Neutralisation of factor VIII inhibitors by anti-idiotypes isolated from phage-displayed libraries

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Summary
Following replacement therapy with coagulation factor VIII (FVIII), up to 30% of haemophilia A patients develop FVIII-specific inhibitory antibodies (FVIII inhibitors). Immune tolerance induction (ITI) is not always successful, resulting in a need for alternative treatments for FVIII inhibitor-positive patients. As tolerance induction in the course of ITI appears to involve the formation of anti-idiotypes specific for anti-FVIII antibodies, such anti-idiotypes might be used to restore haemostasis in haemophilia A patients with FVIII inhibitors. We isolated anti-idiotypic antibody fragments (scFvs) binding to murine FVIII inhibitors 2–76 and 2–77 from phage-displayed libraries. FVIII inhibitor/anti-idiotype interactions were very specific as no cross-reactivity with other FVIII inhibitors and isotype controls was observed. ScFvs blocked binding of FVIII inhibitors to FVIII and neutralised their cognate inhibitors in vitro and a monoclonal mouse model. In addition, scFv JkH5 specific for FVIII inhibitor 2–76 stained 2–76-producing hybridoma cells. JkH5 residues R52 and Y226, located in complementary determining regions, were identified as crucial for the JkH5/2–76 interaction using JkH5 alanine mutants. SPR spectroscopy revealed that JkH5 interacts with FVIII inhibitor 2–76 with nanomolar affinity. Thus, FVIII inhibitor-specific, high-affinity anti-idiotypes can be isolated from phage-displayed libraries and neutralise their respective inhibitors. Furthermore, anti-idiotypic scFvs might be utilised to specifically target inhibitor-specific B cells. Hence, a pool of anti-idiotypes could enable the reestablishment of haemostasis in the presence of FVIII inhibitors in patients or even allow the depletion of inhibitors by targeting inhibitor-specific B cell populations.

Keywords
Haemophilia A, inhibitors, anti-idiotypes, phage display, FVIII

Introduction
Mutations in the gene of blood coagulation factor VIII (FVIII) result in congenital haemophilia A. Patients usually receive regular injections of recombinant or plasma-derived FVIII to restore haemostasis. Unfortunately, this treatment leads to the development of inhibitory FVIII-specific antibodies (FVIII inhibitors) in 25–30% of patients with severe haemophilia A (1). To induce tolerance against FVIII, affected patients receive frequent, usually high-dose transfusions of FVIII for many months and up to years. This process called immune tolerance induction (ITI) leads to eradication of inhibitors in about 70% of the patients (2). However, for remaining patients with ITI failure, there is currently no alternative treatment available to eradicate FVIII inhibitors. Thus, the development of new treatment strategies is of high importance.

Anti-idiotypes are antibodies that bind to another antibody’s paratope. Niels Jerne introduced the idea that anti-idiotypes can modify the immune response by forming antibody-anti-idotype networks in the 1970s (3). Anti-idiotypes specific for anti-FVIII antibodies were first detected in intravenous immunoglobulin preparations (ivIg) following the observed therapeutic benefit of ivIg in patients with FVIII autoantibodies (4). FVIII inhibitor-specific anti-idiotypes were also identified in healthy individuals (5), acquired haemophilia A patients after recovery (6), and haemophilia A patients with successful ITI (7).

To elucidate the therapeutic potential of anti-idotypic antibodies for FVIII inhibitor-positive haemophilia A patients, anti-idiotypes were raised against the human C2 domain-specific FVIII inhibitor BO2C11 in mice (8). The BO2C11-specific anti-idotype 14C12 neutralised BO2C11 in functional clotting assays and a
Materials and methods

Cells

The human embryonic kidney cell line 293T (HEK293T) was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 4 mM L-glutamine. Hybridoma 76–113, kindly provided by Pete Lollar (Atlanta, GA, USA), was maintained in DMEM, supplemented with 10% FBS (Gibco, Life technologies Darmstadt, Germany), 100 units/ml penicillin, 0.1 mg/ml streptomycin, 2% Hybridoma cloning supplement (Roche, Basel, Switzerland), 1% GlutaMax (Life technologies), 1% OPI media supplement, 2% HEPES buffer. Media and supplements were purchased from Sigma Aldrich (St. Louis, MO; USA) if not indicated otherwise.

Cloning and expression of scFv-hFc constructs

Amber stop codons in the scFv sequences of JkH5, JkE5, JC2, and JF6 were mutated to CAG coding for glutamine using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's instructions. Phagemids containing the corrected scFv sequences were digested with NcoI and NotI and respective Ncol/Notl fragments were ligated into the expression vector pCMV2.5-hlgG1-Fc provided by Stefan Dübel (TU Braunschweig, Germany). The vector encodes for the hinge, C_{H}2, and C_{H}3 region of the human IgG1 heavy chain (9), resulting in the generation of scFv-human Fc (hFc) constructs.

HEK293T cells were transiently transfected with scFv-hFc constructs using 45 kDa polyethyleneimine (Polysciences GmbH, Eppelheim, Germany). Medium was replaced by DMEM containing Insulin-Transferrin-Selenium (Life technologies) instead of FBS 24 hours (h) after transfection. Cell culture supernatants were collected for three consecutive days. Purification of scFv-hFc proteins was performed on an ÄKTAprime system (GE Healthcare, Munich, Germany) using HiTrap Protein A HP columns (GE Healthcare) as previously described (10).

Titration of anti-idiotypic binding to FVIII inhibitors

Microtitre plates were coated with 0.67 pmol of monoclonal antibodies (mAbs) 2–76 (GMA-8021) or 2–77 (GMA-8006; both Green Mountain Antibodies, Burlington, VT, USA) per well overnight at 4°C. After blocking with MPBST (PBS, 5% (w/v) milk powder, 0.05% (v/v) Tween20), different dilutions of scFv-hFc preparations were added for 2 h at room temperature (RT). Bound scFv-hFcs were detected with HRP-conjugated goat-anti-human IgG (CalTAG, Buckingham, UK). Antibody binding was visualised by addition of o-phenylenediamine (OPD, Sigma Aldrich) and absorbance measurements at 492 nm and 620 nm (reference).

Sequence alignment of the JkH5 scFv with FVIII

Homology of JkH5 and FVIII was evaluated by aligning the amino acid sequences of scFv JkH5 to the sequence of the FVIII epitope of mAb 2–76 (FVIII 484–508). We used the EBLUMat62 matrix for pairwise sequence alignment with standard settings for gap penalty and extend penalty.

Modelling of the JkH5 structure

To predict the three-dimensional structure of JkH5, V_{H} and V_{L} chains with high sequence homology to the variable chains of JkH5 were identified using the Fold & Function Assignment server (http://ffas.godziklab.org). Homologue chains were used for structure modelling utilising the program SWISS-MODEL (http://swissmodel.expasy.org). The JkH5 structure model was visualised with MacPyMOL v1.3 (Schrödinger LLC, Portland, OR, USA).

Binding studies with JkH5-hFc variants

JkH5 amino acid residues were mutated to alanines using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. JkH5-hFc variants were produced in HEK293T cells as above. Different dilutions of cell culture media were incubated on
microtitre plates coated with 0.1 µg of mAb 2–76 captured by 0.5 µg anti-mouse IgG (Dianova, Hamburg, Germany) or with 0.5 µg goat-anti-human IgG (Dianova) to verify the presence of JkH5-hFc protein. After 2 h incubation at RT, bound JkH5-hFc was detected with HRP-conjugated goat-anti-human IgG (Caltag). Antibody binding was visualised as described above.

Surface plasmon resonance (SPR) spectroscopy

SPR measurements were performed using a Biacore T200 system (GE Healthcare) at a constant temperature of 25°C. Streptavidin-coated sensor chips SAP (XanTec bioanalytics, Düsseldorf, Germany) were used for analysis. Experiments were carried out in HBST buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM CaCl₂, and 0.05% (v/v) Tween-20). Monoclonal antibodies 2–76 and 2–77 were biotinylated with Sulfo-NHS-LC-Biotin using the EZ-Link® Sulfo-NHS-LC-Biotinylation Kit (Thermo Scientific, Waltham, MA, USA) according to the supplier’s instructions. Biotinylated mAbs 2–76 and 2–77 were immobilised on SAP sensor chips according to manufacturer’s instructions. Briefly, a constant flow rate of 30 µl/minute (min) was used for all coupling steps. Sensor chips were pre-conditioned with 1 M NaCl, 50 mM NaOH for 600 seconds (s). Biotinylated mAbs (200 nM in PBS) were captured for 600 s, which resulted in a nearly complete saturation of the SAP sensor chip with the respective ligand (data not shown). Free streptavidin binding sites were blocked with biotin (41 µM in PBS) for 360 s. For quantitative interaction analysis different concentrations of analytes (JkH5-, JC2-, and JF6-hFc) were passed over the mAb-coated sensor chip surface. Association and dissociation time of analytes were set to 300 s each at a constant flow rate of 10 µl/min. After each interaction cycle, regeneration of the sensor chip surface was performed with 100 mM glycine-HCl (pH 2.2) for 30 s at a flow rate of 10 µl/min. The sensorgrams were corrected for nonspecific analyte-binding to the biotinylated sensor chip surface. Baseline-corrected binding curves were evaluated with a 1:1 binding model for kinetic rate constant determination. Alternatively, equilibrium dissociation constants (K_D) were calculated from the maximal response signals (Response_max) and analysed with a steady-state affinity model.

Blocking of inhibitor-binding to FVIII in ELISA

Human recombinant FVIII (hFVIII, 0.25 IU, Bayer Healthcare, Leverkusen, Germany) was immobilised on microtitre plates. Concentrations of mAbs 2–76 (1.3 nM) and 2–77 (3.6 nM) leading to about 75% of maximal mAb binding to hFVIII were incubated with different dilutions of scFv-hFcs for 1 h at 37°C prior to incubation on hFVIII-coated microtitre plates for 2 h at RT. Binding of mAbs was detected with HRP-conjugated rat-anti-mouse IgG (Dianova). Bound antibodies were detected as above. Absorption in the absence of anti-idiotypes was set to 0% neutralisation and absorption in the presence of anti-idiotypes was expressed as % neutralisation of mAb binding.

Anti-idiotype mediated restoration of FVIII activity in vitro

Measurements of the activated partial thromboplastin time (aPTT) to determine FVIII activities were performed on a BCT® (Siemens Healthcare Diagnostics, Marburg, Germany) using manufacturer’s reagents. 6.7 nM of mAb 2–76 were mixed with varying concentrations of scFv-hFcs (3.34 nM to 668 nM) in HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM CaCl₂, 0.01% (v/v) Tween20). FVIII-deficient plasma (Siemens Healthcare Diagnostics) was utilised for mAb 2–77 (4.1 nM), which was mixed with scFv-hFc dilutions ranging from 390 nM to 6.25 µM. After 1 h incubation at 37°C, FVIII was added to a final concentration of 1 IU/ml. FVIII activity was determined after a 30 min incubation as previously described (13). Briefly, samples were mixed with an equal amount of FVIII-deficient plasma and aPTT reagent. After 3 min incubation at 37°C calcium chloride was added and clotting times were measured. FVIII activity was calculated using standard curves from standard human plasma dilutions. The percentage of neutralisation was calculated as follows

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\% \text{neutralisation} = \frac{\text{FVIII activity}_{\text{anti-id}} - \text{FVIII activity}_{0}}{\text{FVIII activity}_{\text{max}} - \text{FVIII activity}_{0}} \times 100
\]

where FVIII activity_{anti-id} corresponds to FVIII activity in the presence of anti-idiotype, FVIII activity_{0} is the activity in the absence of anti-idiotype, and FVIII activity_{max} corresponds to FVIII activity in the absence of FVIII inhibitor.

In vivo neutralisation of FVIII inhibitors by anti-idiotypes

Breeding pairs of B6; 129S4-F8<tm1Kaz>/J (FVIII−/−) mice with a targeted deletion in exon 16 of FVIII were purchased from Charles River Laboratories (Sulzfeld, Germany) and bred under SPF conditions. All experiments performed were approved by the local authority for animal welfare (Regierungspräsidium Darmstadt, Gen.Nr. F 21/05).

Residual FVIII activities in murine plasma samples were determined in a chromogenic assay. The assay was performed on a BCS® (Siemens Healthcare Diagnostics) according to the manufacturer’s instructions using manufacturer’s reagents and human normal plasma for calibration.

To analyse the potential of scFv-hFcs to neutralise FVIII inhibitors in vivo, scFv-hFcs were analysed in a monoclonal inhibitor model as described before (8). Briefly, FVIII activity in plasma of FVIII−/− mice reconstituted with 1 IU hFVIII was evaluated. Therefore, 100 µl PBS or FVIII inhibitor were injected intravenously (i.v.), followed by 1 IU hFVIII in 100 µl PBS 30 min later. Varying concentrations of scFv-hFcs were either applied with hFVIII (therapeutical setting) or with the FVIII inhibitor after a 30 min incubation at 37°C (experimental setting). Blood was collected for FVIII activity measurements 30 min after FVIII injection and mixed with 1/10 volume of 3.8% trisodium citrate solution as anticoagulant. Plasma was collected after centrifugation at 1500g for
15 min. One-way ANOVA test and Tukey’s multiple comparisons test were used for statistical analysis as normal distribution was assumed.

**FACS analysis of 2–76 producing hybridoma 76–113**

Monoclonal antibody 2–76-producing hybridoma 76–113 and an off-target hybridoma specific for c-myc were stained in parallel. JkH5-hFc as well as M699-hFc, a 100 bp peptide-hFc fusion, were conjugated with Alexa Fluor 488 using the Alexa Fluor 488 Protein Labelling Kit (Invitrogen, Paisly, UK) according to the manufacturer’s instructions. 5x10^5 cells were stained with Alexa Fluor 488-labelled JkH5-hFc, M699-hFc (each 0.85 pmol/µl) or Alexa Fluor 647-labelled hamster anti-CD79b (AbD Serotec, Kidlington, UK) for 20 min at 4°C. Data of 3x10^5 viable hybridomas were acquired on a FACSCanto 10 for each setting (Becton Dickinson, Franklin Lakes, NJ, USA) and analysed using FlowJo software (FlowJo LLC, Ashland, OR, USA).

**Results**

**Selection of FVIII inhibitor-specific anti-idiotypes**

The selection of FVIII inhibitor-specific scFvs from Tomlinson libraries was successfully accomplished for A2 domain-specific FVIII inhibitor 2–76 (GMA-8021) and C2 domain-specific 2–77 (GMA-8006). Seven phage clones specific for monoclonal antibody (mAb) 2–76 and four mAb 2–77-specific clones were chosen for further characterisation based on their mAb binding intensity. To identify anti-idiotypic scFvs, binding of phage clones to FVIII inhibitors was compared in the presence and absence of FVIII. For five mAb 2–76-specific clones binding was reduced more than 50 % in the presence of FVIII and mAb 2–77-binding was FVIII-sensitive for three of the four specific phage clones analysed (Suppl. Figure 1, available online at www.thrombosis-online.com). ScFv-bearing phage clones with FVIII-sensitive binding were considered as anti-idiotypes as they probably compete with FVIII for binding to the FVIII inhibitors’ paratope.

For each FVIII inhibitor two phage clones with potential anti-idiotypic scFvs were chosen for further characterisation. For that, scFvs were fused to the Fc domain of human IgG1 (hFc). Both mAb 2–76-specific scFv-hFcs, JkH5 and JkE5, showed concentration-dependent binding to mAb 2–76 in ELISA with EC50 values of 0.2 nM (JkH5) and 4.0 nM (JkE5) (Figure 1A). MAb 2–77-specific scFv-hFcs JC2 and JF6 bound to mAb 2–77 with EC50 values of 37.0 nM (JC2) and 3.5 nM (JF6) (Figure 1B).

To analyse the specificity of our isolated anti-idiotypes, we tested cross-reactivity of mAb 2–76-specific JkH5 with other A2 domain-specific antibodies as well as reactivity of mAb 2–77-specific JC2 and JF6 with a set of C2 domain-specific antibodies. ScFv-hFcs JkH5 and JkE5 showed to be very specific for 2–76 and did not bind to any other immobilised FVIII-specific antibody (Figure 1C) and comparable results were obtained for JC2 and JF6 (Figure 1D). In accordance to these results, no interaction of JkH5, JC2 and JF6 with polyclonal inhibitor-positive murine plasma samples was observed in immunoprecipitation experiments (Suppl. Figure 2A, available online at www.thrombosis-online.com). Further, a total of 30 inhibitor-positive human plasma samples were tested for JkH5-binding in immunoprecipitation experiments. However, no JkH5-specific antibody population was detected (data not shown). In addition, no neutralising effect of anti-idiotypes on FVIII inhibitors was seen in five human plasma samples (Suppl. Figure 2B, available online at www.thrombosis-online.com). Taken together, we neither observed cross-reactivity towards murine or human antibodies nor a neutralising effect of the selected scFvs on human FVIII inhibitors.
Kinetics of the JkH5/mAb 2–76 interaction

Affinity and kinetic analysis of the anti-idiotypic/FVIII inhibitor interaction pairs was performed with surface plasmon resonance (SPR) spectroscopy. For that, biotinylated mAb 2–76 or 2–77 was immobilised on a streptavidin-coated sensor chip surface and binding of scFv-hFc was measured in real time at different concentrations. Only negligible binding signals were detected for the two mAb 2–77-specific scFv-hFc JC2 and JF6. Hence, no reliable equilibrium dissociation constant could be calculated for these two analytes. Accessibility of the biotinylated mAbs’ paratopes by FVIII and anti-idiotypes was addressed by ELISA (data not shown). Biotinylation did not result in a masking of the anti-idiotypes’ epitopes on their respective targets and therefore could be excluded as a reason for the insignificant binding of JC2 and JF6 to mAb 2–76.

In contrast, scFv-hFc JkH5 showed specific binding to mAb 2–76 (Figure 2). Nonlinear regression analysis of the data fitted to a 1:1 Langmuir binding model provided an association rate constant \( (k_{on}) \) of \( 1.93 \pm 0.14 \times 10^{5} \text{M}^{-1} \text{s}^{-1} \) and dissociation rate constant \( (k_{off}) \) of \( 2.13 \pm 0.02 \times 10^{-3} \text{s}^{-1} \). The equilibrium dissociation constant \( KD \) derived from these data is \( 11.0 \pm 1.4 \text{nM} \).

Mapping of the mAb 2–76 epitope on scFv JkH5

As scFv JkH5 and FVIII both bind to mAb 2–76, these proteins might have a structural homology with regard to their mAb 2–76 binding site. MAb 2–76 binds to region R484-I508 on the A2 domain (11). Within this region, residues R489, Y495, and/or F501 are crucial for mAb binding (13). To examine the potential homology between scFv JkH5 and the 2–76 epitope, a linear sequence alignment was performed. The scFv region Y226-Q232 showed good homology to FVIII residues F501-E508 (Figure 3A).

In vitro effects of anti-idiotypes on FVIII inhibitors

JkE5 and JkH5 were able to inhibit binding of mAb 2–76 to FVIII (Figure 4A). However, inhibition was much stronger by JkH5, which blocked mAb 2–76-binding to about 90% with an \( EC_{50} \) of 27 nM. In contrast, both mAb 2–77-specific anti-idiotypes comparably blocked binding of 2–77 to FVIII to about 90% with \( EC_{50} \) values of 157 nM (JC2) and 79 nM (JF6) (Figure 4B). We therefore tested if
these anti-idiotypes share the same epitope on mAb 2–77. Binding of biotinylated JF6 to immobilised mAb 2–77 was analysed in the presence of JF6 or JC2. While binding was strongly reduced in the presence of JF6, excessive amounts of JC2 did not block JF6 binding (Suppl. Figure 4, available online at www.thrombosis-online.com). Thus, JF6 and JC2 to bind to distinct epitopes on mAb 2–77.

JkH5-specific for mAb 2–76 as well as both mAb 2–76-specific scFv-hFcs were next analysed for their capacity to restore FVIII activity in the presence of their respective inhibitor. For this purpose, FVIII was supplemented with a fixed concentration of FVIII inhibitor and varying concentrations of anti-idotypic scFv-hFcs. Addition of mAb 2–76 at a final concentration of 6.7 nM resulted in about 10% residual FVIII activity. Addition of a 50-fold molar excess of JkH5 neutralised the inhibitory effect of mAb 2–76 by 60% (Figure 4C). The addition of 4.1 nM mAb 2–77 to FVIII resulted in 30% residual FVIII activity. Here, addition of 3.1 µM JC2 representing a 760-fold molar excess resulted in 50% neutralisation (Figure 4D). In contrast, 6.3 µM JF6 (1520-fold molar excess) completely neutralised mAb 2–77. Thus, scFv-hFcs were able to neutralise their respective FVIII inhibitor in vitro.

Restoration of FVIII activity in a monoclonal mouse model

To address the question if anti-idiotypes specific for mAbs 2–76 and 2–77 can also restore FVIII activity in the presence of their respective inhibitors in vivo, anti-idotypic scFv-hFcs were analysed in a murine monoclonal inhibitor model. Thereby, reconstitution of FVIII+/– mice with 1 IU hFVIII resulted in a mean of about 30% FVIII activity. Injection of 6.7 nM mAb 2–76 or 4.1 nM mAb 2–77 resulted in a reduction of FVIII activity to about 5% (2–76) and 10% (2–77). When anti-idiotypes were incubated with their respective FVIII inhibitor prior to injection (experimental setting), FVIII activity was partially restored in a concentration-dependent manner for all tested scFv-hFcs (Figure 5A–C). For mAb 2–76-specific JkH5, a second setting was examined where mAb 2–76 was injected first, followed by JkH5 in combination with FVIII in order to reflect the situation in an inhibitor-positive patient (therapeutic setting). In this setting JkH5 completely restored FVIII activity with a 140-fold molar excess over 2–76 (Figure 5D). Thus, anti-idiotypes can restore the FVIII activity in the presence of FVIII inhibitors in vivo.

Targeting of mAb 2–76-producing hybridoma cells

As secreted IgGs develop from membrane-anchored B cell receptors (BCR) on naïve or memory B cells, anti-idiotypes targeting FVIII inhibitors might also be used to target FVIII inhibitor-specific B cells. To test this hypothesis, mAb 2–76-producing hybridoma cell line 76–113 was stained with Alexa Fluor 488-conjugated JkH5 or M699-hFc as negative control. While the staining pattern with M699-hFc was comparable to the unstained control, cells were specifically stained with scFv-hFc JkH5 (Figure 6A). To verify the specificity of JkH5, an anti-c-myc antibody-producing hybridoma cell line was stained in parallel. No unspecific binding of JkH5 was observed (Figure 6B). BCRs are typically expressed with the coreceptor CD79b, which could not be detected on the 76–113 cell line (Figure 6C). Hence, staining of hybridoma 76–113 with JkH5 presumably occurs via JkH5-binding to mAb 2–76 in transit through the membrane. Further use of this hybridoma for killing studies was therefore not pursued.

Discussion

For FVIII inhibitor-positive haemophilia A patients with in part repetitive ITI failures, so far no generally accepted treatment alternative for inhibitor eradication is available. Anti-idiotypes binding to FVIII-specific antibodies have been found in intravenous IgG preparations (4), healthy individuals (5), and plasma of haemophilia A patients after successful ITI (7). In this study, we analysed the feasibility to isolate FVIII inhibitor-specific anti-idiotypes from phage-displayed scFv libraries and their potential for inhibitor neutralisation and targeting of inhibitor producing cells using murine monoclonal FVIII inhibitors as model system.
Our results show that scFvs specifically binding to murine mAbs 2–76 and 2–77 were successfully isolated from the Tomlinson scFv libraries. Of note, selections against three other murine FVIII inhibitors were not successful. The Tomlinson I and J libraries each have a diversity of about $1 \times 10^8$ scFvs while the naïve human antibody repertoire probably exceeds $10^{11}$ individual antibody molecules (14). Thus, the library diversity limits the isolation of specific scFvs for any given antigen. In addition, the quality of these commercial libraries decreased over time, eventually leading to a stop of their distribution by the supplier. In general, naïve or synthetic libraries with higher diversities approaching the diversity of the human antibody repertoire might be more suitable for successful anti-idiotype selection.

Cross-reactivity of anti-idiotypes with different FVIII inhibitors might facilitate the use of selected scFvs for a reestablishment of haemostasis in inhibitor-positive patients. In contrast to the BO2C11-specific anti-idiotype 14C12 (8), JkH5 and JkE5 showed no specificity for any other A2 domain-specific antibody tested. Similarly, JF6 and JC2 only recognised mAb 2–77 (4.1 nM, D) were mixed with varying concentrations of scFv-hFcs and added to hFVIII after pre-incubation. After 30 min incubation at 37°C, residual FVIII activity was determined. A target-non-specific scFv-hFc was used as negative control. Absorption in the absence of scFv-hFcs was set to 100% to convert reductions in absorption to % neutralisation of binding. C-D) Monoclonal antibodies 2–76 (6.7 nM, C) and 2–77 (4.1 nM, D) were mixed with varying concentrations of scFv-hFcs and added to hFVIII after pre-incubation. After 30 min incubation at 37°C, residual FVIII activity was determined. A target-non-specific scFv-hFc was used as negative control. The percentage of neutralisation was calculated by dividing the increase in FVIII activity resulting from the addition of anti-idiotypes by the maximal increase in FVIII activity that was obtained from a sample containing FVIII but no FVIII inhibitor or anti-idiotype. A-D) Errors bars represent the SD of the mean of duplicates. Experiments were performed twice (A, B) or in triplicates (D) with similar results. C) Results from the least effective of three independent experiments are shown.

As a consequence of the high target specificity of the selected scFvs we further characterised the anti-idiotype/FVIII inhibitor interactions and were able to determine the kinetics of the mAb 2–76/JkH5 interaction. The association rate constant $k_{\text{on}}$ of JkH5-hFc for mAb 2–76 ($1.93 \times 10^5$ M$^{-1}$ s$^{-1}$) determined in our study is comparable to the $k_{\text{on}}$ of 14C12 for BO2C11 ($5 \times 10^5$ M$^{-1}$ s$^{-1}$) (8). However, the dissociation rate constant $k_{\text{off}}$ of the JkH5/mAb 2–76 complex ($2.13 \times 10^{-3}$ s$^{-1}$) is about 40-fold higher than the $k_{\text{off}}$ of the 14C12/BO2C11 complex ($5 \times 10^{-5}$ s$^{-1}$) indicating that the JkH5-hFc/mAb 2–76 complex is not as stable as the 14C12/BO2C11 complex.

Besides analysing the JkH5/2–76 binding kinetics we also analysed the 2–76 epitope on JkH5. Exposed amino acid residues R52 and Y226 of JkH5 that might resemble R489 and F501 of the 2–76 epitope on FVIII showed to be crucial for JkH5-binding to 2–76. Thus, the selected anti-idiotypes proofed to be very specific for their FVIII inhibitor, minimising the risk of off-target effects.

Figure 4: Anti-idiotype-mediated reduction of FVIII inhibitor-binding to FVIII and restoration of FVIII activity in vitro. A-B) Recombinant hFVIII was immobilised on microtitre plates and 1.3 nM mAb 2–76 or 3.6 nM mAb 2–77 were pre-incubated with different scFv-hFc dilutions. JkH5, JC2, and JF6 inhibited binding of their respective FVIII inhibitor to FVIII to more than 90% with EC$_{50}$ values of 27 nM (JkH5), 157 nM (JC2), and 79 nM (JF6). BSA-specific scFv-hFc was used as negative control. Absorption in the absence of scFv-hFcs was set to 100% to convert reductions in absorption to % neutralisation of binding. C-D) Monoclonal antibodies 2–76 (6.7 nM, C) and 2–77 (4.1 nM, D) were mixed with varying concentrations of scFv-hFcs and...
pocket of 50–70 amino acids in size, the participation of only a few essential residues in the antibody/antigen interaction is a common phenomenon (reviewed in [15]). For the lysozyme-specific antibody D1.3 it has been shown for instance, that the mutation of one single CDR residue results in a 1000-fold decrease in affinity for lysozyme (16).

Isolated scFvs inhibited binding of their respective mAbs to FVIII and partly restored FVIII activity in a functional clotting

Figure 5: Restoration of FVIII activity in vivo. A-C) Mixtures of scFv-hFc s JC2 (A) or JF6 (B) with mAb 2–77 and JkH5 (C) with mAb 2–76 were intravenously injected into FVIII−/− mice, followed by injection of hFVIII. D) MAb 2–76 was injected into FVIII−/− mice, followed by injection of mixtures of hFVIII and JkH5-hFc. A-D) Blood was collected after 30 min incubation and residual FVIII activity in the plasma was measured in a chromogenic assay. P-values are indicated. * p < 0.05; ** p < 0.01; *** p < 0.001; n.s., not significant.

Figure 6: Flow cytometric analysis of JkH5 binding to mAb 2–76-producing hybridoma. A) Hybridoma 76–113 producing mAb 2–76 was stained with Alexa Fluor 488-labelled JkH5-hFc and M699-hFc and staining intensities were compared to unstained cells. B) Specificity of JkH5-hFc for hybridoma 76–113 was shown by staining of hybridoma 76–113 and an off-target hybridoma (/uni03B1)-c-myc) with Alexa Fluor 488-labelled JkH5-hFc. C) Hybridoma 76–113 cells were stained with a CD79b-specific antibody to analyse BCR expression. A-C) Plots show the data acquired for 3×10⁴ viable hybridoma cells.
In summary, we could show that FVIII inhibitor-specific anti-idiotypes can be successfully isolated from phage-display scFv libraries and be used to restore FVIII activity in vitro as well as in a monoclonal disease model. As anti-idiotype JkH5 could also specifically stain the FVIII inhibitor-producing hybridoma 76–113, inhibitor-specific anti-idiotypes might serve as a tool for the depletion of FVIII inhibitor-producing cells in the future.

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What is known about this topic?

- Anti-idiotypic antibodies specific for FVIII inhibitors can be found in haemophilia A patients following successful immune tolerance induction.
- Mice develop anti-idiotypes after immunisation with FVIII inhibitors that can be used to neutralise these inhibitors in vitro and in vivo.

What does this paper add?

- FVIII inhibitor-specific anti-idiotypic can be isolated from phage-displayed scFv-libraries.
- Anti-idiotypes can be used to specifically target FVIII inhibitor-producing hybridomas.

2–76-specific hybridoma cell line as well as A2 and C2 domain-specific mAbs.

Author contributions

A.S., K.B., J.K., M.B., R.T., JSCH., C.K. designed research. A.S., K.B., A.O., K.B.P., D.S., M.B. performed research. A.S., K.B., J.K., JSCH., M.B., C.K. analysed data. A.S., D.SCH., C.K. wrote the manuscript. All authors critically reviewed and edited the manuscript.

Conflicts of interest

None declared.

References


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