Low Molecular Weight Peptides Restore the Procoagulant Activity of Factor VIII in the Presence of the Potent Inhibitor Antibody ESH8*

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Following repeated administration of factor VIII (FVIII), a significant number of hemophilia A patients develop antibodies (Abs), inhibiting the procoagulant activity of infused FVIII. We have designed an approach based on the blocking of the deleterious activity of these Abs by peptide decoys mimicking the anti-FVIII Ab epitopes. Here, the well characterized inhibitory monoclonal Ab ESH8 served as a model. Several phage peptide libraries were screened for specific binding to ESH8. Seven constrained dodecapeptide sequences were obtained. Six sequences carried the consensus motif, hydrophilic-(Y/F)GKTXL. This motif showed a certain similarity with the 2231-QVD-FQKTMKV2240 sequence of the C2 domain. In the seventh sequence, YCNPSIGDKNCR, the residues GDKN are similar to the sequence 2267-DGHQ2270. Upon inspection of the C2 domain crystallographic structure, the two stretches QVD-FQKTMKV and DGHQ appeared close together in space and might constitute a discontinuous epitope. Corresponding synthetic peptides were able to inhibit the binding of ESH8 to FVIII in a specific and dose-dependent manner. Moreover, the ability of the selected peptides to neutralize the inhibitory activity of ESH8 was demonstrated in functional tests as well as in vivo in a murine model of hemophilia A. This study demonstrates the potential of this approach to neutralize the activity of potent inhibitory Abs.

Coagulation defects attributed to the absence or dysfunction of blood coagulation factor VIII (FVIII)1 are observed in 0.01–0.02% of the male population (1). This recessive X-linked bleeding disorder is known as hemophilia A. The adequate treatment of this disease relies on infusions of human monoclonal Ab ESH8 served as a model. Several phage peptide libraries were screened for specific binding to ESH8. Seven constrained dodecapeptide sequences were obtained. Six sequences carried the consensus motif, hydrophilic-(Y/F)GKTXL. This motif showed a certain similarity with the 2231-QVD-FQKTMKV2240 sequence of the C2 domain. In the seventh sequence, YCNPSIGDKNCR, the residues GDKN are similar to the sequence 2267-DGHQ2270. Upon inspection of the C2 domain crystallographic structure, the two stretches QVD-FQKTMKV and DGHQ appeared close together in space and might constitute a discontinuous epitope. Corresponding synthetic peptides were able to inhibit the binding of ESH8 to FVIII in a specific and dose-dependent manner. Moreover, the ability of the selected peptides to neutralize the inhibitory activity of ESH8 was demonstrated in functional tests as well as in vivo in a murine model of hemophilia A. This study demonstrates the potential of this approach to neutralize the activity of potent inhibitory Abs.

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‡ The abbreviations used are: FVIII, factor VIII; Abs, antibodies; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; Fmoc, N-(9-fluorenyl)methoxycarbonyl; TG, thrombin generation.

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tial of the peptides is broad as mimotopes of linear, conformational, and even non-proteinaceous epitopes have been reported (14).

To evaluate the possibility of disrupting the interaction between the FVIII molecule and its inhibitors by peptide decoys to restore the procoagulant activity of FVIII, a model system was investigated, namely, the well characterized murine inhibitor mAb ESH8 (15). We used this mAb to screen four different libraries displaying foreign peptides at the surface of the major coat protein, pVIII. We present here evidence both in vitro and in vivo that mimotopes of the C2 domain can efficiently neutralize the inhibitory activity of mAb ESH8 and as such could represent an efficient method for the treatment of hemophilia A patients with inhibitory antibodies.

MATERIALS AND METHODS

Screening the Phage Library with mAb ESH8—The different M13 phage libraries used expressing 15-mer (XCV), 30-mer (XV), 17-mer including a fixed cysteine residue (XXCXX), and 12-mer peptides including two fixed cysteine residues (XXCXXCXX) were all described by Bonny et al. (16) and were obtained from Novabiochem, Inc. (San Jose, CA). MAb ESH8 was obtained from American Diagnostica Inc. (Greenwich, CT) and has been characterized previously (15). Pannings were performed as described by et al. (9). mAb ESH8 was coated onto a polystyrene Petri dish (10 × 1.5 cm, Falcon 1029) overnight at 4 °C at a concentration of 5 µg/ml for the first two pannings and a concentration of 0.5 µg/ml for the last panning in 100 mM NaHCO₃, pH 8.6, on a shaking platform. For the first panning, 5 × 10¹² transducing units of each library were incubated with the adsorbed mAb. After incubation, unbound phages were washed and bound phages were removed by acidic elution. Eluted phages were then used to infect Escherichia coli K92 cells. After three rounds of enrichment, individual phage clones were isolated and further analyzed (17).

Phage Binding Analysis by ELISA—ELISA microtiter plates were coated with 0.5 µg/ml ESH8 or control mAbs in 100 mM NaHCO₃, pH 8.6, overnight at 4 °C. Plates were washed with PBS, 0.1% Tween 20 (v/v) and then blocked with PBS, 0.1% Tween 20, 2% nonfat dried milk (w/v) for 30 min at 37 °C. The mixture of ESH8 and synthetic peptide was incubated 2 h at 37 °C. mAb binding was revealed by adding a peroxidase-conjugated anti-mouse IgG antibody (Sigma) diluted 1:3000 for 30 min at 37 °C. The resulting absorbance was measured at 490 nm after the addition of 50 µl of 4-N-EtSOF₄. The reference absorbance was obtained with the adsorbed ESH8 alone.

Modified Bethesda Assay—This assay was based on the Bethesda assay (21) with the Nijmegen modification. ESH8 at a fixed concentration giving 50% FVIII activity inhibition was incubated overnight at 4 °C with the different peptides at various concentrations diluted with 0.05 M imidazole buffer, pH 7.3. Equal volumes (250 µl) of this sample and normal plasma buffered with 0.1 mM imidazole, pH 7.4, were mixed together and incubated for 2 h at 37 °C. One-stage FVIII potency assays were then carried out. The inhibitory activity was read in Bethesda unit/ml from a semilogarithmic plot representing the correlation between residual FVIII activity (logarithmic) and inhibitor activity (linear).

A minimum of three dilutions was made for each condition.

Thrombin Generation Test—This assay is a modification of that carried out by Pitney and Docie (22). Normal platelet-poor plasma was used as substrate, 375 µl of this normal platelet-poor plasma substrate was added to a 100-µl preincubated solution of ESH8 (175 nM) mixed with either Tris borate saline buffer (control) or with a 1000 molar excess of the different peptides. This mixture was incubated at 37 °C for 20 min prior to the addition of 80 µl of FIXa (14 nM) and further incubated for 1.5 min at 37 °C. To this mixture, 400 µl of phospholipid (3.1 µg/ml) and 400 µl of CaCl₂ (7.5 mM) were then added to start the reaction. At different time intervals, 50-µl aliquots were then removed and placed in 200 µl of fibrinogen in cups on a Deca coagulometer (Diagnostic Grifols S.A., Barcelona, Spain). The clotting times were then converted into thrombin units by use of a α-thrombin standard curve. The peak thrombin level and the time taken to reach half-maximal (1/2max) coagulation were deduced from the thrombin generation curves.

Peptide Stability—The peptides were incubated for 0, 1, 2, 3, 4, 5, and 24 h at 37 °C at 300 µM in cell culture medium containing 10% fetal calf serum. Following incubation, the samples (150 µl) were centrifuged for 8 min at 11,000 rpm through an ultrafiltration membrane (10 kDa, Amicon Microcon) to remove serum proteins. 100 µl aliquots of the filtrate were then analyzed by reverse phase-high pressure liquid chromatography and quantified.

In Vivo Experiments—The FVIII knock-out mice used here were as described previously (23–25). All of the mice were used in groups of three individuals. 0.5 IU of human FVIII (Kogenate, Bayer Inc., Berkeley, CA) diluted to 100 µl in physiological medium was injected in the tail vein. A blood sample obtained by cardiac puncture after 15 min was taken, and the concentration of FVIII was evaluated in a chromogenic assay (Dade Behring). A group of mice was injected in the tail with 0.25 µg of ESH8 followed 30 min later by an injection of 0.5 IU of FVIII. Preliminary experiments had demonstrated that this amount of ESH8 was sufficient to inhibit 80% FVIII activity. Lastly, ESH8 was preincubated overnight at 4 °C with 1 mg of peptide 46 or an irrelevant peptide before injection in the tail vein, and the residual FVIII activity was determined as described above.

RESULTS

Selection of Phages Displaying Peptides Recognized by mAb ESH8—To identify peptide binding to mAb ESH8, we screened four different phage libraries, two of which expressed linear indolylphosphate, 3-(4,5-dimethoxyphenyl)-2-yl)-2,5-diphenyltetrazolium bromide (Sigma), which gives a blue precipitate on peptide spots recognized by ESH8. A plot of spot intensities was obtained with the Scion Image software after scanning the membrane. The membrane was further treated to remove precipitated dye and bound antibodies and was visualized (19).

Synthesis of Soluble Peptides—All of the soluble peptides were synthesized on an Abimed AMS42 synthesizer by Fmoc chemistry (20). Peptides were deprotected and released from the resin by trifluoroacetic acid treatment in the presence of appropriate scavengers. Peptides were lyophilized, and their purity assessed by reverse phase high pressure liquid chromatography and mass spectrometry. When necessary, peptides were purified by high pressure liquid chromatography to reach a purity greater than 90%.

Competitive ELISA Assay with Synthetic Peptides Derived from Phages—ESH8 (0.05 µg/ml) was preincubated in PBS-0.1% Tween 20, 2% nonfat dried milk (w/v) with synthetic peptides at increasing concentrations overnight at 4 °C. ELISA plates were coated with 1 µg/ml recombinant FVIII overnight at 4 °C in PBS. Plates were washed and blocked for 1 h at 37 °C. The mixture of ESH8 and synthetic peptide was incubated 2 h at 37 °C. mAb binding was revealed by adding a peroxidase-conjugated anti-mouse IgG antibody (Sigma) diluted 1:3000 for 1 h at 37 °C. The resulting absorbance was measured at 490 nm after the addition of 50 µl of 4-N-EtSOF₄. The reference absorbance was obtained with the adsorbed ESH8 alone.

Restoration of FVIII Procoagulant Activity with Peptides

Thrombin Generation Test

Peptide Stability

In Vivo Experiments

RESULTS

Selection of Phages Displaying Peptides Recognized by mAb ESH8
peptides of either 15 (Xn) or 30 amino acids (Xn), whereas the other two phage libraries displayed peptides including either one or two fixed cysteines and whose sizes were 17 (CXn) or 12 amino acids (CXnC). These random peptides are fused to the pVIII coat protein of filamentous phage. A significant enrichment for phage binding to the target Ab was obtained after three rounds of panning on the immobilized mAb ESH8. Three hundred phage clones were randomly picked from the third round of selection and were checked by ELISA for their ability to bind mAb ESH8. The specificity of the signal was tested by evaluating their reactivity on mAb ESH4 (15), another anti-C2 domain mAb, and 11E12 (26). Thirty positive clones giving ELISA signals at least four times higher than background were further characterized. All of them bound to mAb ESH8 in a dose-dependent manner and in a specific way, because none of them cross-reacted with each of the control mAbs. The results obtained with the most reactive phage clone are shown in Fig. 1A. This phage was further used in a competition experiment with FVIII (Fig. 1B). FVIII competed with the phage for mAb ESH8 binding in a dose-dependent manner with a IC50 of 4 μg/ml at 10^11 phages/ml. Reciprocally, the clone was able to inhibit FVIII binding to mAb ESH8. However, because of limitation in the available quantity of phages, no complete inhibition curve could be obtained (data not shown). Taken together, these results show that phage peptides specifically recognizing mAb ESH8 have been obtained.

**Sequence Determination and Comparison** —The phage DNA coding for foreign peptide was sequenced. The deduced peptide sequences are shown in Table I. A sequence analysis revealed striking features. Despite exhaustive screening of four phage peptide libraries, all of the selected peptides originated only from the dodecapeptide cysteine-constrained library (CXnC), suggesting that both the primary sequence and the conformational context in which the sequence is presented are critical for binding. From the thirty selected clones, only seven distinct sequences were obtained with six peptides sharing the consensus motif GKTX. These four invariant amino acids suggested that they might be important for binding either by providing residues contacting mAb ESH8 or by participating in the appropriate peptide folding. A closer inspection of the six related sequences revealed that the second position in the loop was exclusively occupied by a hydrophobic residue, a valine (3 times), a threonine (2 times), or a leucine (1 time). The third position in the loop corresponded to two aromatic residues, tyrosine or phenylalanine. Consequently, the final consensus motif could be the following: XCXV/L/I/Y/P)GKTXLCX with X being any amino acids. The seventh sequence (YCNPSIGD-KNCR, peptide 73) is totally distinct from the others. To assess whether the selected peptides could still be recognized by mAb
ESH8 out of phage context, they were synthesized on a cellulose membrane by the Spot technique. All of these sequences were recognized by mAb ESH8, suggesting that the binding activity was an intrinsic property of the peptide (Fig. 2A). In this format, the unique sequence, YCNPSIGDKNCR (peptide 73), as well as the sequences ECIVYGKTALCT (peptide 46) and QCQTFGKTMLCT (peptide 47) were the most reactive.

Localization of Mimotopes within the C2 Domain Sequence—To identify peptide residues critical for binding to mAb ESH8, a mutational analysis based on the Spot technique was performed. A set of peptides was generated in which each residue of the lead sequence was replaced in turn by each one of the other nineteen naturally occurring amino acids. Fig. 2B shows the results on the analysis conducted on peptide 46, ECIVYGKTALCT. The four conserved residues, Gly, Lys, Thr, and Leu, of the consensus motif appeared important functionally and/or structurally as any substitution totally abrogated the binding. Similarly, the substitution of the two cysteines by two alanines or serines resulted in a complete loss of reactivity, underlying the possible importance of the disulfide bridge for binding. With regards to the second position, only the amino acid with hydrophobic properties (Ile, Leu, Val, Met, Trp, Tyr, and Thr) was tolerated, and a phenyl functionality at the third position was crucial because no substitution even by tryptophan was allowed. The same reactivity profile was obtained with the five other related sequences (data not shown). However, the same study conducted on the sequence YCNPSIGDKNCR (peptide 73) showed that the critical amino acids were isoleucine, glycine, aspartic acid, lysine, and the last aspartic acid residue as well as the two cysteines (data not shown). The sequence alignment between the C2 domain of FVIII and the consensus motif indicated a putative three residue homology (2234FKT2237) in the sequence QVDFQKTMKV (residues 2231–2240) of the C2 domain (Table I). By inspecting the three-dimensional structure of the C2 domain, these three residues appeared to belong to an exposed loop, which faces residues 2267DGHQ2270 (Fig. 3A). On the other hand, although peptide 73 presented no apparent homology with the FVIII sequence, we observed possible similarities between its sequence, YCNPSIGDKNCR, and the sequence 2267DGHQ2270 of the C2 domain. The aspartic acid and the glycine residues were present in both of them. His2269 can be replaced by conservative substitution with lysine and Gln2270 with asparagine. Therefore, it is possible that peptide 73 functionally mimicked the region 2267DGHQ2270 of FVIII, a region that is close to residues 2234FKT2237 in the three-dimensional structure of the C2 domain (Fig. 3B). We postulate that the mAb ESH8 epitope is

<table>
<thead>
<tr>
<th>Prevalence</th>
<th>Sequence related FVIII</th>
<th>Peptide*</th>
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<tbody>
<tr>
<td>11</td>
<td>SCTXVGKTPLCG</td>
<td>45</td>
</tr>
<tr>
<td>1</td>
<td>RCXTVGKTPLCL</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>ECIVYGKTALCT</td>
<td>46</td>
</tr>
<tr>
<td>8</td>
<td>QCQTFGKTMLCT</td>
<td>47</td>
</tr>
<tr>
<td>1</td>
<td>RCKTFGKTTLCS</td>
<td>48</td>
</tr>
<tr>
<td>1</td>
<td>SCRLFGKTYLCH</td>
<td>72</td>
</tr>
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**FVIII homology**

\[ \text{Sequence unrelated to FVIII} \]

| 3         | YCNPSIGDKNCR |

* Number of identical sequences found.
Inhibition of the Interaction between mAb ESH8 and FVIII in Vitro and in Functional Assays—The seven sequences were chemically synthesized in a soluble form and tested for their ability to inhibit the interaction of mAb ESH8 with FVIII in ELISA. To this end, the binding of mAb ESH8 to FVIII was examined in the presence of increasing amounts of specific or unrelated peptides. As shown in Fig. 4A, the different peptides derived from the phage display selection efficiently inhibited the binding of mAb ESH8 to FVIII in a dose-dependent manner, which was not the case for an irrelevant peptide. At a concentration of ~10 μM, all of the six peptides were able to inhibit 50% mAb ESH8 binding, and complete inhibition was obtained at a concentration higher than 100 μM. Therefore, these results suggest that the different peptides derived from the phage display selection mimicked the C2 domain for its binding properties to mAb ESH8.

The capacity of peptides to neutralize the inhibitory activity of mAb ESH8 was measured in two different functional tests. First, in a modified Bethesda assay (21) in which mAb ESH8 was used at a concentration resulting in 50% inhibition of FVIII cofactor activity, the peptides were all able to neutralize mAb ESH8 inhibitory activity in a dose-dependent manner with complete neutralization achieved by peptides 46 and 47 when used in a 10⁶ molar excess, corresponding to a final concentration of 200 μM, over mAb ESH8 (Fig. 4B). The neutralization was specific, because an irrelevant peptide had no measurable activity in the same range of concentrations (Fig. 4B). The peptides by themselves had a minimal effect in the assay (data not shown). Interestingly, when the test was repeated with a concentration of mAb ESH8 giving 70% inhibition of FVIII activity, complete neutralization was still reached by using a 10⁶ molar excess of peptides 46 and 47.

These peptide properties were confirmed in a second functional assay based on thrombin generation (TG). Fig. 5 shows the TG profiles of normal platelet-poor plasma in the absence and presence of mAb ESH8. A lag phase was observed in the presence of mAb ESH8, although the peak thrombin produced remained very similar to the profile obtained in absence of mAb ESH8. The inhibition of FVIII by mAb ESH8 gave a t1/2max value of 220 s compared with the t1/2max value of 116 s for normal plasma alone (Fig. 5). When this test was repeated with mAb ESH8 that had been preincubated with the various peptides, the inhibition of the TG profile was corrected by all of the peptides with the exception of the control peptide, which showed only a slight correction of the lag phase. This finding suggests that neutralization of the inhibitory activity of mAb ESH8 by peptides 45, 46, 47, and 48 is indeed of physiological relevance.

Efficiency of the Peptide in Vivo—Based on these in vitro experiments, a potential application of such peptides for neutralizing FVIII inhibitor Abs in vivo was considered. We first assessed the stability of peptides 46 and 73 upon incubation in 10% fetal calf serum at 37 °C for various lengths of time using high pressure liquid chromatography. No degradation was observed for either peptide after 2 h of incubation, in contrast with the fate of a linear peptide that was already 50% degraded (data not shown). Over a longer period of time (24 h), peptide 73 was shown to be particularly resistant with only 50% degradation, whereas 84% peptide 46 was degraded. Under the same experimental conditions, the linear peptide was fully degraded.

These results encouraged us to examine whether or not such peptides could neutralize the FVIII inhibitory activity of mAb ESH8 in an in vivo model. We used FVIII-deficient mice obtained by targeted disruption of exon 16 of the FVIII gene (23, 27).
An injection of 0.5 IU of human FVIII in the tail vein resulted in a FVIII level of 0.3 IU/ml after 10 min as measured by a chromogenic assay. 2 groups of 3 mice were pretreated with either 0.25 μg of mAb ESH8 or a mixture of 0.25 μg of mAb ESH8 with 1 mg of peptide 46 or control peptide preincubated overnight at 4 °C (this represents a 2 x 10² molar excess of the peptide). Thirty minutes later, all of the mice were reconstituted with 0.5 IU of human FVIII. From Fig. 6, it can be seen that pretreatment with mAb ESH8 alone reduces FVIII activity by 88%, whereas mice treated with the mAb ESH8-peptide mixture had only a 58% reduction, indicating that peptide 46 exerted some neutralizing activity on mAb ESH8-mediated FVIII inhibition. Such neutralization was specific for peptide 46, because a control peptide was unable to restore FVIII activity.

**DISCUSSION**

A significant proportion of hemophilia A patients develops inhibitor Abs following repeated FVIII administration (28). Such Abs inhibit the procoagulant activity of FVIII and present a serious clinical complication in that they preclude FVIII replacement therapy. Several treatments have been attempted to overcome this problem, but these are therapeutically or economically not suitable, emphasizing the need to evaluate new approaches. One such attempt could be to prevent FVIII binding to the inhibitor Abs using epitope-mimicking peptides. By combining to the variable parts of these Abs, such peptides would neutralize the inhibitory capacity of anti-FVIII Abs. In an attempt to evaluate the feasibility of this approach, we chose an anti-C2 antibody, mAb ESH8, which was used to identify peptides that were able to divert mAb ESH8 from FVIII. The choice for mAb ESH8 was motivated by several criteria. First, this mAb is well characterized (15, 29, 30) and is used as a reference inhibitor Ab by a number of research teams. Second, its epitope is mapped to residues 2248–2285 on the C2 domain of FVIII, a domain frequently recognized by human anti-FVIII Abs (31). Third, it is an effective high titer inhibitor Ab (6300 Bethesda unit/mg) (15). Finally, as monoclonal and human Abs that map to C2 epitope 2218–2307, its inhibition mechanism is based on the slow release of thrombin-cleaved FVIII from von Willebrand factor, which reduces FVIII binding to phospholipid-
The peptides binding to mAb ESH8 were selected by the phage display technology, a method allowing the identification of peptides with significant mimicry potential for a broad spectrum of applications (14). We have identified from a mixture of several library peptides, two that are able to functionally mimic mAb ESH8 epitope. By combining the information obtained from the consensus motifs of the two sequences with that derived from the three-dimensional structure of the C2 domain (27), we were able to identify two distinct regions of the C2 domain likely to be involved in the interaction with mAb ESH8. The two ligand peptides are postulated to mimic non-consecutive solvent-exposed stretches brought together in the three-dimensional structure of the C2 domain and constituting a discontinuous epitope for mAb ESH8. One of the two components (DGHQ270) belongs to a region that had already been described by Scandella et al. (15). They performed an epitope mapping using truncated forms of recombinant C2 domain and identified a region encompassing residues 2248–2285 as the putative mAb ESH8 epitope. The second epitopic component (residues belonging to the fragment 2227–2241) was identified in this study. mAb ESH8 was found to have some affinity for each individual peptide sequence. The two unrelated and separated peptides were each able to block in an efficient and specific manner the in vitro binding of mAb ESH8 to FVIII. The simultaneous addition of the two peptides improved the inhibition (data not shown). An inspection of the crystallographic structure of the C2 domain (27) indicates that the two parts of the epitope are in fact spatially close together (7.3 Å) and may form a coherent epitope. Our data indicate that the two contributing parts are equally involved in mAb ESH8 recognition. Scandella et al. (15) carried out the analysis using the N-terminal truncated C2 domain in which the second antigenic part could not be identified. Therefore, our results do not stand in contradiction with this previous report. Consistent with these epitope-mimicking properties, the peptides identified here also exert functional properties in vitro. In the Bethesda test, the peptides completely neutralized the inhibitory activity of mAb ESH8, thereby restoring full FVIII cofactor activity. This neutralizing effect was also observed in the inhibition of the thrombin generation test in which peptides representative of the two epitopes were able to neutralize the mAb ESH8-dependent inhibition of thrombin generation. In both cases, 200 μM of peptides were required to completely neutralize the inhibitory activity of mAb ESH8. This is probably related to the fact that each peptide represents only a part of the epitope to which mAb ESH8 binds. This is in line with the findings of Reinke et al. (32) in a study on the reconstruction of a discontinuous binding site on interleukin 10. These authors (32) demonstrate that each of the peptides mimicking either one of the two parts of the binding site was only slightly efficient when used alone. However, combining these peptides representative of the two binding sites by a linker molecule resulted in a biological activity within the nanomolar range. The capacity to prevent the binding of antibodies to FVIII by specific epitope-like peptides has also been shown with another anti-FVIII mAb, mAb F7b4.2 In such a case, full neutralization of the FVIII inhibitory activity was obtained with only a 50-fold molar excess (14 μM). Whether or not the combination of peptides would be required for improved neutralization, our data already demonstrate the feasibility of the approach, because peptides can efficiently neutralize in vitro the inhibitory activity of an anti-FVIII even when they mimic only part of the epitope.

An in vivo experiment using single peptides was carried out to establish the basis of a possible therapeutic application. Because mAb ESH8 does not inhibit the cofactor activity of mouse FVIII,3 we used the model of hemophilia A mice reconstituted by human recombinant FVIII (25). The inhibition of FVIII as measured in a chromogenic assay was compared using either ESH8 alone or in a combination with one of the peptide. A significant yet limited neutralization of the inhibitory capacity of ESH8 was observed. The efficacy of a peptide in vivo depends on a number of parameters and, in particular, on its stability. For example, it is commonly accepted that L-peptides are rapidly degraded in serum (33). Preliminary data had shown that peptide 46 exhibited an unusual resistance to protease degradation upon incubation in serum, which was deemed to be dependent on the presence of a disulfide bridge between Cys-2 and Cys-11, and to the derivatization of both peptide ends, acetylation of the N-terminal end, and amidation of the C-terminal end (34). These characteristics prompted us to use the peptide as such with no attempt to further increase its stability. However, this could be achieved by the replacement of L-amino acids by D-derivatives or by the addition of unnatural ones and/or modification of the peptide bond itself. In addition, the dosing regimens used for the in vivo bioassays were based on an extrapolation from in vitro results, which might not be optimal for animal administration.

The efficiency of a molecular decoy approach similar to the one described in this paper has already been studied in two models of human pathology, diabetes mellitus (35) and myasthenia gravis (36). In both cases, short RNA sequences were selected from a large random RNA library on the basis of their ability to specifically bind to a mAb representative of autoantibodies. The authors (35, 36) demonstrated that the selected decoys were able to inhibit the binding of a few human sera to their target antigen, insulin, and acetylcholine receptor, respectively. However, the efficacy of such RNA decoys had not yet been validated in an animal model. Provided that technical improvements would increase peptide stability and capacity to neutralize inhibitory Abs, a number of clinical applications can already be envisioned. Moderate and mild hemophilia A patients with inhibitor could be more prone to benefit from peptide-based therapy insofar, because the anti-FVIII immune response of such patients appears to involve only a limited number of B cell clones, the specificity of which is directed essentially against the epitope corresponding to the region containing the FVIII mutation. In severe hemophilia A cases, although the immune response is usually more heterogeneous, the B cell epitopes cluster to only a limited number of regions, i.e., residues Arg404-Ile508 on the A2 domain (37), residues Glu2181-Val2243 and His2315-Asp2332 on the C2 domain (30, 38), and finally residues Gln1772-Met1923 (39). The present approach will now be repeated using Abs of human origin of both monoclonal and polyclonal origins. The optimal combination of peptides will be determined to obtain a "universal" decoy or a mixture of decoys that is able to inhibit a majority of anti-FVIII Abs. If full neutralization of inhibitor Abs cannot be obtained, we can expect that it will significantly help reduce significantly the antibody titers of the patients.

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