In vivo neutralization of a C2 domain–specific human anti–Factor VIII inhibitor by an anti-idiotypic antibody

Jean Guy G. Gilles, Sabrina C. Grailly, Marc De Maeyer, Marc G. Jacquemin, Luc P. VanderElst, and Jean-Marie R. Saint-Remy

Factor VIII (FVIII) administration elicits specific inhibitory antibodies (Abs) in about 25% of patients with hemophilia A. The majority of such Abs reacts with FVIII C2 domain. mAbBO2C11 is a high-affinity human monoclonal antibody (mAb) directed toward the C2 domain, which is representative of a major class of human FVIII inhibitors. Anti-idiotypic Abs were raised to mAbBO2C11 to establish their neutralizing potential toward inhibitors. One mouse anti-idiotypic mAb, mAb14C12, specifically prevented mAbBO2C11 binding to FVIII C2 domain and fully neutralized mAbBO2C11 functional inhibitory properties.

Introduction

Hemophilia A is characterized by a functional deficiency of coagulation Factor VIII (FVIII), a cofactor for the formation of the intrinsic tenase complex, which is required for thrombin generation. Patients with hemophilia A suffer from spontaneous or posttraumatic bleeding into joints, muscles, or soft tissues. Replacement therapy using either plasma-derived or recombinant FVIII is complicated in about 25% of the patients by the development of specific antibodies (Abs) that neutralize FVIII function, also called inhibitors.1,2 The presence of an FVIII inhibitor precludes further specific replacement, thereby threatening the patient’s life. To date, patients with FVIII inhibitor are treated by administration of bypassing agents, including recombinant FVIIa.3 Administration of high doses of FVIII over extended periods of time is considered in some cases, but such a therapy is extremely costly and limited to patients with recent and low inhibitor titers.4 Neutralizing anti-FVIII inhibitors nevertheless remains a major challenge and alternative; more specific strategies are warranted.

One of such strategies would be to directly neutralize the function of inhibitor Abs. Methods to inhibit the binding of inhibitory Abs to FVIII have been proposed, in which FVIII-derived peptides compete for the binding to inhibitor,5 but they have been limited practically by the absence of precise molecular information on such Abs and the risk of interfering with normal FVIII activity.

Despite its high molecular weight, FVIII presents only a limited number of B-cell epitopes, which are directly or indirectly involved in the function of the molecule.6 The majority of Abs react toward the C2 domain of FVIII, which mediates interactions with von Willebrand factor (VWF) and phospholipids (PLs). We recently derived a human monoclonal antibody (mAb), mAbBO2C11, from the peripheral memory B-cell repertoire of a patient with severe hemophilia and a FVIII inhibitor,7 and we demonstrated that mAbBO2C11 reacts with high affinity to the C2 domain of FVIII. Furthermore, the crystal structure of a complex of C2 and mAbBO2C11 Fab fragment demonstrated that the antibody recognizes a large conformational C2 epitope.8 Thus, mAbBO2C11 belongs to a prominent class of FVIII-specific Abs that inhibit the binding of FVIII to VWF and PL. Neutralizing anti-FVIII inhibitors nevertheless remains a major challenge. Alternative, more specific strategies are warranted.

Such neutralization could be achieved by using specific anti-idiotypic Abs. An Ab idiotype refers to the ensemble of determinants that are located within its variable part. Anti-idiotypic Abs are second-generation Abs directed toward the variable part of pathogenic Abs and, as such, are highly specific. On the basis of previous findings that anti-idiotypic Abs may neutralize FVIII inhibitor Abs in the plasma of healthy individuals9,10 and of patients with hemophilia A under tolerance induction by administration of high doses of FVIII,11 it was predicted that idiotypic regulation could be used therapeutically to interfere with the function of FVIII inhibitors.12 Recent evidence has indicated that only a proportion of anti-FVIII Abs exert an inhibitory activity13 and that FVIII inhibitors could emerge from only a limited number of B-cell precursors, at least with regard to light chain-specific Abs.14 Taken
together, this information suggests that a limited number of anti-idiotype Abs could be all that is required to control FVIII inhibitors.

Starting from mAbB02C11, we have generated mouse anti-idiotype Abs, and we provide in the present paper the in vitro and in vivo proof of concept, establishing the basis for idotype-mediated neutralization of a major class of FVIII inhibitors.

Materials and methods

**Reagents**

Full-length human recombinant FVIII (rFVIII) was obtained as material for laboratory use only (rFVIII; Bayer, Berkeley, CA). Plasma-derived VWF was obtained from the Département Central de Fractionnement (DCF; Brussels, Belgium) and further purified as described.15 mAbB02C11 IgG4 specific for the FVIII C2 domain has been fully described.7,8 Human mAbLE2E9 specific for the C1 domain15 and mouse mAbs directed toward different human FVIII domains or VWF were produced in our center (Center for Molecular and Vascular Biology [CMVB]).

**Animals**

C57BL/6 FVIII−/− with a target deletion in exon 16, initially obtained from Dr. S. Antonarakis (Geneva, Switzerland), and BALB/c mice were bred in our animal facilities. All animal experiments were conducted according to the Ethical Committee Rules for Animal Experiments of the University.

**Anti-idiotype mAb production and characterization**

mAbB02C11 (10 μg) emulsified in complete Freund adjuvant (CFA) were injected subcutaneously in BALB/c mice, followed by 2 boost injections in incomplete Freund adjuvant (IFA) made at 2-week intervals. Antibody titer were measured by direct binding to mAbB02C11-coated microtitration plates. Clones were obtained after fusing spleenocytes with the SP2 myeloma cells and expanded under limiting dilution. mAb14C12 Fab fragments were prepared by digestion with papain agarose beads (Pierce, Rockford, IL) and purified by passage over a protein-A Sepharose column. Real-time kinetic interaction between mAb14C12 and mAbB02C11 was evaluated by surface plasmon resonance by using a Protein MicroSensor BLAcore instrument (Pharmacia Biotech AB, Uppsala, Sweden). Purified mAbB02C11 (5 μg/mL in 10 mM sodium acetate buffer, pH 5.0) was immobilized on the activated surface of a CM5 sensor chip. mAb14C12 was infused at various concentrations over the mAbB02C11-immobilized sensor chip surface. Association and dissociation rate constants were determined as reported.4 For sequencing, mRNA from the mAb14C12 hybridoma was isolated by using the Quick Prep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). CDNA was synthesized with First-strand CDNA Synthesis Kit (Amersham Pharmacia Biotech). The cDNA encoding the heavy (H) and light chain (L) variable regions were amplified by polymerase chain reaction (PCR) using specific primers. PCR products were isolated from 1.5% agarose gel using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned using the PGEM-T Easy Vector system (Promega, Madison, WI). Plasmid DNA from positive colonies was isolated by using the High Pure Plasmid Isolation Kit (Roche Diagnostics, Mannheim, Germany) and sequenced in both directions with Sequenase (US Biochemical, Cleveland, OH).

**Binding of mAb14C12 to mAbB02C11 and its neutralization**

**Binding of mAb14C12 to mAbB02C11.** Microtitration plates were coated with 2 μg/mL mAbB02C11. Samples containing mAb14C12 were added to the plate, and bound mAb14C12 was detected by addition of a horseradish peroxidase (HRP)-labeled goat antimouse IgG (Bio-Rad, Hercules, CA). Optical density (OD) was evaluated at 490 nm in a microtitration plate reader Emax ( Molecular Devices, Sunnyvale, CA).

**Inhibition of mAbB02C11 binding to FVIII by mAb14C12.** The concentration of mAbB02C11 required to reach 80% of maximal binding to FVIII-coated plates was determined. This determination was carried out by using plates coated with 2 μg/mL rFVIII overnight at 4°C. All Ab solutions were diluted in casein buffer (citrate buffer containing 0.5% casein, 9 g/L NaCl, pH 7.2). Bound mAbB02C11 was detected by using an HRP-conjugated mAb specific for human Fc (Southern Biotechnology, Birmingham, AL). For inhibition assays, 60 μL of 2 μg/mL mAbB02C11 was mixed with an equal volume containing various concentrations of mAb14C12. The mixture was preincubated for 1 hour at 21°C. Aliquots of 50 μL were applied in duplicates to FVIII-coated plates for a further incubation of 2 hours at 21°C. mAbB02C11 binding was detected as earlier, and inhibition was calculated from the ratio of OD values.

**Neutralization of mAbB02C11 inhibitory activity in a functional FVIII assay.** To evaluate the capacity of mAb14C12 or of its Fab fragment to restore FVIII function in the presence of the inhibitor mAbB02C11, we first determined the concentration of mAbB02C11 required to inhibit 80% of FVIII activity in a functional chromogenic assay (Dade Behring, Marburg, Germany) by using 1 IU/mL rFVIII. The same determination was made with a total immunoglobulin G (IgG) fraction and with affinity-purified polyclonal anti-FVIII Abs obtained from the mAbB02C11 donor. The amount of mAbB02C11 (or of polyclonal Abs) required to inhibit 80% of FVIII activity was mixed with an equal volume of various concentrations of mAb14C12 or of its Fab fragment. The mixtures were incubated for 1 hour at 37°C before addition of rFVIII. An aliquot of the mixture was retrieved after a further 1-hour incubation at 37°C and added to the chromogenic assay reagents. Control experiments included rFVIII incubated alone or with an Ab of unrelated specificity.

**3-D modeling and alignment of mAb14C12 with the C2 domain**

The 3-D model of the variable parts of mAb14C12 was established by submitting the corresponding amino acid sequences to the Web Antibody Modelling (WAM; University of Bath, Swindon, United Kingdom; http://antibody.bath.ac.uk/). This algorithm takes into account both conserved structures of framework regions corrected for variations in particular beta strands and the large sequence and structure variability of complementarity determining regions (CDRs), which fall into one of the canonical classes,16 as well as the particular sequence of the CDR3. The method combines database and conformational searches, energy screening, and structural-determining residue filtering. Alignment of C2 domain and variable parts of mAb14C12 was carried out by combining manual alignment of secondary structures with the use of the Kabsch-Sander algorithm,17 and global sequence alignment was calculated with the Myers-Miller algorithm.18 The result was then refined by juxtaposing the 3-D structure of C2 domain19 and the WAM model of mAb14C12 Fv.

**Inhibition of FVIII binding to VWF and PL**

The capacity of mAb14C12 to interfere with the binding of FVIII to either VWF or PL was investigated as follows. Microtitration plates were coated with an anti-VWF Ab followed by purified VWF as described.15 mAb14C12 (1.25 μg/mL in phosphate-buffered saline [PBS] containing 0.5% casein) was mixed with an equal volume of FVIII at a final concentration of 1 IU/mL. Fifty microliters of the mixture was then added to the VWF- or PL-coated plates, and the plates further were incubated for 2 hours at 21°C. The binding of the FVIII to VWF was detected by the addition of 2 μg/mL biotin-labeled mAb15 (an anti–heavy chain–specific antibody), followed by avidin-peroxidase and a specific substrate. To assess FVIII binding on PL, plates were coated with phosphatidyl serine diluted at 10 μg/mL in methanol, and the capacity of mAb14C12 to inhibit the binding of FVIII to PL was investigated by following the same procedure as for the VWF assay. Control experiments included the substitution of mAb14C12 by mAbBo2C11, known to inhibit the binding of FVIII to both VWF and PL.7

**Binding of mAb14C12 to anti-FVIII antibodies of unrelated donors and mouse**

Cloning of the FVIII C2 domain cDNA fragment and expression in a reticulocyte transcription/translation system. The DNA fragment encoding for the C2 domain was generated by PCR using primers bound by the restriction sites HindIII and Not1. Sense primer, named according to the first FVIII amino acid, 478/18528.
Acid residue encoded, was as follows: 2124, 5'-GATGCGAGGCTGTCCTTTGGCAATGGGGATC-3'. The antisense primer, named according to the last encoded amino acid residue, was as follows: 2352, 5'-TTCTGCAATTGCAGCCGCGTCGAGGCTCCTGGCACCC-3'.

The PCR product was subcloned in frame into Signal plgplus (R&D Systems Europe Abingdon, United Kingdom) and controlled by sequencing in both directions with the use of an ABI genetic analyser 3.10 (Applied Biosystems, Foster City, CA).

A mixture containing all amino acids except methionine was added, together with 35S-methionine (Amersham, Bucks, United Kingdom). The presence of C2 in the supernatant was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography, showing a single band with the expected molecular mass of 23 kD.

**Immunoprecipitation assay.** The capacity of polyclonal anti-FVIII human Abs to recognize C2 was evaluated as described.3 The assay was adapted to determine whether mAb14C12 could prevent the binding to the C2 domain. Assays were carried out for plasma samples from 7 unrelated patients with hemophilia A inhibitor, including the patient from whom mAbBO2C11 was derived. Thus, mAb14C12 was diluted to final concentrations of 50, 150, or 450 μg/mL in 50 μL 50 mM Tris (tris(hydroxymethyl)aminomethane)-HCl buffer, pH 7.5, containing 150 mM NaCl, 0.1% Nonidet NP-40, 1 mM EDTA (ethylenediaminetetraacetic acid), 0.25% gelatin, and 5% bovine serum albumin (BSA; NET-gel buffer). Undiluted plasma (20 μL) containing polyclonal anti-FVIII antibodies was added to each vial, and the mixtures were incubated for 1 hour at 4°C. From 1 to 3 μL L-35S-methionine-labeled FVIII C2 domain was then added to the mixture for a further incubation of 1 hour at 4°C. Protein-A Sepharose (20 μL; Amersham Pharmacia Biotech) was then added to each vial, and the suspensions were again incubated for 1 hour at 4°C. Sepharose beads were centrifuged and washed twice with NET-gel buffer. Bound antigen/antibody complexes were eluted from beads by boiling for 2 minutes in 30 μL SDS gel-loading buffer. Supernatant (2 μL) was taken and diluted in 20 μL Lumasafe (Lumac LSC, Groningen, The Netherlands) for radioactivity counting.

As positive control, an aliquot of 2 μL mAbBO2C11 (1 mg/mL in PBS) was added to each of the mAb14C12 solutions, and the experiment was carried out as described earlier for polyclonal antibodies.

**Binding of mAb14C12 to anti-FVIII mouse mAbs.** Microtitration plates were coated with PBS containing 2 μg/mL mouse mAb to various FVIII domains, including the C2 domain. Plates were washed and further incubated for 2 hours at room temperature with biotin-labeled mAb14C12, followed by washing and sequential addition of streptavidin peroxidase and its substrate. mAb14C12 binding was evaluated by OD reading described earlier.

**In vivo assays**

**Effect of mAb14C12 on FVIII clearance.** To ensure that mAb14C12 did not modify FVIII clearance, C57BL/6 FVIII−/− mice were injected with 10 μg/mL mAb14C12 or an IgG2a mAb of unrelated specificity, followed by an intravenous injection of 1 IU rFVIII. Blood samples were collected by cardiac puncture. Three mice were used under each experimental condition. Mice were injected intravenously with 100 μL mAbBO2C11 at concentrations that ranged from 10 to 2.5 μg/mL in PBS followed 30 minutes later by 1 IU rFVIII, and the concentration resulting in 90% inhibition of FVIII activity (ie, 0.5 μg) was selected for neutralization assays. Controls included injection of a human IgG4 antibody of unrelated specificity. In a first series of experiments, complexes of mAbBO2C11 and mAb14C12 were formed prior to injection. Thus, 10, 1, or 0.1 μg mAb14C12 was mixed with 0.5 μg mAbBO2C11 in 100 μL PBS for 30 minutes at 37°C before the injection, followed by 1 IU rFVIII. Control experiments included mice injected with saline, mAbBO2C11 alone, mAb14C12 alone, and mAbBO2C11 mixed with an IgG2a of unrelated specificity. In a second series of experiments, the capacity of mAb14C12 to combine with, and neutralize, mAbBO2C11 in vivo was evaluated. Thus, groups of 3 FVIII−/− mice were injected intravenously with 0.5 μg mAbBO2C11 or saline. After 30 minutes, each mouse received a second 100 μL intravenous injection of a mixture containing 1 IU rFVIII plus various amounts of mAb14C12, ranging from 0.1 to 10 μg. Blood samples were collected as described earlier for assessing residual FVIII activity.

**Results**

**Anti-idiotypic Ab, mAb14C12, neutralizes in vitro the FVIII inhibitory activity of human mAbBO2C11**

BALB/c mice were immunized with mAbBO2C11, a human anti-FVIII inhibitor Ab that recognizes a major determinant of the C2 domain.3 The supernatants of 8 clones reacted with mAbBO2C11 but not with an unrelated IgG4 or with another human mAb specific for FVIII C1 domain. Two of these clones produced Abs that inhibited the binding of mAbBO2C11 to FVIII in a dose-dependent manner and neutralized the FVIII inhibitory activity of mAbBO2C11 in a functional assay. Figure 1 shows the results for one of the 2 mAbs, mAb14C12 (IgG2a). It can be seen that mAb14C12 specifically binds to mAbBO2C11, whereas it does not bind to a human anti-C1 Ab (Figure 1A). mAbBO2C11 binding to FVIII can be inhibited in a dose-dependent manner (Figure 1B). Fifty percent of the neutralization of the inhibitory activity was obtained in a chromogenic FVIII activity assay at a 1:2 mAbBO2C11/mAb14C12 molar ratio (Figure 1C). Fab fragments were prepared from mAb14C12 and shown to neutralize 50% of mAbBO2C11 inhibitory activity at a 1:8 mAbBO2C11/mAb14C12Fab molar ratio (Figure 1C), thereby confirming that the neutralization properties of mAb14C12 were brought about by residues located in the Ab variable parts.

![Figure 1](https://example.com/figure1.png)
The efficient neutralization of mAbBO2C11-mediated inhibitory activity prompted us to measure the binding affinity of mAb14C12 for mAbBO2C11. This measure was carried out by surface plasmon resonance, which showed association and dissociation of mAb14C12 for mAbBO2C11. This measure was carried out by second site (Met2199 and Phe2200) is not represented. Taken together these observations indicate that the VH region of mAb14C12 in its germ line configuration carries an extended homology of the C2 domain.

mAb14C12 shows extended homology with the C2 domain

The sequence of mAb14C12 was aligned with that of the FVIII C2 domain. A significant homology was found between the C2 domain and the VH region. Figure 2 shows that 31 amino acid residues were found either identical or similar. This included 13 residues associated with CDR regions, but clustering to the CDR1 (6 residues) and CDR3 (6 residues). Interestingly, a significant contribution is observed from residues located in framework regions (18 amino acids). Moreover, only 2 residues (marked with an asterisk) of the VH region are mutated from the germ line sequence of mAb14C12. All 31 residues are surface exposed on C2, according to the crystal structure of the C2 domain (Figure 2), as well as on mAb14C12 VH region. A salient feature of this alignment is that it involves 1 of the 2 PL binding sites made of Leu2251 and Leu2252 (total surface of 180 A), which correspond to Leu102 and Leu103 in mAb14C12 VH CDR3, whereas the second site (Met2199 and Phe2200) is not represented. Taken together these observations indicate that the VH region of mAb14C12 in its germ line configuration carries an extended homology of the C2 domain.

mAb14C12 does not interfere with FVIII binding to either VWF or PL and does not reduce FVIII clearance rate from the circulation

The extended homology between the C2 domain and mAb14C12 VH, which involved FVIII VWF and PL binding sites, prompted us to verify whether mAb14C12 was able to inhibit the binding of FVIII C2 domain to VWF and/or to PL. No such inhibition was observed for either VWF or PL in the presence of mAb14C12, whereas control experiments carried out in the presence of mAbBO2C11 showed complete inhibition in both cases (Table 1). As mAb14C12 carries only one of the PL binding sites of C2 (made of Leu2251 and Leu2252), we also checked whether the antibody could interfere with the binding of a mutant of FVIII with a deletion of Ala2201, known to alter the PL-binding capacity through Met2199 and Phe2200. No inhibition was found (data not shown), indicating that absence of inhibition of C2 binding to PL was not solely related to absence of the Met2199-Phe2200 residues.

The clearance of FVIII is at least in part due to the binding of the C2 domain to the lipoprotein-receptor-related protein (LRP) receptor and could, therefore, be reduced by mAb14C12. This was examined in FVIII−/− C57BL/6 mice reconstituted by tail injection of 1 IU human rFVIII. Previous calculation has shown that the average human FVIII t1/2 in this model was 180 minutes. The
experiment was repeated twice with mice that were first injected with 10 μg mAb14C12, followed 15 minutes later by 1 IU FVIII. No significant difference in FVIII t½ was observed in the presence of mAb14C12 after either 180 minutes (Table 1) or 360 minutes. Despite extensive homology between mAb14C12 VH and the C2 domain, the former does not seem to interfere with the physiologic activity and clearance of FVIII.

mAb14C12 binds to unmutated CDR and framework residues of mAbBO2C11
Aligning mAb14C12 VH and VL regions with those of mAbBO2C11 identified a total of 40 putative contact residues between the 2 antibodies (data not shown). Five of mAb14C12 residues are homologous to the C2 domain. Interestingly, only 12 of the 40 putative contact residues are located within CDR of mAb14C12. Residues located in the framework regions of mAb14C12, therefore, contribute significantly to the recognition of mAbBO2C11. Again, mutations contribute very little to this interaction, as only 3 of 40 amino acid residues of mAb14C12 are mutated, suggesting that mAb14C12 reacts in its germ line configuration with mAbBO2C11. However, 5 of the 40 putative contact residues within the variable parts of mAbBO2C11 are involved in the binding to C2. The majority (24 of 40) of such residues in mAbBO2C11 is located in framework regions, and only 6 mutations are observed in putative contact residues. This finding suggests that mAb14C12 reacts with the canonical sequence of the subfamily of Abs to which mAbBO2C11 belongs.

Altogether, the neutralization properties of mAb14C12 on mAbBO2C11 binding to, and inhibition of, FVIII, therefore, result from a combination of an extended homology with the C2 domain and recognition of mAbBO2C11 variable part residues not involved in C2 binding. However, attempts to raise anti-C2 inhibitory Abs by subcutaneous (SC) immunization of BALB/c mice with mAb14C12 in CFA/IFA have failed.

mAb14C12 inhibits the binding to C2 of polyclonal antibodies from unrelated patients
The extended complementarity between variable parts of mAb14C12 and those of mAbBO2C11 in germ line configuration suggests that mAb14C12 could neutralize anti-C2 inhibitory antibodies that carry a canonical sequence identical or close to that of mAbBO2C11. This finding is further stressed by the observation that a major proportion of anti-C2 inhibitory antibodies belong to the DP5 subfamily.14 We, therefore, evaluated the capacity of mAb14C12 to neutralize the inhibitory activity of anti-C2 domain polyclonal antibodies of unrelated patients with hemophilia A. To this end, we used an immunoprecipitation assay in which a radiolabeled C2 domain, obtained by combined transduction with rabbit reticulocytes, was allowed to react in solution with samples of plasma containing inhibitory Abs to the C2 domain. This assay detects all anti-C2 Abs, irrespective of their capacity to inhibit the function of FVIII.

Figure 3 shows that mAb14C12 neutralized up to 50% of the binding capacity of polyclonal anti-C2 Abs purified from the patient from whom mAbBO2C11 was derived (patient G). In such an assay, mAb14C12 also significantly neutralized polyclonal anti-FVIII Abs obtained from 3 of 6 unrelated patients (patients B, D, E). Idiotypes carried by mAbBO2C11 are, therefore, present on a significant proportion of anti-C2 Abs from unrelated patients. However, the capacity of mAb14C12 to neutralize FVIII inhibition was not paralleled by the results obtained in the immunoprecipitation assay (data not shown), indicating that, even in the presence of anti-C2 Abs, the latter are not necessarily the prominent inhibitors.

Additional experiments were carried out to determine whether the idiotype recognized by mAb14C12 was also presented on mouse anti-FVIII antibodies. A total of 50 mAbs were assayed in a direct binding ELISA, including 3 inhibitory mAbs with C2 specificity; none of such mAbs was, however, competing with mAbBO2C11. mAb14C12 did not recognize any of these mAbs (data not shown).

mAb14C12 neutralizes mAbBO2C11-mediated inhibiting activity in vivo
To determine whether mAb14C12 had the capacity of neutralizing the inhibitory activity of mAbBO2C11 in vivo, FVIII−/− mice were reconstituted with rFVIII and mAbBO2C11.20 The capacity of mAb14C12 to restore the function of FVIII was examined by using various concentrations of mAb14C12. Preliminary experiments have established that the t½ of mAbBO2C11 and mAb14C12 in FVIII−/− mice were of 3 and 5 days, respectively.

Injection of 1 IU FVIII is sufficient to obtain an average concentration of 0.5 IU/mL. Intravenous administration of 0.5 μg mAbBO2C11 30 minutes before FVIII completely inhibits FVIII activity (Figure 4). The capacity of mAb14C12 to neutralize mAbBO2C11 in vivo and, therefore, to restore FVIII function was evaluated by mixing 10, 1, and 0.1 μg mAb14C12 to 0.5 μg mAbBO2C11 before injection to mice that were injected with 1 IU FVIII 30 minutes later. Figure 4A shows that the mAbBO2C11 inhibitory activity is neutralized in a dose-dependent manner, with 50% neutralization obtained at approximately a one-to-one molar ratio. A maximum of 88.3% (± 23.8%) inhibition was observed at a 50-fold molar excess of mAb14C12.

A second series of experiments was carried out in which FVIII−/− mice were first injected with mAbBO2C11 (0.5 μg) followed 30 minutes later by a mixture of FVIII (1 IU) and various concentrations of mAb14C12. Results similar to the ones reported above with the preincubation of mAb14C12 and mAbBO2C11 were observed with sequential administration of the 2 antibodies (Figure 4B), which indicates that in vivo the affinity of mAb14C12 for mAbBO2C11 is high enough to prevent the binding of mAbBO2C11 to FVIII.
Discussion

Selective neutralization of inhibitor Abs represents one of the hoped-for outcomes of immunotherapy strategies. This outcome could in theory be achieved by using FVIII polypeptides containing Ab-binding sites, as proposed early after the cloning of FVIII was obtained.\textsuperscript{5,25} However, Ab epitopes are frequently located in regions of FVIII by which it interacts with physiologic partners such as VWF and/or PL. Hence, fragments of FVIII could interfere with such binding and reduce the procoagulant activity of FVIII. To circumvent this risk, alternative strategies have been sought, notably through the use of synthetic peptides mimicking conformational epitopes with a limited number of contact residues.\textsuperscript{20} Although promising, this approach suffers from the fact that small peptides have a short $t_{1/2}$ and usually exhibit a low affinity, requiring large doses to achieve a physiologic effect.

A valuable compromise between these 2 possibilities would be to produce large mimotopes, namely FVIII epitopes mimicking natural ones but divergent in amino acid sequence. This would potentially offer better stability and prolonged $t_{1/2}$ as compared with small peptides. Moreover, such large mimotopes can be altered in such a way as to eliminate the risk of interfering with FVIII function, while maintaining a strong, high-affinity reaction with inhibitorAbs. Obtaining large mimotopes by synthesis is cumbersome and impractical. However, anti-idiotypic Abs exhibiting extended homology with the antigen could serve as such, or as a template to prepare large mimotopes.

The production of suitable anti-idiotypic Abs for this purpose has been delayed until specific human mAbs with FVIII inhibitory activity were obtained by derivatization of peripheral memory B cells from patients with hemophilia A with inhibitors.\textsuperscript{7} This, combined to the elucidation of the crystal structure of FVIII domains,\textsuperscript{8} has opened the possibility of exploring at the clonal level the therapeutic potential of anti-idiotypic Abs for FVIII inhibitors.

The results reported here establish the proof of concept that this strategy is appropriate for designing efficient mimotopes for FVIII inhibitors. Starting from a unique human mAb representative of a major class of inhibitors we have produced an anti-idiotypic Ab that combines several properties: (1) it carries an extensive sequence homology with the C2 domain, spanning more than 90 amino acid residues; (2) it shows an affinity for the variable regions of the anti-C2 Ab comparable to that of the inhibitor Ab for C2; (3) it does not interfere with the binding of FVIII to either VWF or PL, nor does it alter the clearance rate of FVIII; and (4) it fully neutralizes the inhibitory activity of mAbBO2C11 in a reconstituted FVIII/\textsuperscript{11} mouse model.

The sequence homology between the variable parts of mAb14C12 and the C2 domain of FVIII is primarily carried by the VH region. Remarkably, the latter contains 31 putative surface-exposed residues identical or similar to C2, including one PL binding site (Leu2251-Leu2252). Interestingly, the homology includes a significant number of conserved residues of C2, suggesting significant structural homology between the 2 molecules. This homology is illustrated functionally by the observation that neutralization of mAbBO2C11 inhibiting activity is obtained at molar equivalence between mAbBO2C11 and mAb14C12, both in vitro and in vivo, in keeping with affinity measurement. mAb14C12 can, however, not be considered as carrying a complete internal image of the C2 domain, insofar as it does not bind to VWF or PL, it does not interfere with the binding of FVIII to either VWF or PL, and does not elicit anti-C2 Abs by conventional immunization.

An additional reason as to why the homology between the C2 domain and mAb14C12 VH region is interesting is related to the strong participation of residues located in mAb14C12 framework regions (ie, 45%), with only 6 mutated residues over a total of 31 (21%). In other words, the C2 domain presents a sequential and structural homology with some families of Abs in their germ line configuration.\textsuperscript{26} The C2 domain general organization is made of a barrel of 2 series of 4 beta sheets linked by flexible loops and is reminiscent of the organization of immunoglobulin domains, with loops represented by hypervariable regions, or CDRs, constituting the antigen-binding site.

The results obtained in vivo with FVIII \textsuperscript{11}/\textsuperscript{11} mice reconstituted with human FVIII suggest that mAb14C12 Abs and the like have a therapeutic potential. Full humanization of the Ab would reduce its potential immunogenicity but will also prolong its $t_{1/2}$ to up to 3 weeks. A single injection could, therefore, exert a significant effect for more than 1 month, which represents a definite advantage over small peptides. The risk of inducing Abs cross-reacting with the C2 domain on administration of mAb14C12 is likely to be remote, because of the use of such a fully humanized Ab and the fact that administration would be made by the intravenous route only. Besides, only a single or a small number of injections would be all that is required. Finally, the anti-idiotypic Ab variable part can be used as a template for the design of molecules, which would be even more adapted to the neutralization of inhibitors, such as multimeric versions of mAb14C12 VH region,\textsuperscript{27} or combination of V regions from different anti-idiotypic Abs.

The results presented here show that idiotypes recognized by mAb14C12 are expressed by a significant number of patients that have anti-C2 inhibitory Abs. This seemingly reflects the observation that the interaction between anti-C2 inhibitors similar to mAbBO2C11 and mAb14C12 depends on residues present in the Ab germ line sequence. C2 inhibitors are prominent in the production of anti-FVIII inhibitors: mAb14C12 or a derivative of it might, therefore, be helpful for a significant proportion of patients with inhibitor. However, current knowledge suggests that a second C2 epitope is recognized by inhibitor Abs,\textsuperscript{13} which would mean that full neutralization of C2 inhibitors would require another set of anti-idiotypic Abs. We are currently extending our approach to identify such a second epitope.

Currently, 5 main clusters of B-cell epitopes have been described, although the vast majority of antibodies bind to either the
C2 or the A2 domain. Efficient use of anti-idiotypic antibodies for patients with inhibitors would, therefore, require in addition to neutralize antibodies toward at least the main A2 domain. Whether anti-A2 antibodies derive from a small number of precursors as do anti-C2 antibodies is not fully understood. Obviously, this should be determined through clonal analysis of anti-A2 antibodies derived from patients’ memory cell repertoire. Whatever the case, it is likely that efficient neutralization of inhibitors would require a set of anti-idiotypic antibodies or of their derivatives. However, full neutralization might not be an absolute requirement, as a reduction of inhibitor titers to levels of 10 BU/mL or less is sufficient to render patients amenable to tolerance induction using infusions of FVIII. Another possible difficulty might emerge from the fact that inhibitor antibodies change specificity over time and could, therefore, “escape” control by anti-idiotypic antibodies. Specificity can change for a number of reasons, associated to the FVIII product used, the conditions under which FVIII is administered (bleeding, infection), but also as the result of an evolving immune response. The latter can be brought about by somatic hypermutation, receptor editing, or epitope spreading. The efficacy of anti-idiotypic neutralization of inhibitors will depend on the possibility of generating reagents interacting with inhibitors in germ line configuration, as it is suggested in the present study.

Potential therapeutic applications include the use of anti-idiotypic Abs for short-term neutralization of inhibitors, for instance before a surgical procedure. Such Abs have the potential of interacting with B cells carrying the corresponding idiotype. The consequence of this binding can be a functional neutralization of the cell producing the inhibitor or even the translation of a signal leading to target cell apoptosis. Besides, the heavy chain of the Ab can be engineered in such a way as to favor its interaction with complement or with the Fcy receptor present on natural killer (NK) cells. Therefore, it can be expected that anti-idiotypic Abs would induce a long-lasting elimination of inhibitor production.

References

14. van den Brink E, Bril W, Turenhout E, et al. Two classes of germline genes both derived from the VH1 family direct the formation of human anti-bodies that recognize distinct antigenic sites in the C2 domain of factor VIII. Blood. 2002;99:2828-2834.
22. d’Orión R, Lavergne J-M., Lavend H18528. From www.bloodjournal.org by guest on October 22, 2017. For personal use only.
In vivo neutralization of a C2 domain–specific human anti–Factor VIII inhibitor by an anti-idiotypic antibody

Jean Guy G. Gilles, Sabrina C. Grailly, Marc De Maeyer, Marc G. Jacquemin, Luc P. VanderElst and Jean-Marie R. Saint-Remy