-FVIII inhibitor antibodies.

Case	Type of Ab	Epitope	Type of kinetics	Inhibitor titre (BU/ml)
1	alloAb	A2	1	114
2	alloAb	A2	1	56
3	alloAb	C2	1	128
4	alloAb	C2	1	580
5	alloAb	C2	1	23
6	alloAb	C2	2	4
JR8	mAb	A2	1	620
ESH4	mAb	C2	1	33
ESH8	mAb	C2	2	840

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Plasma samples (80 μl) were preincubated for 10 min with 20 μl of trigger reagent containing TF, PL, and ellagic acid (f.c. 0·5 pmol/l, 4 μmol/l, and 0·3 μmol/l, respectively). Measurements were then recorded after the addition of 20 μl reagent containing $CaCl_2$ and fluorogenic substrate (f.c. 16·7 mmol/l and 2·5 mmol/l, respectively). The development of fluorescent signals was monitored using a Fluoroskan Ascent microplate reader (Thermo Fisher Scientific, Boston, MA, USA). Data analyses were performed using the manufacturer's software to derive the standard parameters; peak thrombin, lagtime, and time to peak. It was difficult to evaluate the small changes in endogenous thrombin potential in the present study, and hence total thrombin generation at intervals from the beginning to peak level was quantified. These values (nmol/l) were divided by the sample times (min, time to peak – lagtime), and represented the mean velocity of thrombin generation until the reach to peak level, expressed as mean velocity to peak thrombin (MV-peak thrombin).

Clot waveform analysis

Activated partial thromboplastin time (PTT) measurements were performed using the MDA-II™ Haemostasis System (Trinity Biotech, Dublin, Ireland). The clot waveforms obtained were computer-processed using the commercial kinetic algorithm (Matsumoto *et al*, 2000). The minimum value of the first derivative (min1) was calculated as an indicator of the maximum velocity of coagulation. The second derivative of the transmittance data reflected the acceleration of the reaction at any given time point. The minimum value of the second derivative (min2) was calculated as an index of the maximum acceleration of the reaction. As the minimum of min2 is derived from negative changes, the data were expressed as |min2|. Given that |min2| values were well-correlated with the levels of FVIII:C (Matsumoto *et al*, 2000), |min2| was analysed in the present study.

Data analyses

All experiments were performed at least four separate times, and the average and standard deviation values are shown. Nonlinear least squares regression analyses were performed using KALEIDAGRAPH (Synergy Software, Reading, PA, USA).

Results

Neutralizing effects of FVIII in in vitro *models of HA with inhibitors*

To examine whether the different epitopes of anti-FVIII inhibitor alloAbs affected the neutralizing ability of FVIII, *in vitro* models reflecting potential therapeutic protocols were prepared using mixtures of FVIII and anti-FVIII alloAbs, as described in Materials and methods. We considered that anti-FVIII inhibitors at 2·5 BU/ml were neutralized by FVIII at 1 U/ml in circulating blood. Coagulation function at various reaction times was evaluated by thrombin generation assays. Fig 1 shows representative curves for the different classes of inhibitor. Case 2 illustrates an anti-A2 epitope with type 1 pattern, Case 3 an anti-C2 type 1, and Case 6 an anti-C2 type 2 inhibitor. With both anti-A2 type 1 (Case 2) and anti-C2 type 1 (Case 3) a time-dependent reduction in thrombin generation (a decrease of peak thrombin and a delay of time to peak) was evident. In particular, thrombin generation in the presence of anti-C2 type 1 was significantly lower than that in anti-A2 type 1. However, the anti-C2 type 2 inhibitor (Case 6) showed a small decrease in thrombin generation over a 2-h incubation period. Results with other

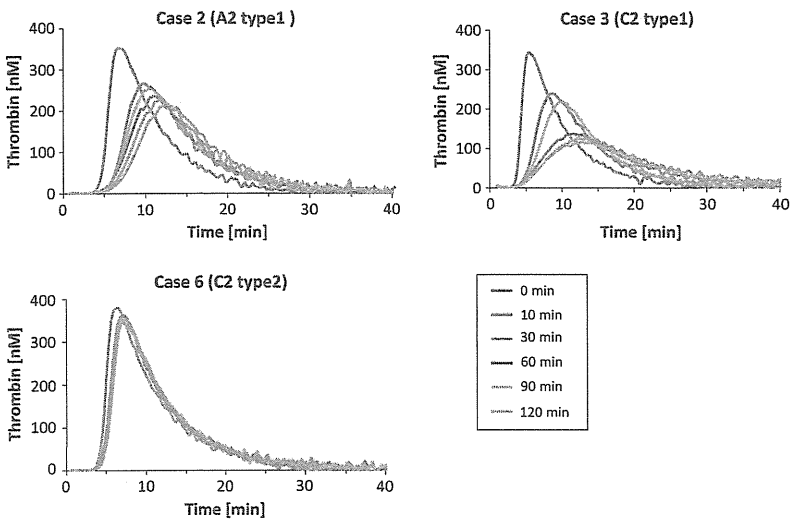

Fig 1. Thrombin generation in time-related reactions of FVIII with anti-FVIII alloantibodies – Inhibitor IgG samples [final concentration (f.c.) 2·5 BU/ml] from Cases 2, 3, and 6 were added to FVIII-deficient plasma, and incubated with FVIII (f.c. 1 U/ml). Samples were obtained at the indicated reaction times (0, 10, 30, 60, 90, and 120 min) and were mixed with tissue factor (0·5 pmol/l), ellagic acid (0·3 μmol/l), and phospholipid (4 μmol/l), prior to the addition of fluorogenic substrate and $CaCl_2$ at the start of the assay as described in Materials and methods. Representative curves from each case are illustrated.

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inhibitor samples (Cases 1, 4, and 5) showed similar patterns based on epitope classification (data not shown).

Kinetics of thrombin generation in the presence of anti-FVIII alloAbs with different epitopes

To further examine the relationship between inhibitor epitopes and the neutralizing effects of FVIII in thrombin generation assays, peak thrombin and MV-peak thrombin obtained from thrombin generation curves were analysed using the formula of single exponential decay (Fig 2A-a,b, respectively). The results are summarized in Table II. In both instances, mixtures without or with the addition of FVIII (1 U/ml) prior to incubation with inhibitor IgGs (2·5 BU/ml) were regarded as 0% or 100%, respectively. Levels of peak thrombin with anti-C2 type 1 inhibitors (Cases 4 and 5) were reduced to *c.*10% and 20%, respectively within 30-min incubation. Using another antibody with similar properties (Case 3), peak thrombin was reduced to *c.*50% of the initial level at 30-min incubation, and was decreased to *c.*30% at 2-h incubation. With both of the anti-A2 type 1 inhibitors (Cases 1 and 2), peak thrombin was >50% of the initial level after 2 h. In contrast, the anti-C2 type 2 inhibitor (case 6) produced a small decrease in peak thrombin generation over the 2-h incubation period. Compared to normal IgG (control), therefore, anti-C2 type 1 and anti-A2 type 1 inhibitors depressed the peak thrombin by 10- to 30-fold and *c.* 5-fold, respectively, whilst anti-C2 type 2 moderated activity by only *c.* 1·5-fold (Table II). Analyses of the alternative parameter, MV-peak thrombin, showed similar results, and illustrated a good correlation between peak thrombin and MV-peak thrombin measurements. It appeared, therefore, that the inhibition of thrombin generation by anti-C2 type 1 and anti-A2 type 1 inhibitors were, respectively, 10- to 20-fold and *c.* threefold more potent than that in anti-C2 type 2 in these assays. The findings suggested that the different epitopes of FVIII inhibitors could significantly affect the neutralizing ability of therapeutic FVIII infusions.

Physiologically, FVIII circulates as a complex with VWF, a macromolecule that protects and stabilizes the cofactor. Experiments as described above were repeated, therefore, using FVIII-VWF complex (1 U/ml) in place of FVIII alone. There appeared to be less decrease in peak thrombin formation (Fig 2B-a) and MV-peak thrombin measurements (Fig 2B-b) in the presence of VWF, in the order of anti-C2 type 1 (Cases 3, 4 and 5), anti-A2 type 1 (Cases 1–2), and anti-C2 type 2 (Case 6). In particular, with all three anti-C2 type 1 inhibitors, the thrombin generation measurements were less affected in the presence of FVIII-VWF (Fig 2B) compared with FVIII alone (Fig 2A). The ratios of these parameters in the presence of VWF relative to its absence (B)/(A) ranged between 0·59 and 0·89 (Table II), supporting the protective

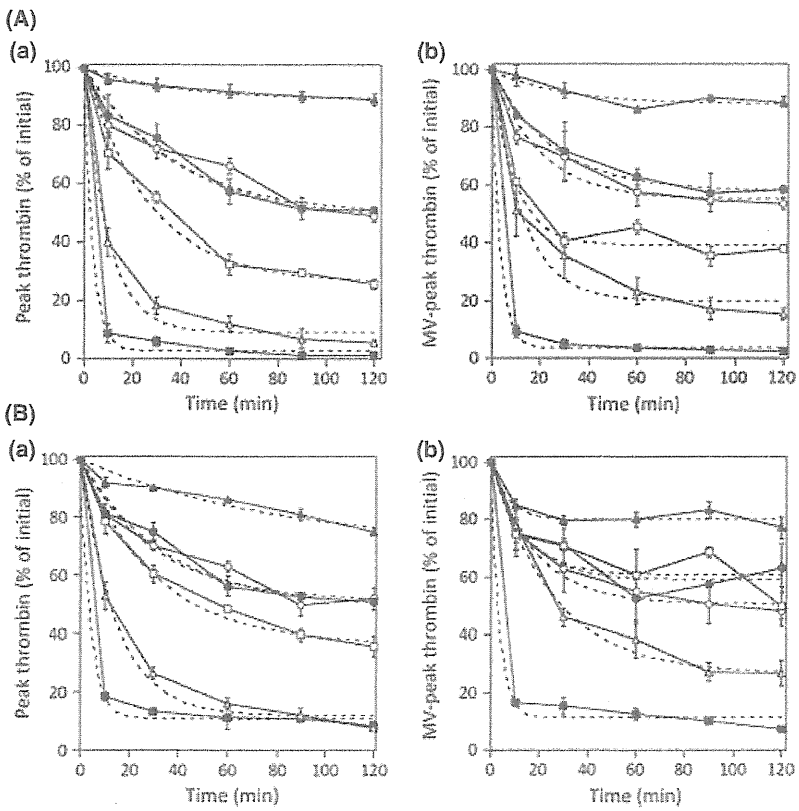

Fig 2. Time-dependent decreases in peak thrombin or mean velocity (MV)-peak thrombin in mixtures of FVIII or FVIII-VWF with anti-FVIII alloantobodies (alloAbs)- Mixtures with alloAb IgG [final concentration (f.c.) 2·5 BU/ml] and FVIII-deficient plasma were incubated with FVIII (*panel* A) or FVIII-VWF (*panel* B; f.c. 1 U/ml). Thrombin generation was measured at the indicate times as described in Materials and methods. The symbols used are, ○; Case 1, ●; Case 2, □; Case 3, ■; Case 4, △; Case 5, and ▲; Case 6, respectively. The values of peak thrombin (*panel* a) or MV-peak thrombin (*panel* b) in the absence or presence of FVIII (or FVIII-VWF) prior to incubation with inhibitor IgGs were regarded as 0% or 100%, respectively. Peak thrombin and MV-peak thrombin measurements at time zero were 300–400 nmol/l and 500–700 nmol/l/min, respectively. The data obtained in (A and B) were fitted using the formula of single exponential decay (*dotted lines*).

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function of VWF. In contrast, the results with the anti-C2 type 2 inhibitor (Case 6) suggested higher decrease rate of thrombin generation in the presence of FVIII-VWF compared with FVIII alone ((B)/(A); *c.* 2·0), whereas with both of the anti-A2 type 1 inhibitors (Cases 1 and 2), the presence of VWF had little effect on the kinetics ((B)/(A); *c.* 1·0). The VWF-interactive sites on the FVIII molecule are located in the acidic regions of the A3 and C2 domains (Foster *et al*, 1988; Saenko *et al*, 1994), and our results supported the concept that the characteristics of the anti-FVIII alloAbs with different epitopes governed interactions with FVIII and FVIII-VWF.

Effects of anti-FVIII mAbs in the in vitro *neutralization models*

Anti-FVIII inhibitor alloAbs are polyclonal, and to confirm specificity, therefore, similar experiments were repeated using anti-FVIII mAbs with well-defined epitopes. The measurements of peak thrombin (Fig 3A) and MV-peak thrombin (Fig 3B) observed with anti-C2 mAbESH4 (anti-C2 type 1) were rapidly depressed to *c.*30% of the initial level after a 30-min incubation. In contrast, thrombin generation was maintained at *c.*75% after 30 min, and decreased to *c.*65% after 2 h with anti-A2 mAbJR8 (anti-A2 type 1), and was reduced by only *c.*20% after 2 h with mAbESH8 (anti-C2 type 2). These results were similar to those obtained with polyclonal FVIII antibodies, and demonstrated that compared to normal IgG, these sensitive parameters of thrombin generation were significantly inhibited by mAbESH4, (*c.* 20-fold), but were less affected by mAbJR8 (*c.* three to sixfold) and mAbESH8 (*c.* twofold; Table III).

Inhibition of thrombin generation with mAbESH4 was slightly weaker in assays using FVIII-VWF than with FVIII alone, and VWF had very little effect on the results with mAbJR8 (Fig 3). The ratios of these parameters in the

Table II. Decrease rates of peak thrombin and MV-peak thrombin with anti-FVIII alloAbs.

	Peak thrombin			MV-peak thrombin		
Case	(A) FVIII (-fold) ×10/min	(B) FVIII-VWF(-fold) ×10/min	(B)/(A) ratio	(A) FVIII (-fold) ×10/min	(B) FVIII-VWF(-fold) ×10/min	(B)/(A) ratio
1	0·30 ± 0·08 (5·0)	0·31 ± 0·08 (5·1)	1·03	0·35 ± 0·10 (5·0)	0·36 ± 0·12 (5·1)	1·02
2	0·29 ± 0·04 (4·8)	0·30 ± 0·07 (5·0)	1·03	0·36 ± 0·06 (5·1)	0·39 ± 0·10 (5·6)	1·07
3	0·63 ± 0·06 (10)	0·50 ± 0·06 (8·3)	0·79	0·94 ± 0·14 (13)	0·55 ± 0·09 (7·9)	0·59
4	2·03 ± 0·31 (33)	1·65 ± 0·30 (27)	0·81	2·30 ± 0·19 (33)	2·05 ± 0·46 (29)	0·89
5	1·19 ± 0·19 (19)	0·89 ± 0·07 (14)	0·74	1·09 ± 0·22 (16)	0·69 ± 0·07 (9·9)	0·63
6	0·09 ± 0·02 (1·5)	0·18 ± 0·04 (3·0)	2·00	0·12 ± 0·05 (1·7)	0·29 ± 0·04 (4·1)	2·40
Control	0·06 ± 0·01 (1·0)	0·06 ± 0·01 (1·0)	—	0·07 ± 0·01 (1·0)	0·07 ± 0·01 (1·0)	—

Values were calculated by nonlinear least squares regression from the data shown in Fig 2 using single exponential decay.

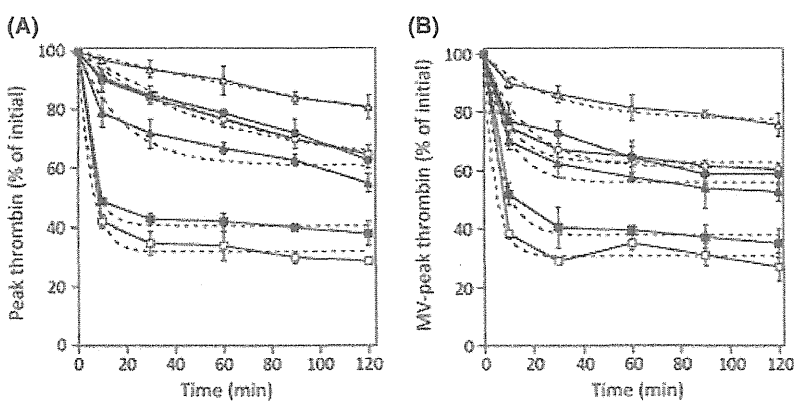

Fig 3. Time-dependent decreases in thrombin generation parameters in mixtures of FVIII or FVIII-VWF with anti-FVIII monoclonal antibodies (mAbs) – Mixtures with mAb IgG [final concentration (f.c.) 2·5 BU/ml] and FVIII-deficient plasma were incubated with FVIII or FVIII-VWF (f.c. 1 U/ml) and thrombin generation parameters determined at the indicated times. The symbols used are, ○; JR8/VWF(−), ●; JR8/VWF(+), □; ESH4/VWF(−), ■; ESH4/VWF(+), △; ESH8/VWF(−), and ▲; ESH8/VWF(+). Peak thrombin (*panel* A) or MV-peak thrombin (*panel* B) in the absence or presence of FVIII (or FVIII-VWF) prior to incubation with inhibitor IgGs were regarded as 0% or 100%, respectively. Peak thrombin and MV-peak thrombin measurements at time zero were 300–400 nmol/l and 500–700 nmol/l/min, respectively. The data in (A and B) were fitted using the formula of single exponential decay (*dotted lines*).

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Table III. Decrease rates of peak thrombin and MV-peak thrombin with anti-FVIII mAbs.

	Peak thrombin			MV-peak thrombin		
Case	(A) FVIII (-fold) ×10/min	(B) FVIII-VWF(-fold) ×10/min	(B)/(A) ratio	(A) FVIII (-fold) ×10/min	(B) FVIII-VWF(-fold) ×10/min	(B)/(A) ratio
JR8	0·18 ± 0·04 (3·0)	0·17 ± 0·05 (2·9)	0·97	0·42 ± 0·12 (6)	0·46 ± 0·80 (6·5)	1·09
ESH4	1·03 ± 0·29 (17)	0·86 ± 0·12 (14)	0·82	1·56 ± 0·35 (22)	1·10 ± 0·11 (15)	0·70
ESH8	0·09 ± 0·03 (1·5)	0·25 ± 0·06 (4·1)	2·78	0·18 ± 0·07 (2·6)	0·50 ± 0·14 (7·0)	2·77
Control	0·06 ± 0·01 (1·0)	0·06 ± 0·01 (1·0)	—	0·07 ± 0·01 (1·0)	0·07 ± 0·01 (1·0)	—

Values were calculated by nonlinear least squares regression from the data shown in Fig 3 using single exponential decay.

presence of VWF relative to its absence (B)/(A) were 0·7–0·8 and *c.*1·0, respectively (Table III). In contrast, the thrombin assays using mAbESH8 appeared to be significantly enhanced in the presence of FVIII-VWF compared FVIII alone ((B)/(A); *c.* 2·8), probably showing a lower neutralizing effect of FVIII-VWF. These data demonstrated a similar tendency to those obtained with polyclonal alloAbs, and again indicated that the different epitopes of anti-FVIII inhibitors could affect the outcome of neutralization therapy using either FVIII or FVIII-VWF complex concentrates.

Clot waveform analysis in mixtures of FVIII or FVIII-VWF with anti-FVIII inhibitors

The neutralizing effects of FVIII and FVIII-VWF on the FVIII inhibitors with different epitopes were further compared using another global coagulation assay, clot waveform analysis. The anti-FVIII alloAbs or mAbs were incubated with FVIII or FVIII-VWF for 2 h, and measurements of coagulation acceleration (|min2|) were derived from clot waveforms obtained in samples as described in Materials and methods (Fig 4). The |min2| values observed with Cases 1 and 2 and mAbJR8 (anti-A2 type 1) were approximately half (0·27–0·29) of those with normal IgG (*c.* 0·55), and were little affected by the presence of VWF. In contrast, the |min2| observed with Cases 3–5 and mAbESH4 (anti-C2 type 1) were much lower (0·06–0·15) than those with anti-A2 type 1, and were significantly elevated in the presence of VWF (1·4–2·0-fold higher) compared with FVIII alone. The |min2| data obtained for Case 6 and mAbESH8 (anti-C2 type 2) were modestly lower, relative to control (0·5 and 0·35, respectively), but were further reduced in the presence of VWF, reflecting a potentially lower neutralizing ability of FVIII-VWF with this type of antibody. These results were consistent with those obtained in thrombin generation assays.

The levels of FVIII:C in samples obtained after a 2 h incubation, measured by one-stage clotting tests, did not appear to be significantly related to the different epitopes of anti-FVIII alloAbs and mAbs, although FVII:C levels obtained in Case 6 were relatively high. The findings suggested, therefore, that conventional assays of this nature would be unlikely to provide suitable laboratory data for evaluating the clinical effects of neutralization therapy.

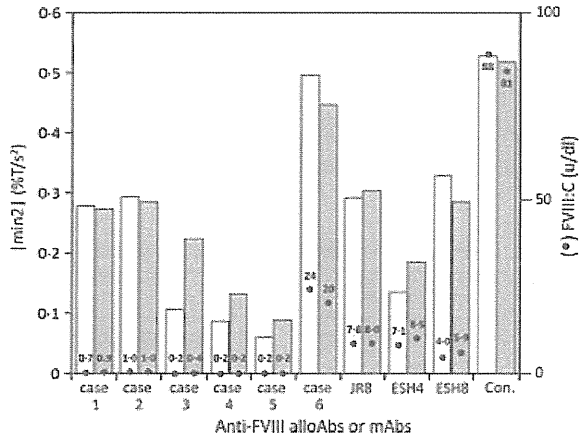

Fig 4. Clot waveform analysis after a 2-h incubation of FVIII or FVIII-VWF with anti-FVIII antibodies – Mixtures of anti-FVIII alloantibody (alloAb) or monoclonal antibody (mAb) IgG [final concentration (f.c.) 2·5 BU/ml] and FVIII-deficient plasma were incubated with FVIII or FVIII-VWF (f.c. 1 U/ml) for 2 h, prior to clot waveform analysis as described in Materials and methods. The values of |min2| obtained from mixtures containing FVIII or FVIII-VWF are represented by *white bars* or *grey bars* respectively. The figures above the symbols (•) within the bars indicate the levels of FVIII:C coagulant activity (FVIII:C) measured in one-stage clotting assays. Con., control.

Discussion

Anti-FVIII inhibitors are classified into two groups, based on the immunological response in patients with inhibitors treated with FVIII concentrates. So called 'low responders' are those in which inhibitor titres remain at <5 BU/ml even after considerable FVIII therapy. Conversely, 'high responders' are those in which inhibitor levels increase significantly above 5 BU/ml after administration of FVIII. In general, haemostatic treatment in HA patients with inhibitors depends on neutralization therapy or bypassing therapy, for low responders or high responders, respectively. The clinical effectiveness of neutralization therapy, however, could depend on differences between the reactivity of exogenous FVIII with specific anti-FVIII inhibitors, and changes in established strategies may be beneficial in some circumstances. This concept remains to be fully explored, however,

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and for this purpose in the present study, we have devised *in vitro* models of neutralization therapy using mixtures of FVIII (1 U/ml) and anti-FVIII inhibitor alloAbs or mAbs (2·5 BU/ml) in FVIII-deficient plasma.

Differences in the *in vitro* neutralizing effects of FVIII observed in this study were dependent on the particular epitope of anti-FVIII inhibitors. The addition of FVIII alone appeared to decrease the potency of the inhibitor in the order of anti-C2 type 2 ≫ anti-A2 type 1 > anti-C2 type 1. Anti-C2 and anti-A2 type 1 inhibitors showed similar rapid decreases of FVIII:C (<1 U/dl) in one-stage clotting assays (data not shown). In both the thrombin generation assay and one-stage clotting assay, anti-C2 inhibitors showed the rapid decrease even at lower concentrations, but anti-A2 inhibitors showed moderate decrease (data not shown), suggestive of the presence of different inhibitory mechanisms (probably due to FVIIIa-PL interaction and FVIIIa-FIXa interaction, respectively). This potent effect could contribute to the much greater inhibition seen with anti-C2 type 1. On the other hand, equivalent effects of FVIII-VWF were observed in the order of anti-C2 type 2 ≫ anti-A2 type 1 ≥ anti-C2 type 1. Furthermore, with the anti-C2 type 2 inhibitors, FVIII-VWF appeared to have diminished haemostatic properties compared with those of FVIII alone. These different effects identified using the *in vitro* coagulation model inferred that clinical responses in HA patients with low responding inhibitors could be optimized by making a choice between FVIII and FVIII-VWF. The data suggest that the durability of therapeutic FVIII and FVIII-VWF might be predicted by determining the precise epitopes of the individual inhibitors.

The current evidence suggests that the different neutralizing effects of FVIII and FVIII-VWF were centred on precise inhibitory mechanisms related to the functional properties of FVIII. The anti-C2 type 1 antibodies prevent FVIIIa binding to PL surfaces (Shima *et al*, 1993), and, hence, intrinsic FXase complexes that are responsible for the propagation phase of blood coagulation could not be formed. The anti-A2 type 1 and anti-C2 type 2 antibodies prevent FVIIIa-FIXa association (Fay & Scandella, 1999) and thrombin (and FXa)-catalysed activation of FVIII (Meeks *et al*, 2007; Matsumoto *et al*, 2012), respectively. It seems likely, therefore, that although both coagulation mechanisms are essential for the expression of intrinsic FXase activity, the overall effects of these specific inhibitors were less prominent than those of the anti-C2 type 1 antibodies, and it may be that FVIIIa-PL interactions play the dominant role in the expression of FXase activity.

VWF binds non-covalently to the N-terminus of A3 and C2 domains in FVIII (Foster *et al*, 1998, Saenko *et al*, 1994). The neutralizing effects of FVIII-VWF complex in our experiments, therefore, could be especially relevant. The epitopes of anti-C2 type 1 inhibitors overlap with the VWF-binding site(s) on FVIII, and as could be expected, the time-dependent neutralization of anti-C2 type 1 antibodies by FVIII-VWF was enhanced compared with FVIII alone, reflecting the protective role of VWF. Moreover, the results with anti-A2 type 1 inhibitors were little affected by the presence of VWF, whilst the neutralization with anti-C2 type 2 inhibitors was more attenuated with FVIII-VWF than with FVIII alone. This apparent paradox might be explained by the effects of the inhibitors on thrombin-catalysed proteolysis of FVIII. Thrombin-catalysed cleavage of FVIII at Arg^{1689} liberates FVIII from VWF, resulting in the generation of FVIIIa cofactor activity (Lollar *et al*, 1988). The anti-C2 type 2 antibodies (case 6 and mAbESH8) could, therefore, have inhibited the release of FVIII from VWF (Saenko *et al*, 1996) and disturbed thrombin-catalysed FVIII activation. Our findings suggest that this type of antibody might retain full inhibitory activity in patients treated with FVIII alone, but could be expected to have reduced potency in therapeutic protocols using FVIII-VWF.

Accurate measurements of blood coagulation *in vitro* are essential for complete clinical assessment of clotting function. Measurements of FVIII:C using conventional activated partial thromboplastin time-based one-stage clotting assays demonstrated little difference between the various types of inhibitor, but the global coagulation assays, thrombin generation and clot waveform analysis, facilitated identification of epitope-dependent reactions with FVIII and FVIII-VWF. The one-stage assays have been utilized for many years for routine laboratory assessment of clotting factor activity, but only partially reflect coagulation in a non-physiological environment. In the present study, the inhibitor titre used (2·5 BU/ml), was determined by the sample after a 2-h incubation in one-stage clotting assay. However, in global coagulation assays (thrombin generation assay and clot waveform analysis), coagulation function after a 2-h incubation revealed mild reduction, probably representing the quite different implication of inhibitor titre, especially anti-C2 type 2 inhibitors, between one-stage clotting assay and comprehensive coagulation assay. The global assays have recently been utilized for monitoring haemostatic treatment using bypassing agents in HA patients with inhibitors (Turecek *et al*, 2003; Barrowcliffe, 2008), and it seems evident that these techniques could also provide useful data for the clinical management of neutralization therapy using FVIII and FVIII-VWF concentrates.

Our previous results have shown that both rFVIIa and APCC, used as bypassing agents, could activate FVIII directly even in the presence of anti-FVIII inhibitor alloAbs (Yada *et al*, 2011, 2013). In patients with anti-C2 type 1 inhibitors, in particular, inactivation of FVIIIa by APCC was moderated by a delay in cleavage at Arg^{336}, and resulted in relatively persistent levels of activated FVIII. Our present findings add further details regarding epitope-dependent reactions with FVIII and FVIII-VWF, and indicate that further studies of this nature could lead to the development tailor-made treatment protocols. In addition, the data emphasize that detailed analysis of inhibitor epitopes could help to establish important guidelines in general for the haemostatic treatment of HA patients with inhibitors.

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Conflict of interest

The authors have no direct or indirect conflicts of interest.

Authorship contributions

KY performed the research, KY and KN analysed the data and wrote the paper, KN designed the research study, KN and MS interpreted the data.

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Repression of factor VIII inhibitor development with apoptotic factor VIII-expressing embryonic stem cells

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Abstract

Development of factor VIII (fVIII)-neutralizing antibodies, called inhibitors, is a challenging problem in the management of hemophilia A patients. We explored the possibility of pretreatment with apoptotic fVIII-expressing embryonic stem (ES) cells to prevent the development of fVIII inhibitors. Murine ES cells integrated with the human *F8* gene were differentiated into embryoid bodies, dissociated to a single cell suspension, subjected to hypo-osmotic shock to induce apoptosis, and intraperitoneally injected into hemophilia A mice. Inhibitors were induced by periodic intraperitoneal injections of recombinant human fVIII (rhfVIII). In the groups in which intraperitoneal injections of rhfVIII began at 1-3 weeks after pretreatment, the titers of inhibitors were significantly lower after the third administration of rhfVIII compared with that in the control group in which apoptotic Ainv18 ES cells (without the human *F8* gene) were used for pretreatment, and continued to show lower levels until the sixth administration of rhfVIII. These results suggest that pretreatment with apoptotic hfVIII-expressing ES cells might be promising for the prevention of fVIII inhibitor development in hemophilia A patients.

Introduction

Hemophilia A is an X-linked bleeding disorder resulting from an abnormality in the coagulation factor VIII (fVIII) gene. Hemophilia A patients require life-long supplementation with intravenous fVIII. The risk of infectious diseases has dramatically decreased as a result of the development of highly pure plasma-derived and recombinant fVIII. Nonetheless, development of fVIII-neutralizing antibodies, called fVIII inhibitors, remains a challenging problem for the treatment of hemophilia A. Approximately 25-35% of severe type hemophilia A patients develop inhibitors that reduce or completely negate the benefits of replacement therapy.[1,2] Furthermore, a major issue is that all gene therapy trials for hemophilia are subject to the risk of developing inhibitory antibodies.[3] Although immune tolerance induction therapy (ITI) using high amounts of fVIII is effective for eradicating inhibitors, the extremely high cost required for this type of therapy hampers its worldwide application. Moreover, ITI is ineffective in about 30% of hemophilia A patients with inhibitors.[4] Thus, it is desirable to develop a novel method to prevent the development of fVIII inhibitors. To this end, several attempts have been made as follows. Madoiwa *et al.* successfully induced immune tolerance for human fVIII (hfVIII) by intrathymic injection of hfVIII into neonatal hemophilia A mice.[5] Furthermore, induction of long-term fVIII tolerance can be achieved by administration of lentiviral vectors carrying a canine fVIII transgene to neonatal hemophilia A mice.[6] Although these approaches were effective, they have several disadvantages such as difficulties in the procedures or the risk of viral vectors causing adverse genetic modification of patients.

The use of apoptotic cells for immune tolerance induction is in line with the current perception that dendritic cells induce peripheral tolerance by capturing cells that normally die during cell turnover. Because apoptotic cells are immunologically *silent*,[7] they exert a tolerogenic influence in adaptive immune responses.[4,8-10] Therefore, a method to prevent antibody production with apoptotic cells has an immunological rationale and is sufficiently promising based on recent studies.[11,12] However, such an approach has several disadvantages that should be overcome for practical use in the clinical setting of hemophilia A treatment. One of the most concerning issues is the source of fVIII-expressing cells. While the most appropriate cells are autologous cells from an immunological viewpoint, patient-derived cells have a genetically abnormal *F8* gene. Thus, *ex vivo* transfection of the *F8* gene into patient-derived fibroblasts has provided a breakthrough for this approach. Su *et al.* demonstrated that injection of fVIII expression vector-modified apoptotic syngeneic fibroblasts achieves suppression of fVIII inhibitor development in hemophilia A mice.[12] However, the significant issues in this approach are the complex procedures such as collection of fibroblasts from each patient and transfection of the *F8* gene into these cells. Therefore, fVIII-expressing cells derived from histocompatible stem cells [such as embryonic stem (ES) cells or induced pluripotent stem (iPS) cells] will be the most suitable. Although we have observed that iPS cells can be differentiated into liver-like populations that show a strong expression level of fVIII mRNA,[13] fVIII protein expression levels have not yet been examined. Therefore, we attempted to use genetically engineered (hfVIII-transgenic) ES cells as fVIII-expressing stem cells. We explored the possibility of fVIII inhibitor prevention by pretreatment with apoptotic fVIII-secreting ES cells in anticipation of the common and broad usage of iPS cells.

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Key words: hemophilia, fVIII inhibitors, prevention, embryonic stem cells, apoptosis.

Contributions: YS, conception and design of the study, interpretation of data, and revisions of the manuscript; SK and KT, animal experiments, interpretation of data, literature searches, and preparation of manuscript; TK and JK, cell culture, animal experiments, and collection of data; AK, preparation of ES cells and supervising genetic manipulation; MS supervising study and critical revisions of the manuscript.

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Materials and Methods

Mice

fVIII-deficient mice carrying a stop mutation in exon 16 of the *F8* gene were generously given to us by Prof. Yoichi Sakata (Research Division of Cell and Molecular Medicine, Jichi Medical University, Shimotsuke, Japan) and used as a model of hemophilia A.[14] These hemophilic mice were mated with C57BL/6 mice for at least eight generations to create C57BL/6 fVIII knockout mice. The genotypes of hemophilic mice were

confirmed by polymerase chain reaction analysis of genomic DNA extracted from peripheral leukocytes.[14] The mice were maintained as an inbred colony and were between 8 and 12 weeks of age at the beginning of the study. All mice were housed under specific pathogen-free conditions in the animal facility of Nara Medical University Research Center. Blood samples were obtained by orbital plexus bleeding and anticoagulated (9:1) with 0.105 M sodium citrate. Plasma samples were obtained by centrifugation of the blood at 3600 g for 10 min at room temperature, divided into aliquots, and frozen at –80°C until analysis. All experiments using mice were approved by the Nara Medical University Animal Use Committee and performed in accordance with the applicable guidelines and regulations.

Induction of factor VIII inhibitors

Development of fVIII inhibitors in C57BL/6 fVIII knockout mice was induced by weekly intraperitoneal administrations of 4 IU recombinant hfVIII (rhfVIII; Kogenate FS, Bayer Yakuhin, Osaka, Japan) (approximately 100 IU/kg body weight).

Assay for factor VIII inhibitors

The titers of fVIII inhibitors were assessed by the Bethesda assay using the obtained plasma samples according to a previous report with minor modification.[15,16] In brief, 50 μL of doubling diluted mouse plasma with Owren's Veronal Buffer (more diluted if needed) was incubated with 50 μL of normal pooled human plasma at 37°C for 2 h. Residual human fVIII activity was measured in a one-stage assay using 50 μL of FVIII-deficient human plasma (Sysmex, Kobe, Japan) and a 50 μL sample from the previous incubation. Samples were mixed with 100 μL of phospholipid activator (APTT-SLA, Sysmex), incubated at 37°C for 3 min, and then mixed with 100 μL of 20 mM/L $CaCl_2$. Clotting times were measured with a coagulometer (KC 10, Amelung, Lemgo, Germany). Coagtrol N (Sysmex) was diluted with Owren's Veronal Buffer to produce a standard curve of fVIII activity. The measurements were made in the linear portion of the response range.

Cell preparations

Murine Tet-WT-F8 ES cells, in which human *F8* gene expression was induced by the tetracycline analog doxycycline, were prepared as described previously.[17] Briefly, Ainv18 ES cells were transfected with the WT-F8-plox targeting plasmid (wild-type human fVIII gene-loading, lox-targeting plasmid) by electroporation to yield tet-WT-F8,[18] after which the transfectants were selected with G418 as described previously.[19] Tet-WT-F8 ES cells were maintained on mouse embryonic fibroblasts as a feeder layer, passaged twice on gelatin-coated dishes, and then induced to form embryoid bodies (EBs) for 6 days. The EBs were stimulated with doxycycline for the last 2 days. Then, EBs were dissociated to a cell suspension by digestion with 0.25% trypsin/EDTA. As a control, Ainv18 ES cells without the human *F8* gene were treated in the same manner.

Apoptosis of the dissociated ES cells was induced according to the osmotic shock method by Liu *et al.*[20] Briefly, 1×10^6 cells were washed in RPMI 1640 twice, and resuspended in 1 mL hypertonic medium (0.5 M sucrose, 10% wt/vol polyethylene glycol 1000, and 10 mM Hepes in RPMI 1640, pH 7.2) for 10 min at 37°C. Then, 10 mL prewarmed hypotonic medium (40% H_2O, and 60% RPMI 1640) was added, and the cells were incubated for an additional 2 min at 37°C. Immediately after the incubation, the cells were centrifuged, washed twice with ice-cold phosphate-buffered saline (PBS), and used as apoptotic cells. Induction of apoptosis of the dissociated ES cells was confirmed by flow cytometry analysis with annexin V/7-amino-actinomycin D staining (*data not shown*).

Administration of apoptotic embryonic stem cells

Dissociated and apoptosis-induced Tet-WT-F8 ES cells or control Ainv18 ES cells in 200 μL PBS (2×10^5 cells) were intraperitoneally injected into hemophilia A mice. Intraperitoneal injections of rhfVIII for the induction of fVIII inhibitor development were started at 3 days, 1, 2, and 3 weeks after administration of the apoptotic Tet-WT-F8 ES cells (group D3, W1, W2, and W3, respectively) and after administration of Ainv18 ES cells (control). After the first intraperitoneal injection of rhfVIII, blood sampling was performed just prior to the next intraperitoneal injection of rhfVIII (Figure 1).

Statistical analysis

Data were presented as the mean ± standard error of the mean (SEM). Differences between groups were assessed by the Kruskal-Wallis test, followed by Dunn's multiple-comparison post hoc analysis. A P value of less than 0.05 was defined as statistically significant. All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Results

Development of factor VIII inhibitors in hemophilia A mice

Figure 2 shows the progressive increase of the fVIII inhibitor titer with weekly intraperi-

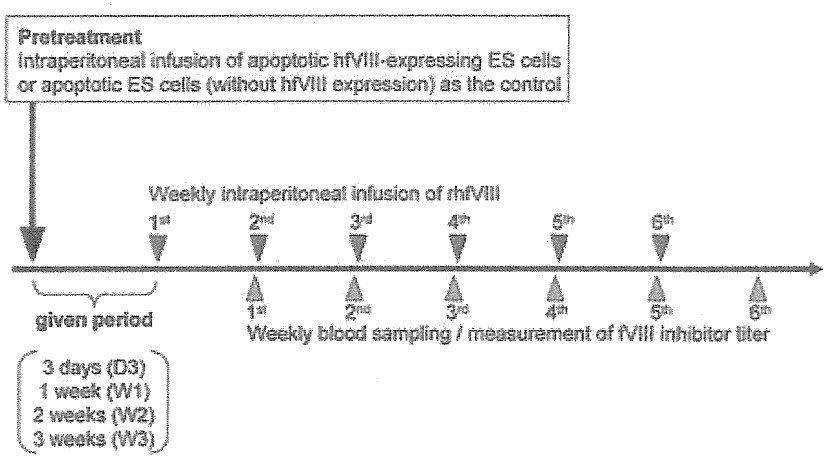

Figure 1. Timeline of the experimental procedure.

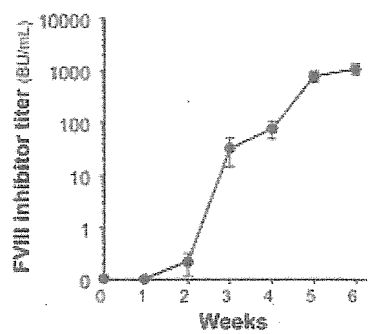

Figure 2. Progressive increase of factor VIII (fVIII) inhibitor titers in hemophilia A mice by weekly intraperitoneal administrations of recombinant human fVIII (rhfVIII) (n=6-8). Inhibitor titers reached a plateau after the fifth administration of rhfVIII.

toneal administration of rhfVIII to non-pretreated hemophilia A mice (n=8). The fVIII inhibitor titers appeared after the second administration of rhfVIII (0.22±0.38 BU/mL). Then, inhibitor titers increased with every following rhfVIII administration and reached 1687±309 BU/mL after the six administrations.

Intraperitoneal injection of apoptotic factor VIII-expressing cells attenuates the development of factor VIII inhibitors

The fVIII inhibitor titers were undetected in both treated and untreated groups before intravenous rhfVIII administration. Those of pretreatment groups at each blood collection point are shown in Figure 3. Pretreatment with apoptotic Ainv18 ES cells (without the human *F8* gene), regardless of the period between pretreatment and the first administration of rhfVIII, resulted in no difference in fVIII inhibitor development compared with that in non-pretreatment groups (data not shown). The fVIII inhibitor titers of a group in which intraperitoneal injections of rhfVIII were started at 1 week after administration of Ainv18 ES cells were shown in Figure 3 as a representative control group (Cont).

The fVIII inhibitor titers observed in pretreatment group D3 appeared higher than that in the control group after the second blood sampling but without significance. However, in W1, W2, and W3 groups, the fVIII inhibitor titers were significantly lower than that in the control group at the third blood sampling ($P<0.05$, $P<0.01$, and $P<0.05$, respectively). The results obtained during the rest of the observation period also showed a similar tendency of lower fVIII inhibitor titers in W1, W2, and W3 groups than those in the control group.

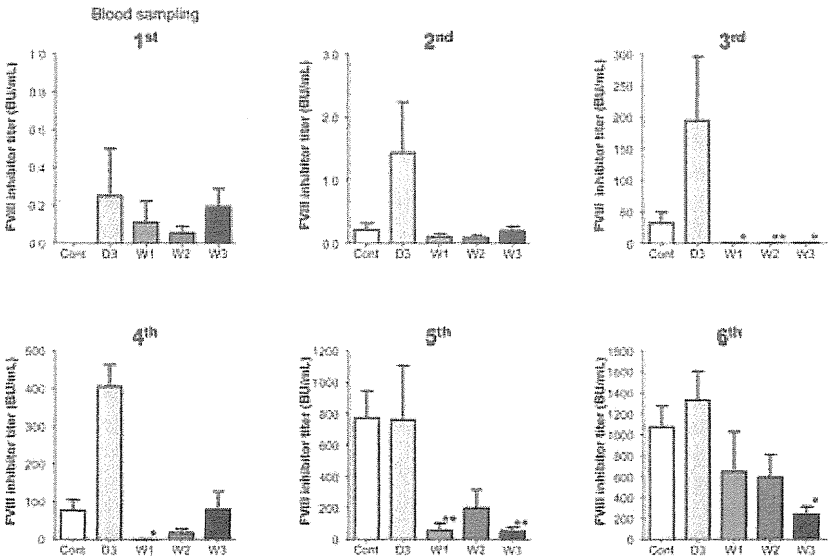

Figure 3. Effects of intraperitoneal injection of apoptotic factor VIII (fVIII)-expressing embryonic stem cells on fVIII inhibitor development. Intraperitoneal administrations of recombinant human fVIII (rhfVIII) for the induction of fVIII inhibitor development were started at 3 days, 1, 2, and 3 weeks after administration of the apoptotic cells (D3, W1, W2, and W3 groups, respectively) (n=4-8) and 1 week after administration of Ainv18 cells (Cont) (n=6). Data are presented as the mean ± standard error of the mean. *P<0.05, **P<0.01, compared with the control group.

Discussion

In the current study, we examined the possibility of pretreatment with hfVIII-expressing ES cells for the prevention of fVIII inhibitor development. The efficacy for the reduction of fVIII inhibitor titers depended on the period between pretreatment with apoptotic hfVIII-expressing ES cells and the first administration of rhfVIII. In the D3 group, pretreatment with apoptotic hfVIII-expressing ES cells resulted in rising inhibitor titers rather than a reduction. However, in groups with a greater than 1 week interval, significant suppressive effects on fVIII inhibitor development were achieved by the pretreatment. These observations suggest that a specified period is required to suppress immune response to fVIII by prior injection of apoptotic hfVIII-expressing ES cells.

The molecular weight of the hfVIII protein is very large, as much as 280 kDa, and multiple epitopes in this protein are recognized by fVIII inhibitors.[21] Thus, it is a critical issue to clarify whether the fVIII inhibitors that bind multiple epitopes of the fVIII protein can be suppressed by apoptotic hfVIII-expressing cells. Lei *et al.* found that full-length antigen expressed by B cells can induce immune tolerance for multiple epitopes in a target protein,[22] because proteolytic cleavage of the protein, selection of the resulting peptides, and peptide presentation are executed by the host's own APCs. Our successful results in the present study support this previous notion. We have no need for identification of the precise peptide epitopes of the fVIII inhibitors or the establishment of ES cells that express each epitope corresponding to a fVIII inhibitor.

The mechanism by which pretreatment with apoptotic hfVIII-expressing ES cells suppresses fVIII inhibitor development remains to be elucidated. Although further investigations are required to address this issue, it might be attributed to the inhibitory effects of the apoptotic ES cells on the proliferation of fVIII-responsive effector T cells by antigen-specific $CD4^+CD25^+$ regulatory T cells as described by Su *et al.*[12]

In this study, rhfVIII was injected not intravenously but intraperitoneally. A previous report describes that inhibitor titer elevated to 122.5 BU/mL after five biweekly intravenous injections of 50 U/kg rhfVIII (total amount of 250 U/kg) in hemophilia mouse.[5] In our study, inhibitor titer did not exceeded 122.5 BU/mL until total amount of administered rhfVIII reached 500 U/kg, suggesting that our protocol may be inefficient compared to that of the previous report. The difference of administration route as well as applied dose and administration interval may exert influence. Large size of molecules are absorbed slowly through the peritoneal lymphoid system through the stomata of the milky spot.[23] As intraperitoneally injected fVIII with a molecular size of more than 280 kDa would be therefore absorbed more slowly than intravenously injected fVIII, immune response against rhfVIII might wane. Furthermore, the peritoneal immune system, which contains distinct immune cells such as B1 cells,[24] may respond to foreign rhfVIII differently from the circulating immune system. Apoptotic ES cells were also administered intraperitoneally. Intraperitoneal administration of apoptotic cells could be applied as intraperitoneal catheters have been used for intraperitoneal chemotherapy. A comparative controlled study of administration routes, intravenous and intraperitoneal should be required to clarify which route is more effective. Furthermore, modification of the protocol for administration of apoptotic fVIII-expressing ES cells, including changes of the administrating cell dose or administration frequency, would be required for complete suppression of fVIII inhibitor development.

Thus, our methodology is not necessarily directly applicable to hemophilia patients with

inhibitors. Nevertheless, our study opens up a whole new avenue for the prevention of fVIII inhibitor development. Although the ES cells used in this study were genetically engineered (hfVIII transgenic), iPS cell-derived fVIII-secreting cells will be established in the near future.[25] In addition, establishment of an iPS cell bank may allow the prevalence of this approach for fVIII inhibitor prevention.[26]

Conclusions

Our results in this study suggest the effectiveness of pretreatment of apoptotic fVIII-expressing ES cells on repression of factor VIII inhibitor development. In addition, a specific period might be required to induce immune tolerance by pretreatment with such apoptotic cells. We believe that this approach has potential as a future preventive therapy against fVIII inhibitor development in hemophilia A patients.

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Two haemophilia patients with inhibitors who became ambulatory after physiotherapy under haemostatic cover with bypassing agents

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For haemophilia patients who develop inhibitory antibodies to factor VIII (FVIII) or FIX, haemostatic therapy becomes more complicated and difficult than for patients without inhibitors. Treatment of haemarthroses should be aggressive to prevent recurrent bleeding and chronic and debilitating joint damage [1]; otherwise severe mobility impairment may be exhibited from a young age. Bypassing agents are the recommended haemostatic treatment for inhibitor patients [2], and currently recombinant activated factor VII (rFVIIa; NovoSeven®, Novo Nordisk, Chiyoda-ku, Tokyo, Japan) and plasma-derived activated prothrombin complex concentrate (pd-aPCC; FEIBA®, Baxter, Chuo-ku, Tokyo, Japan) are available for use in Japan.

In patients with severe haemophilia without inhibitors, prophylactic therapy can reduce bleeding and resultant joint damage [3]. Use of bypassing agents to prevent bleeding in inhibitor patients prior to surgical procedures is well described [4]; however, prophylaxis with bypassing agents in non-surgical scenarios remains under investigation for therapeutic benefit and safety [5]. Here, we report two previously non-ambulatory haemophilia A patients with inhibitors, who became ambulatory following physiotherapy accompanied by haemostatic therapy with bypassing agents.

Patient 1 was a 19-year-old male who had no family history of bleeding disorders, but a haemorrhagic stool 2 days after birth led to a diagnosis of severe haemophilia A, and on-demand replacement therapy was started. An inhibitor [67 Bethesda Units (BU) mL−1] was detected when this patient was 7 years of age, and haemostatic therapy was switched to bypassing agents. Frequent joint haemorrhage and damage resulted in the patient becoming non-ambulatory at approximately 12 years of age. Immune tolerance induction (ITI) was conducted when the patient was 14-years old but was unsuccessful (inhibitor titre rose to 2700 BU mL−1). Subsequently, repeated haemarthroses in the ankle, knee and hip, accompanied by progressive muscular atrophy in both legs, resulted in difficulties standing, and the patient was admitted to hospital for physiotherapy.

The patient's laboratory findings and the Arnold–Hilgartner classification of joint arthropathy at hospital admission are given in Table 1 and Fig. 1 respectively.

Table 1. Laboratory data for patient 1 and patient 2 at hospital admission.

	Patient 1	Patient 2
Haematology		
White blood cell count (cells per μL)	3800	6300
Red blood cell count (cells per μL)	4.68 × 106	5.02 × 106
Haemoglobin (g dL−1)	14.2	14.7
Haematocrit (%)	42.9	43.0
Platelet count (cells per μL)	155 × 103	166 × 103
Coagulation and fibrinolysis		
Prothrombin time (s)	13.5	11.7
Activated partial thromboplastin time (s)	111.0	121.8
FVIII:C (%)	<1	<1
FVII:C (%)	77.0	108.6
FVIII inhibitors (BU mL−1)	84.6	17.9
Thromboelastography		
Reaction time (min)	>150	189.0
Angle (min)	Undetectable	55.6
Maximum amplitude (mm)	Undetectable	Undetectable
Blood chemistry		
γ-glutamyl transpeptidase (IU L−1)	18	26
Aspartate aminotransferase (IU L−1)	19	26
Alanine aminotransferase (IU L−1)	22	22
Lactate dehydrogenase (IU L−1)	126	301
Total bilirubin (mg dL−1)	0.57	1.55
Creatine kinase (IU L−1)	81	81
Sodium (mEq L−1)	143	140
Potassium (mEq L−1)	4.2	4.5
Chlorine (mEq L−1)	102	106
Blood urea nitrogen (mg dL−1)	12.7	11.4
Creatinine (mg dL−1)	0.55	0.67
Total protein (mg dL−1)	7.7	7.0
Total cholesterol (mg dL−1)	170	105
C reactive protein (mg dL−1)	0.05	0.96

FVIII:C, FVIII clotting activity; FVII:C, FVII clotting activity.

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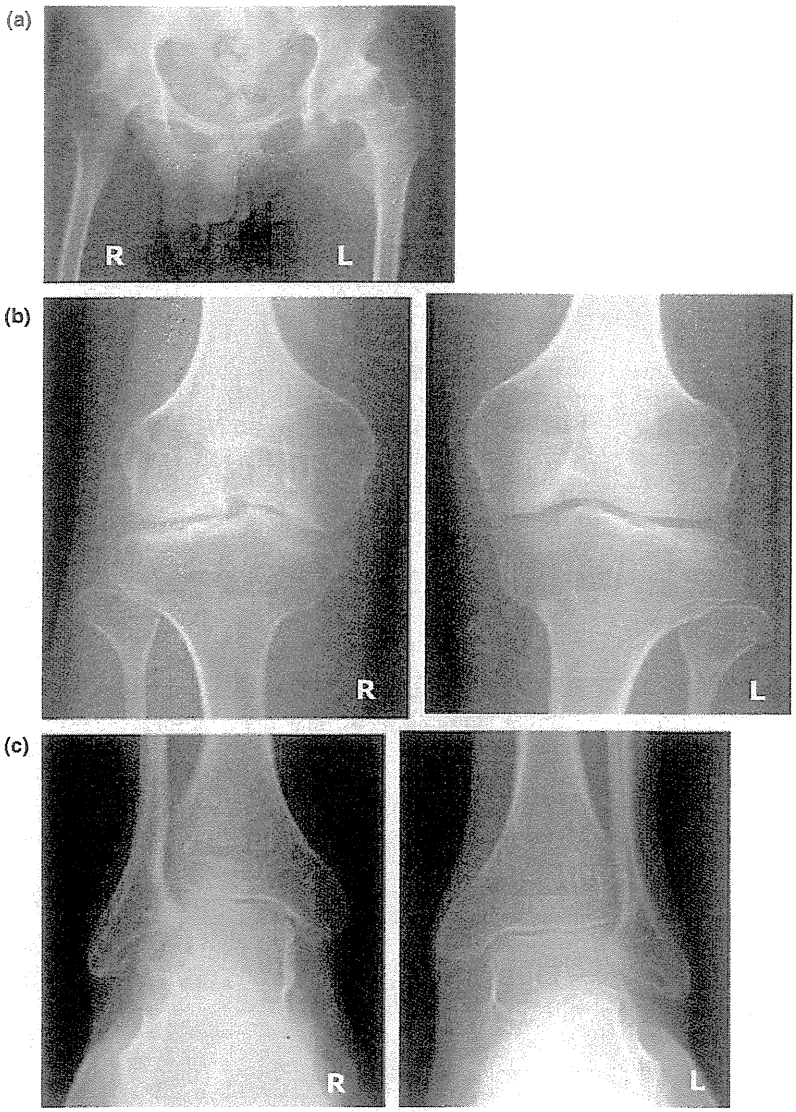

Fig. 1. Patient 1: Degree of progression of arthropathy (Arnold–Hilgartner classification) at hospitalization. (a) Hip joints: right, stage V; left stage III. (b) Knee joints: right, stage III; left, stage II. (c) Ankle joints: right, stage III; left, stage II. R, right; L, left.

Prior to hospital admission, each bleeding episode was treated on-demand with pd-aPCC (59 U kg−1) given 4–9 times (every 6–8 h over 3–4 days), and if haemostasis was not achieved, rFVIIa (94 μg kg−1, 3–4 doses, 3-h intervals) was given. Following haemostasis, no physiotherapy was attempted for 1–2 weeks.

During the patient's hospital stay, physiotherapy (five times per week) focused on strength-training in the lower legs, in combination with pd-aPCC (78 U kg−1 day−1, three times per week). After 21 days in hospital, the patient began to stand up, after 53 days he began walking with a frame, and on day 74 he started to walk with a single crutch. During this time, no intra-articular haemorrhages were observed; however, a right elbow bleed occurred on day 102. As pd-aPCC dosing was time-consuming and the patient's lifestyle improved, on day 116, regular haemostatic coverage was switched to rFVIIa (94 μg kg−1, three times weekly). The patient continued to do well, with no further haemorrhages, and was discharged after 4.5 months in hospital.

After the patient was discharged, prophylaxis with rFVIIa (94 μg kg−1, three times weekly) was continued. The patient used a cane and an assistive device to correct the difference in the length of his legs. He took various independent trips, walking and using public transport. A haemorrhage in his right elbow occurred 1 month after discharge. This and subsequent bleeds were managed with rFVIIa (118 μg kg−1) at 2-h intervals up to three doses. Following haemostasis, the patient rested for 1–2 weeks before continuing rehabilitation. Generally, treatment

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of bleeding episodes with three rFVIIa 118 μg kg−1 doses achieved haemostasis relatively quickly in comparison with the patient's previous history of poorly controlled bleeds.

Patient 2 was a 19-year-old male with a family history of haemophilia; a maternal uncle died at 12 years of age, and the patient had a sibling who was diagnosed with haemophilia in utero. When the patient was 5 months of age, clotting difficulty was observed when bloods were drawn; severe haemophilia A was diagnosed and on-demand replacement therapy was started. An inhibitor (10 BU mL−1) was detected when he was 3 years of age, and treatment was switched to bypassing agents. Repeated haemorrhages in the left knee resulted in joint deterioration.

When the patient was 17 years of age, on-demand treatment with rFVIIa at his local clinic was increased to 228 μg kg−1, with additional 114 μg kg−1 doses two to six times (every 3 h), if required.

At 18 years of age, the patient started having difficulty walking and tried to prevent bleeding by not bearing weight on his left leg. He was subsequently admitted to our hospital for physiotherapy. Laboratory findings and Arnold–Hilgartner classification of arthropathy at hospitalization are shown in Table 1 and Fig. 2 respectively.

Physiotherapy focused on strength-training in the lower legs, combined with rFVIIa prophylaxis (114 μg kg−1 day−1, three times weekly). However, on days when rFVIIa was not administered, haemorrhage occurred after physiotherapy, so dosing was increased to five times per week. Subsequently, the number of bleeding episodes decreased. After 27 days in hospital, the patient began walking with a frame, and after 43 days he could walk with two crutches. After 50 days in hospital, self rehabilitation training was undertaken to enable the patient to take a holiday, and the dosing of rFVIIa was increased to 114 μg kg−1 every day.

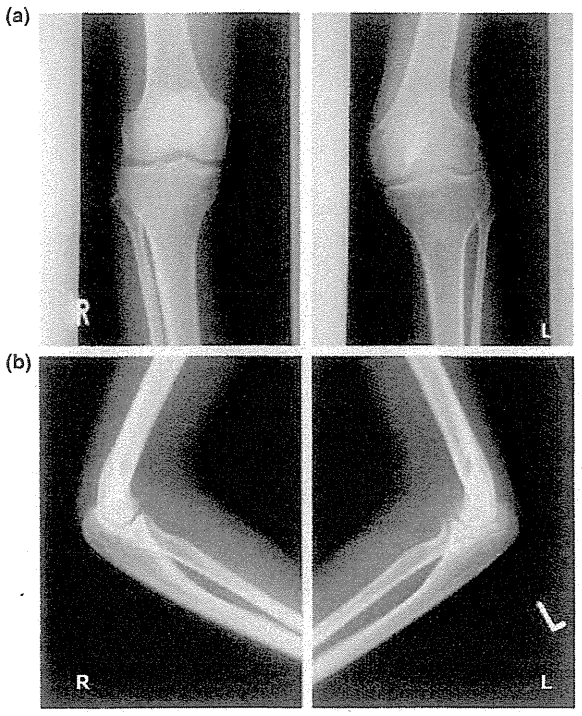

Fig. 2. Patient 2: Degree of progression of arthropathy (Arnold–Hilgartner classification) at hospitalization. (a) Knee joints: left, stage III. (b) Elbow joints: left, stage IV. R, right; L, left.

As self-injection was difficult, and taking into consideration the patient's desired lifestyle, an intravenous Hickman catheter was placed in his right subclavian vein to allow easier administration of rFVIIa, and dosing was gradually reduced. No further joint bleeds occurred and the patient was discharged on day 250.

After discharge, the patient wore a long-leg brace and continued regular treatment with rFVIIa 114 μg kg−1 day−1 (three times weekly). If bleeding occurred, rFVIIa (153 μg kg−1) was administered at 2-h intervals, which achieved haemostasis more quickly than the patient's historical responses.

The experiences described above support those of other groups reporting reduced bleeding frequency using regular haemostatic coverage with bypassing agents [6]. Due to the short half-life of rFVIIa (2.5 h), regular doses of rFVIIa were considered unlikely to prevent bleeding. However, such treatment has been reported to successfully suppress bleeding episodes in inhibitor patients undergoing rehabilitation and ITI [7]. In addition, a randomized, prospective clinical trial showed that 3 months of secondary prophylaxis with rFVIIa (270 or 90 μg kg−1 day−1) reduced bleeding frequency (including joint bleeds) vs. on-demand treatment [8]. Furthermore, such secondary rFVIIa prophylaxis tended to improve the health-related quality of life in the patients in this trial [9]. In patients with more advanced haemarthropathy, suppression of joint deterioration by treatment with bypassing agents may be more difficult [10].

To summarize, our observations demonstrate that after the combined use of physiotherapy and bypassing agents, haemophilia patients with inhibitors who were non-ambulatory due to progressive haemarthropathy, were able to take more control of their daily activities. We believe that this management programme facilitated effective prevention of bleeding as well as improvement in ambulation, and may have helped the patients to differentiate sensations of haemorrhage from those of muscle fatigue (allowing the early administration of bypassing agents if they did experience a haemorrhage). To conclude, management programs combining physiotherapy and haemostatic therapy with bypassing agents can help in the

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