Covalent Inactivation of Factor VIII Antibodies from Hemophilia A Patients by an Electrophilic Factor VIII Analog

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The antigen-binding sites of antibodies (Abs) can express enzyme-like nucleophiles that react covalently with electrophilic compounds. We examined the irreversible and specific inactivation of antibodies (Abs) to Factor VIII (FVIII) responsible for failure of FVIII replacement therapy in hemophilia A (HA) patients. Electrophilic analogs of FVIII (E-FVIII) and its C2 domain (E-C2) were prepared by placing the strongly electrophilic phosphonate groups at surface-exposed Lys side chains of diverse antigenic epitopes. IgG Abs to FVIII from HA patients formed stable immune complexes with E-FVIII and E-C2 that were refractory to dissociation by SDS treatment and boiling, procedures that dissociate noncovalent Ab-antigen complexes. The rate-limiting step in the reaction was formation of the initial noncovalent complexes. Conversion of the initial complexes to the irreversible state occurred rapidly. The antigenic epitopes of E-FVIII were largely intact, and most of the Abs were consumed covalently. E-FVIII expressed poor FVIII cofactor activity in clotting factor assays. Nonspecific interference by E-FVIII in clotting factor function was not evident. Treatment with E-FVIII, and to a lesser extent E-C2, irreversibly relieved the FVIII inhibitory effect of HA IgG in clotting factor assays. Small FVIII peptides did not display useful reactivity, highlighting the diverse epitope specificities of the Abs and the conformational character of FVIII epitopes. E-FVIII is a prototype reagent able to attain irreversible and specific inactivation of pathogenic Abs.

Specific antibodies (Abs) to individual antigens are thought to cause harmful effects in autoimmune diseases, transfusion of incompatible blood products, and organ transplantation. Inhibitory Abs to Factor VIII (FVIII) in hemophilia A (HA) are a well characterized example. HA is a chromosome X-linked genetic disorder characterized by the synthesis of functionally inactive FVIII. This impairs the intrinsic pathway of blood coagulation. The primary therapy for control of bleeding in HA patients is infusion of recombinant or plasma-derived FVIII (1). About 20–30% of patients receiving FVIII replacement therapy produce antibodies (Abs) to FVIII that inhibit FVIII cofactor activity. These are referred to clinically as “inhibitors.” The inhibitory effect is thought to derive from reversible steric hindrance of FVIII interactions with phospholipids and other coagulation factors, including thrombin, Factor IXa (FIXa), and von Willebrand factor (2). In addition, some Abs inactivate FVIII permanently by catalyzing its proteolytic breakdown (3). Epitope mapping studies using FVIII fragments (heavy chain, light chains, and A2, A3, C1, and C2 domains) and FVIII hybrid molecules have suggested that many Abs are directed to conformational epitopes (4, 5). Most inhibitor positive patients mount a highly diverse immune response consisting of Abs to multiple FVIII epitopes located in the A2, C1, C2, and A3 domains (2, 6, 7).

The Abs pose major problems in managing acute bleeding episodes and surgical procedures in the patients. Short term bleeding in inhibitor-positive patients can be controlled by infusing activated prothrombin complex concentrates or recombinant factor VIIa, agents that bypass the requirement for FVIII in the coagulation pathway (8, 9). Refractory bleeds occur in about 20% of inhibitor-positive HA patients receiving bypass therapy, and an overdose carries the risk of inducing thrombotic events (9). In principle, FVIII itself could be infused to saturate the Abs and restore the coagulation pathway. However, massive quantities of FVIII are required to overcome the inhibitory effect of the circulating Abs even for a short duration. An important clinical advance has been the development of immune tolerance protocols in which high dose FVIII infusions are administered over prolonged periods to suppress Ab production by memory B lymphocytes (10, 11). Experimental peptides (5, 12) and anti-idiotypic Abs (13) have been reported to block FVIII inhibitory Abs by mimicking the structure of certain FVIII epitopes. Regrettably, there is no single immu-
nondominant FVIII epitope, and these approaches do not adequately address the problem of diverse epitope reactivities of the Abs.

The combining sites of certain Abs contain enzyme-like activated nucleophiles. The Ab nucleophilic reactivities were evident from formation of covalent complexes with electrophilic phosphate diesters (14–16), compounds that were originally developed as class-specific inhibitors of serine proteases (17). The phosphonates react with activated nucleophiles generated by intramolecular interactions between certain amino acids. For instance, the Ser side chain acquires enhanced nucleophilicity by virtue of the hydrogen-bonded network in the Ser–His–Asp catalytic triads of serine proteases (18). The nucleophilic sites permit certain Abs to catalyze the hydrolysis of their cognate antigens (19). Ser–His–Asp and Ser–Arg–Glu catalytic triads have been identified in proteolytic Abs by site-directed mutagenesis (20) and crystallography studies (21). Nucleophilic catalytic Abs that hydrolyze FVIII and inhibit FVIII cofactor activity are found in HA patients (3). However, only a subset of nucleophilic Abs displays catalytic activity (14), indicating that additional events in the catalytic cycle occurring after the initial nucleophilic attack on the peptide bond carbonyl group can be rate-limiting (e.g., water attack and product release).

We hypothesize that electrophilic FVIII (E-FVIII) analogs may relieve the anti-coagulant effect of Abs by reacting specifically and covalently with their nucleophilic sites. The covalent reaction is predicted to preclude dissociation of the immune complexes, thereby permitting prolonged Ab inactivation. We describe here E-FVIII analogs that relieve the FVIII inhibitory effect of Abs from patients with HA. E-FVIII is a prototypic therapeutic reagent for control of bleeding in inhibitor-positive HA patients. The observed properties of E-FVIII suggest that electrophilic antagonism can potentially be developed as a general basis for attaining specific inactivation of various antigen-specific pathogenic Abs found in immunological diseases.

**EXPERIMENTAL PROCEDURES**

**Electrophilic FVIII Analog**—Recombinant FVIII (Helixate, CSL Behring) in 10 mM HEPES, 150 mM NaCl, 0.025% Tween 20 was derivatized at Lys residues with the 3-sulfosuccinimidyl ester of diphenyl N-suberoyl-amino(4-aminophenyl)methanephosphonate, and unincorporated phosphate was removed by gel filtration (16). The phosphate content of three E-FVIII preparations employed in this study was 52–76 mol of phosphate/mol of FVIII determined by fluorescamine labeling of the residual amine groups (16). To prepare biotinylated E-proteins, biotin was first introduced into recombinant FVIII and C2 protein (22) by partial acylation in 10 mM HEPES, 150 mM NaCl, 0.1 mM CHAPS (14). This yielded FVIII and C2, respectively, containing 8.8 and 0.6 mol of biotin/mol of protein. Phosphonate labeling of biotinylated FVIII was as above (81 mol of phosphate/mol of protein). Fast protein liquid chromatography-gel filtration of E-FVIII was on a Superox-6 column at a flow rate of 0.3 ml/min as described (23). Initial attempts to prepare E-C2 as described above for E-FVIII resulted in precipitation of the protein (>90%). Therefore, a phosphonate reagent with a more hydrophilic linker was prepared by treating diphenyl amino(4-aminophenyl)methane-phosphonate with di(N-succinimidyl)ethylene glycol disuccinate (Sigma), followed by reversed phase HPLC purification (observed m/z 722.5 (MH+5) and calculated MH+ 722.2). Treatment of biotinylated C2 with this reagent yielded E-C2 preparations containing 6.7–8.2 mol of phosphate/mol of C2. The E-VIP preparation has been described (15). The following peptides were prepared by Fmoc (N-(9-fluorenyl)methoxycarbonyl)-based solid phase synthesis: FVIII residues 484–508 with a Cys residue at the N terminus (N-acetyl-CPRLYSRRLPKGVKHLDPIFLPGEL); and the mimotope of a C2 epitope recognized by monoclonal Ab BO2C11 (SCHAWSNRRTCR) (12). The peptides were purified by HPLC (>95% purity) and characterized by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) or electrospray ionization (ESI) mass spectroscopy (FVIII-(484–508), m/z (MALDI) 3076.0 (MH+; calculated MH+ 3075.7); FVIII-(2303–2332), m/z (ESI) 1356.2 (MH+3, calculated MH+3 1356.4), 1018.0 (MH+4, 1017.5), and 814.5 (MH+5, 814.2); FVIII-(1804–1819), m/z (ESI) 677.4 (MH+3, calculated MH+3 677.4), and 508.5 (MH+4, 508.3); BO2C11 epitope: m/z (MALDI) 1474.8 (MH+; calculated MH+ 1474.7)). E-(2303–2332) was prepared by regiospecific acylation (15) as follows. The resin with the 2303–2332 peptide protected at Lys-2320 side chain with the 4-methyltrifluoroacetic acid in dichloromethane to remove this protecting group, and the Lys-2320 side chain amine was acylated with the N-hydroxysuccinimidyl ester of the phosphate reagent used for E-FVIII preparation. Protecting groups and the solid support were removed with trifluoroacetic acid containing 2% phenol, 5% thioanisole, and 5% ethanedithiol, and E-(2303–2332) was purified by HPLC (m/z (ESI) 1529.5 (MH+3, calculated MH+3 1529.5), 1147.3 (MH+3, 1147.4), 917.6 (MH+3, 918.1)). The electrophilic analogs were stored at −80 °C as lyophilized powders. Protein concentrations were measured by the bicinchoninic acid assay.

**Patients and Antibodies**—This study was approved by the University of Texas Institutional Review Board. Plasma was prepared from blood in 3.2% sodium citrate from 8 inhibitor-positive HA patients (our lab codes: HA1828, HA1834, HA1835, HA2084, HA2085, HA2222, HA2223, and HA3112; age range 5–59 years). The patients had a history of FVIII inhibitors for at least 5 years but had not received FVIII replacement therapy for at least 2 months prior to the blood draw. The plasma FVIII titers were determined by Bethesda assay (24) and are reported in Table 1. Control plasma was from a nonhemophilic human subject without known coagulation or autoimmune disorders (code NH1941). Electrophoretically homogeneous IgG from the plasma samples was purified by affinity chromatography using immobilized protein G (25). Fab fragments were prepared by digestion with immobilized papain and chromatography using immobilized protein A (26). Murine monoclonal anti-C2 IgG (clone ESH8) was from American Diagnostica. The isotype-matched control was monoclonal anti-VIP IgG (clone c23.5) (27).

**Hapten Phosphonate Binding**—Synthesis of hapten electrophilic probe E-hapten-1, E-hapten-2, and E-hapten-3 was
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described (14, 23, 28). FVIII (0.5 μM) was treated with the E-hapten probes (100 μM) for 4 h, and formation of irreversible adducts was measured by reducing SDS-electrophoresis as described (14).

E-FVIII and FVIII Binding—Abs were incubated with electrophilic or control polypeptides devoid of electrophilic groups in 10 mM HEPES, pH 7.5, 150 mM NaCl, 0.025% Tween 20 (HEPES/Tween) at 37 °C. The reaction mixtures were boiled (5 min) in 2% SDS and 3.3% 2-mercaptoethanol and subjected to SDS-electrophoresis. Adducts were detected and quantified in gel blots with peroxidase-conjugated streptavidin or goat anti-human IgG (Fc and κ/λ chain-specific, 1:1000; Sigma (16)). ELISAs were done using microtiter plates (Nunc) coated with peptides (4 μg/ml) in 100 mM NaHCO₃, pH 9.5, and blocked with 5% skimmed milk in 10 mM sodium phosphate, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween 20 (PBS/Tween). Ab binding was measured using peroxidase-conjugated goat anti-human IgG as above or goat anti-human Fab (Sigma) followed by peroxidase-conjugated rabbit anti-goat IgG (Pierce) (16). Binding to biotinylated E-(2303–2332) was measured similarly using streptavidin-coated plates (1 μg/ml). To measure irreversible binding, Abs were permitted to bind the immobilized antigens; the fluid was removed, and the wells were incubated for 30 min in PBS/Tween without (total binding) or with 2% SDS (SDS-refractory binding), followed by washing with PBS/Tween (16). Percent residual binding in SDS-treated wells was (A₄₉₀/SDS-treated wells) × 100/(A₄₉₀ PBS/Tween-treated wells). Samples displaying A₄₉₀ > mean ± 3 S.D. for control IgG (from subject NH1941) were considered positive. Binding rate data were fitted to the equation A₄₉₀/A₄₉₀,max = 1 - exp(-Kt), where K is the pseudo-first order rate constant (K = (kₒ × [Ab])/Kd); kₒ is the first order rate constant for covalent bonding; Kd is the equilibrium dissociation constant for noncovalent binding step; [Ab] is the Ab concentration. t½ was computed as ln2/K. In the immunoadsorption experiment, HA IgG (0.7 μM) was incubated in diluent or biotinylated E-FVIII (0.1 μM) for 20 h, and the reaction mixture (0.04 ml) was incubated for 1 h with immobilized streptavidin in spin columns (30 μl of settled gel, UltraLink Plus columns, Pierce). The unbound fraction and three washes (0.05 ml each) were pooled and assayed for binding to FVIII and E-FVIII by ELISA.

Clotting Factor Assays—FVIII and E-FVIII cofactor activity was determined using the Diapharma Coamatic FVIII kit® as instructed by the manufacturer using 0.045-ml solutions of the proteins in HEPES/Tween. The method measures the ability of FVIIIa generated by thrombin cleavage to form the FIXa tenase complex responsible for FXa generation, which in turn hydrolyzes the chromogenic substrate N-acetyl-D-Arg-Gly-Arg-p-nitroanilide. To measure FVIII inhibitor activity of Abs, IgG preparations from patients HA1828 (0.2 mg/ml), HA2222 (0.1 mg/ml), and HA3112 (0.1 mg/ml) were incubated with diluent or the electrophilic FVIII analogs in HEPES/Tween (0.1 ml; 20 h, 37 °C). Unbound electrophilic analogs in the reaction mixtures (0.1 ml) were removed by chromatography on protein G-Sepharose (0.04 ml of settled gel packed in Micro Biospin columns, Bio-Rad). The columns were washed with 5 ml of 50 mM Tris-HCl, pH 7.4, 0.1 mM CHAPS. Bound IgG was eluted with 0.1 M glycine, pH 2.7, 0.1 mM CHAPS (0.2 ml) in tubes containing 0.01 ml of 1 M Tris base, pH 9. The FVIII inhibitory activity of eluates was determined using the Coamatic assay or the one-stage activated partial thromboplastin time (APTT) clotting assay using APPT-SP reagent (29) (Instrumentation Laboratory) and an ACL300 plus coagulometer (Instrumentation Laboratory) according to the manufacturer’s instructions. The standard curve was constructed from the clotting times of reference FVIII-containing plasma diluted in FVIII-depleted plasma (both from George King Bio-Medical, Inc.). Prior to the chromogenic FVIII inhibitor assay, FVIII (0.2 IU/ml, 0.025 ml) was incubated with the IgG eluates from protein G columns (0.025 ml; 1 h). Prior to the APTT clotting assay, pooled plasma from normal subjects (0.06 ml) was incubated with the IgG eluates (0.06 ml; 2 h). The concentrations of the FVIII inhibitory IgG in these assays yielded FVIII inhibition in the linear range of the inhibition curve (25–75% residual activity of reference FVIII-containing plasma).

RESULTS

E-FVIII and E-C2—Multiple electrophilic phosphonate groups were placed on FVIII and C2 Lys residues (E-FVIII, 52 mol/mol; E-C2, 7 mol/mol; total available Lys residues, respectively, 158 and 9; Fig. 1A), producing diverse electrophilic epitopes. Reducing SDS-electrophoresis and silver staining of E-C2 indicated a single band with mass similar to underivatized C2 (18.6 kDa; Fig. 1B, lane 2). SDS gels of E-FVIII revealed major 225- and 86-kDa bands (respectively, intact FVIII and FVIII light chain), minor ~96–200-kDa bands corresponding to known proteolytic FVIII fragments (3, 30), and smeared aggregate bands (nominal mass values ~350 and 580 kDa close to the loading position; Fig. 1B, lane 1). Other than the aggregates, these bands were present in underivatized FVIII obtained from the supplier (Fig. 1B, lane 2). The aggregates constituted 21–32% of the total silver-stainable protein present in three preparations of E-FVIII examined.

We reported recently the presence of nucleophilic sites in various nonenzymatic proteins, evident from their covalent reaction with small molecule phosphate diester compounds (E-haptenes) containing a positive charge neighboring the electrophilic phosphorus atom (31). The presence of a naturally occurring nucleophilic site(s) in FVIII was suggested by the formation of a major 225-kDa adduct and a faint 86-kDa adduct of FVIII treated with a positively charged E-hapten-1 (Fig. 1C, lane 1). Only faint adduct bands were observed in reaction mixtures containing the poorly electrophilic control phosphonic acid (E-hapten-2) or the neutral phosphonate E-hapten-3. Control ovalbumin, a protein with minimal nucleophilic reactivity (31), did not form detectable E-hapten-1 adducts. The E-FVIII aggregates may therefore be interpreted to derive from intermolecular covalent bonding between the electrophilic phosphate and a naturally occurring nucleophilic site(s) of FVIII.

To assess antigenic integrity, the binding of E-FVIII and E-C2 by Abs was determined by ELISA using affinity-purified IgG preparations from eight HA patients positive for FVIII inhibitory antibodies. All of the IgG preparations at 25 μg/ml displayed E-FVIII and E-C2 binding exceeding the mean ± 3 S.D.
values for control IgG from the nonhemophilia subject (code NH1941; A490, respectively, 0.01 ± 0.01 and 0.07 ± 0.01). The IgG concentrations yielding an A490 value of 0.25 computed from dose-response curves are reported in Table 1. Fast protein liquid chromatography-gel filtration of E-FVIII permitted separation of an aggregate peak eluting close to the column void volume (retention time 18.3–21.6 min; 13% of protein loaded on the column) from unaggregated E-FVIII (retention time 43.3–46.0 min). The aggregates did not display reduced reactivity with IgG from an HA patient (HA1828) compared with unfractionated E-FVIII (A490 1.22 ± 0.02 and 0.85 ± 0.10, respectively; 30 μg/ml IgG, 93 ng/well E-FVIII aggregates or unfractionated FVIII), suggesting that the Ab-reactive epitopes aggregates are present in the aggregates. The ability of E-FVIII to consume anti-FVIII Abs was measured following solution phase reactions of HA1828 IgG with biotinylated E-FVIII. Immune complexes were removed using immobilized streptavidin, and free Abs in the unbound fraction were measured. This procedure resulted in essentially complete removal of Abs capable of binding FVIII or E-FVIII (Fig. 2). E-FVIII, therefore, is recognized by the majority of anti-FVIII Abs present in the HA IgG.

**Irreversible Immune Complexation**—Formation of irreversible immune adduct of E-FVIII and Abs was initially studied by
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FIGURE 2. Near-complete consumption of anti-FVIII Abs by E-FVIII. IgG HA1828 (0.7 μM) was incubated in solution phase with diluent or biotinylated E-FVIII (0.1 μM) for 20 h. Immune complexes and free E-FVIII were removed by affinity chromatography using streptavidin-agarose. The flow-through and wash fractions were pooled and assayed for binding to immobilized FVIII in A or E-FVIII in B by ELISA.

Reducing SDS-electrophoresis and detection of large-mass bands by immunoblotting with anti-human IgG. The E-FVIII aggregates do not interfere in immune complex detection, as they are not stained by the anti-IgG reagent. Treatment of an anti-FVIII monoclonal Ab (directed to the C2 domain; clone ESH8) with E-FVIII but not FVIII devoid of the electrophilic groups resulted in formation of two large-mass bands stainable with anti-IgG Ab, one close to the sample loading position and the second with a nominal mass of 500 kDa (Fig. 3A). The theoretical mass of IgG adducts containing one and two FVIII molecules are ~415 and ~680 kDa, respectively. The observed large-mass adducts were absent in E-FVIII treated with a control monoclonal Ab of the same isotype as the anti-FVIII Ab (IgG2a,κ). IgG purified from all eight inhibitor-positive HA patients formed similar immune adducts with E-FVIII but not with FVIII (examples shown in Fig. 3B). Control IgG from the non-HA subjects did not form the adducts. The adducts were observed despite boiling and SDS denaturation, consistent with covalent E-FVIII binding by nucleophilic Ab sites. The electrophoresis studies do not reveal the precise molecular composition of the adducts, but they fulfill our purpose of unambiguously establishing the formation of irreversible and specific immune adducts. As in the case of E-FVIII, treatment of biotinylated E-C2 with the monoclonal anti-FVIII Ab resulted in formation of the predicted large-mass band stainable with streptavidin-peroxidase (nominal mass 187 kDa; anticipated mass of bivalent IgG complexed with 2 E-C2 molecules, 188 kDa; Fig. 3A). No 187-kDa complex was observed by treatment of E-C2 with an equivalent concentration of the control isotype-matched monoclonal Ab. IgG from all eight inhibitor-positive HA patients formed the 187-kDa complex with E-C2 but not C2 devoid of the electrophilic groups. Control IgG from the non-HA subjects did not form the complex or did so at very low levels (example in Fig. 3C). This rules out an indiscriminate covalent reaction of the phosphonate group as the explanation for formation of stable immune adducts by the HA IgGs.

E-FVIII binding by Abs can be modeled as a two-step reaction, in which the initial step generates specific noncovalently associated immune complexes (state 1 in Fig. 4A) followed by conversion of these complexes to irreversible adducts (state 2) via covalent phosphonate bonding with Ab nucleophiles. The model is supported by the following observations. Inclusion of excess FVIII devoid of the phosphonates in the reaction mixture at t = 0 inhibited E-FVIII binding by the HA IgG preparations, indicating specificity typical of conventional Ab-antigen noncovalent binding reactions (Fig. 4B). We measured the dissociation of E-FVIII immune complexes formed by the polyclonal HA IgG by removing the free IgG and incubating the reaction mixture for 20 h in excess FVIII (which precludes reassociation of dissociated complexes). Seventy five percent of the immune complexes remained in the associated state (Fig. 4C). Under these conditions, there was near-complete dissociation of the noncovalent immune complexes formed by the high affinity monoclonal anti-FVIII Ab (clone ESH8, K_D 0.4 nM; Ref. 32) with FVIII devoid of the phosphonates. This is consistent with the observed dissociation rates of other high affinity noncovalent immune complexes (33). As the experimental protocol effectively distinguishes between noncovalent and irreversible
Ab–E-FVIII

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\begin{align*}
\text{Ab} + \text{E-FVIII} & \quad \Rightarrow \quad \text{Ab:::E-FVIII} & \quad k_1 \\
& \quad \Rightarrow \quad \text{Ab–E-FVIII} & \quad k_2 \\
& \quad \Rightarrow \quad \text{Ab} + \text{E-FVIII} & \quad k_3
\end{align*}
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Remove IgG, add excess FVIII

Measure complexes

-4 0 20 hours

FIGURE 4. Specific irreversible E-FVIII binding by HA IgG preparations. A, one-step reaction model for generation of irreversible adducts E-FVIII and Abs. The initial noncovalent binding reaction imparts specificity to the reaction (state 1). The covalent reaction occurs if the electrophilic phosphonate is in register with a naturally occurring nucleophile in the Ab combining site (state 2). The covalent reaction occurs if the electrophilic phosphonate is in register with a naturally occurring nucleophile in the Ab combining site (state 2). B, saturation of E-FVIII binding by HA1828 IgG (0.12 μM) evident from competitive inhibition with excess FVIII (0.25 μM) included in the reaction mixture. Total binding was determined in PBS. Incubation time was 4 h. C, nondissociable E-FVIII complex formation by HA1828 IgG. Top, assay protocol. Left, after formation of immobilized E-FVIII immune complexes with HA1828 IgG for 4 h as in A, free IgG was removed by extensive washing, and the complexes were allowed to dissociate over 20 h in the presence of excess FVIII (0.25 μM). Binding in PBS prior to IgG removal is labeled Binding, t = 0. Binding after removal of IgGs and incubation for 20 h in excess FVIII is labeled Nondissociable binding, t = 20. Right, same experimental protocol was applied to determine nondissociable binding of FVIII devoid of the electrophilic groups with the monoclonal anti-FVIII IgG ESH8 (0.07 μM). Values are corrected for nonsaturable binding in wells that received excess FVIII at t = 0 of the reaction (nonsaturable A_{490} for HA IgG, 0.21; for ESH8 IgG, 0.14).

Irreversible immune complexation was studied further by an ELISA method entailing dissociation of the noncovalent state 1 complexes with 2% SDS (30 min of treatment). The protocol has been validated in our previous report of covalent complexation of human immunodeficiency virus gp120 by Abs (16). Treatment with SDS dissociated the noncovalent complexes of FVIII devoid of the electrophilic phosphonates and the high affinity anti-FVIII monoclonal Ab (<10% residual binding after SDS treatment). After incubation with IgG preparations from HA patients (n = 8) for 2 h, 77.6 ± 9.1% complexes formed by E-FVIII and 66.3 ± 17.6% of the complexes formed by E-C2 (mean ± S.D.) were refractory to dissociation by SDS (Fig. 5A). The proportion of SDS-refractory complexes reached plateau levels within 30 min (Fig. 5B), indicating rapid conversion of the state 1 noncovalent complexes to state 2 irreversible adducts. \( t_{1/2} \) values for formation of the complexes in nondenaturing solvent (PBS; state 1 + state 2 complexes) and SDS (state 2 complexes) were comparable (2.5 and 2.3 h, respectively). This suggests that noncovalent binding of E-FVIII and Abs is the rate-limiting step in accumulation of the covalent adducts. Taken together, it may be concluded that the electrophilic phosphonates are in sufficient proximity with Ab nucleophiles in a majority of noncovalent complexes to allow conversion to the covalently associated state. Approximately 15% of the E-FVIII immune complexes remained SDS-dissociable in Fig. 5B, suggesting that the covalent bonding is disallowed in a minority of complexes. Fab fragments prepared from the IgG of patient HA1828 displayed SDS-refractory binding to E-FVIII equivalent to intact IgG (Fig. 5C). This suggests that an avidity effect because of bivalent IgG binding is not a factor, and the irreversible binding can be attributed to monovalent Ab combining sites.

Irreversible Loss of Antibody Inhibitory Activity—The functional effect of E-FVIII and E-C2 was studied by measuring irreversible loss of FVIII inhibitory activity of IgG from patients HA1828, HA2222, and HA3112 (plasma FVIII inhibitor titer: 800, 1011, and 198 Bethesda assay units/ml, respectively). The IgGs were treated with E-FVIII (0.1 μM), E-C2 (1 μM), or control E-VIP (0.1 and 1 μM). Protein G-Sepharose columns were used to capture free IgG and immune complexes, and free polypeptides were removed by extensive washing. Column eluates from reaction mixtures of IgG and the E polypeptides were tested for the ability to inhibit FVIII cofactor activity by the chromogenic Factor Xa (FXa) generation assay or the one-stage APTT assay. The FXa generation assay measures the ability of FVIII to generate the catalytic complex with FIXa responsible for converting FX to FXa. The APTT assay measures the time to clot formation of plasma after initiating the coagulation cascade by phospholipid contact activators that promote Factor XII conversion to Factor XIIa. Control experiments were conducted prior to studying E-FVIII and E-C2 effects. Dose-dependent inhibition of FVIII cofactor activity was observed in both assays using protein G eluates from control reactions containing increasing HA IgG concentrations (16–100 μg/ml) and dilituent. The recovery of FVIII inhibitory activity in the eluates was 67–95% of the activity predicted from the plasma Bethesda titers of the three HA patients. Identically processed IgG from a non–HA subject treated with dilituent was devoid of FVIII inhibitor activity. Eluates obtained by chromatography of 0.1 μM FVIII in dilituent without IgG did not display detectable FVIII activity, confirming removal of free polypeptides by the chromatography procedure. Similarly processed E-FVIII or E-C2 in dilituent without IgG did not express detectable FVIII activity.

Treatment of the IgG preparations with the irrelevant E-polypeptide E-VIP was without noticeable effect on the FVIII inhibitor activity (<15% loss of activity compared with dilituent-treated inhibitory IgGs). In comparison, treatment with 0.1 μM E-FVIII relieved the FVIII inhibitor activity of the three IgG preparations by an average of 87.9 ± 7.1% (S.D.) and 69.2 ± 5.2%, respectively, determined by the chromogenic FXa and APTT assays (Fig. 6A). E-C2 was less potent than E-FVIII, and higher concentrations of E-C2 were necessary to obtain IgG inactivation. Treatment with 1.0 μM E-C2 relieved the FVIII inhibitor activity of the IgG preparations by 40.1 ± 17.8 and 29.6 ± 13.5%, respectively, determined by the FXa and APTT assays (Fig. 6B). From these results, it may be concluded that
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Irreversibility of E-FVIII complexation with Abs in denaturing solvent. A, SDS-refractory binding of E-FVIII and E-C2 by HA IgG. Plotted are values of residual binding that survived treatment with 2% SDS for 30 min, expressed as percent of total binding without SDS treatment. A<sub>490</sub> values for total E-FVIII and E-C2 binding by IgGs from individual patients were as follows, respectively: HA1828, 1.20 and 1.24; HA1834, 1.20 and 0.68; HA1835, 1.09 and 0.96; HA2084, 0.97 and 1.02; HA2085, 1.30 and 0.84; HA2222, 0.83 and 0.45; and HA3112, 0.79 and 0.36. IgG concentrations, 6.3–100.0 μg/ml. Residual binding was computed as in A. IgG HA1828 was 25 μg/ml. B, time course of total and SDS-refractory E-FVIII binding by HA1828. Total binding was computed as in A. IgG HA1828 was 25 μg/ml. C, irreversible E-FVIII binding by Fab fragments from IgG HA1828. Total binding and SDS-refractory binding by Fab fragments (75 μg/ml) were determined as in A. Inset, Coomasie Blue-stained SDS-electrophoresis gels (nonreducing) of IgG (lane 2) and Fab (lane 3). Molecular mass markers, lane 1.

Irreversible occupancy of the Ab combining sites by E-FVIII and E-C2 results in loss of the IgG FVIII inhibitory activity.

E-FVIII Reactivity with Coagulation Factors—The cofactor role of FVIII in blood coagulation depends on interactions of discrete FVIII regions with various coagulation proteins and phospholipids (34). We compared the ability of E-FVIII and E-FVIII to generate FXa. E-FVIII or FVIII was incubated with a mixture of FIXa, thrombin, calcium chloride, phospholipids, and FX. FXa enzymatic activity was quantified using a chromogenic peptide, FVIII inhibitor activity of the HA IgG preparations (measured as in Fig. 8C; <15% difference for diluent-treated IgG; data not shown). We concluded that the reactivity of the peptides with the Abs is insufficient to afford useful Ab inactivation. Previous reports have also suggested that the inhibitory Abs recognize large FVIII polypeptide fragments (6) better than small peptides, suggesting that the Abs are directed mainly to conformational rather than linear epitopes (12, 35, 36).

DISCUSSION

Infused FVIII is ineffective in correcting defective blood coagulation in a subpopulation of FVIII-deficient HA patients producing inhibitory Abs to the protein. Here we describe electrophilic FVIII analogs that bind specifically and covalently to the nucleophilic sites of Abs. E-FVIII and E-C2 were bound irreversibly by IgG preparations from each of eight inhibitor-positive HA patients studied. The unique mechanism of action of the electrophilic analogs was also evident from irreversible relief of FVIII inhibition by the Abs in coagulation assays. Full-length E-FVIII inactivated the Abs with superior potency compared with E-C2 and synthetic FVIII peptides. This is consistent with findings that HA patients produce Abs directed to diverse FVIII epitopes outside the C2 domain (2, 35). Our studies provide proof-of-principle that targeting of Ab nucleophilic sites can relieve the pathogenic effects of Abs. The strengths of this approach and potential means to address its weaknesses are discussed below.

Irreversible reactions of E-FVIII and E-C2 with Abs from HA patients was detected by ELISA (n = 7 patients; A<sub>490</sub> < 0.1; data not shown). Denaturing electrophoresis of boiled reaction mixtures containing excess biotinylated E-(2303–2332) revealed small amounts of immune adducts formed by IgG from two of the seven HA patients but not control IgG from the non-HA subject (56-kDa IgG heavy chain band, Fig. 8B). However, treatment of these IgG preparations without and with excess E-(2303–2332) (20 μM) did not influence their FVIII inhibitory activity (Fig. 8C). This suggests that E-(2303–2332) binding Abs do not constitute a functionally significant proportion of the FVIII inhibitory Abs in these subjects. Certain other FVIII synthetic peptides are recognized by anti-FVIII Abs found in HA patients with low affinity. In this study, we
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to dissociate the immune adducts. Similarly, a majority of the adducts remained undissociated in non-denaturing solvent after removal of free Abs and prolonged incubation in excess competitor FVIII. There was no evidence that E-FVIII or E-C2 react nonspecifically with irrelevant Abs. This supports a two-step reaction model in which initial noncovalent binding confers specificity to the reaction, followed by covalent bonding of the electrophilic phosphonates with Ab nucleophiles. Conversion of the noncovalent complexes to irreversible adducts was rapid, and noncovalent E-FVIII binding by the Abs was rate-limiting. This is consistent with the strong electrophilicity of phosphonate diesters evident from the study of nucleophilic enzymes. The first order rate constant for the reaction of a phosphonate diester with trypsin is $0.03 \text{ s}^{-1}$ (15, 38). IgG nucleophilic reactions can exceed that of trypsin (14), consistent with rapid conversion of noncovalent E-FVIII immune complexes to irreversible adducts. We did not address in detail the extent of damage to the antigenic structure of FVIII caused by introducing phosphonate groups. However, all eight HA IgG preparations displayed E-FVIII binding. The observed E-FVIII aggregation reaction is likely because of the reactivity of phosphonate groups with an endogenous nucleophile of FVIII. Formation of the aggregates did not appear to impact the reaction with Abs negatively, as the aggregates were bound by an HA IgG preparation comparable to unfractionated E-FVIII. Treatment with excess E-FVIII resulted in near-complete consumption of FVIII binding Abs, indicating that most epitopes recognized by the Abs are expressed on E-FVIII.

To determine the functional consequence of irreversible occupancy of Ab combining sites, free E-FVIII was removed from the E-FVIII/IgG reaction mixtures prior to clotting factor assays. E-FVIII-treated HA IgG preparations displayed reduced FVIII inhibitor activity (by 61–99%). An irrelevant E-polypeptide was without effect, suggesting specific Ab inactivation. The functional results are consistent with biochemical studies indicating that the majority of noncovalent complexes are converted to irreversible adducts. It may be concluded that electrophilic phosphonates are available for covalent bonding in most FVIII epitopes recognized by Abs. This is significant, as diverse Abs directed to various FVIII epitopes must be inactivated irreversibly to obtain a functionally useful effect. The linker attaching the phosphonate groups to FVIII contains single bonds around which rotation is permissible. The resultant spatial freedom enjoyed by the phosphonates should facilitate approach of the electrophilic phosphorus atom within covalent binding distance of Ab nucleophiles. Conversely, the failure of a minority of the reversibly associated complexes to convert to the irreversible state suggests room for improvement in the structure of the E-FVIII. Factors that may limit the covalent reaction are as follows: (a) some epitopes may not be labeled with the electrophilic phosphonate if they do not contain a sufficiently exposed Lys residue; (b) the distance between the phosphonate and Ab nucleophile or the spatial orientation of these groups may be nonpermissive for covalent bonding; and (c) a minority of the Abs may lack nucleophilic sites. We hold the last mentioned possibility unlikely as the nucleophilic reactivity was expressed by the variable domains of every recombinant Ab fragment and monoclonal Ab examined previously (14, 23).
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A.

TRYLRIHPQSWHQLKMEVLGCQYDLK-CONH₂
2303 2332

B.

P

E-2303-2332

C.

HA1834

HA1835

FIGURE 8. Covalent binding of peptide E-(2303–2332) to inhibitory IgG and effect on FVIII inhibitor activity. A, structure of the E-(2303–2332) peptide analog. P and Bt denote, respectively, the phosphonate and biotin groups. B, streptavidin-peroxidase-stained blot of a reducing SDS-electrophoresis gel showing boiled reaction mixtures of biotinylated E-(2303–2332) (10 μM) incubated with HA1834 IgG, HA1835 IgG or control NH1941 IgG (75 μg/ml; 20 h, 37 °C). The bands at the bottom of the lanes and the 56-kDa position represent, respectively, the free E-(2303–2332) and E-(2303–2332) complexed to the heavy chains of the IgG. C, FVIII inhibitory activity of IgG preparations treated with E-(2303–2332). FVIII cofactor activity was measured by the APTT assay after incubation of HA IgG1834 or HA IgG1835 (400 μg/ml) with E-(2303–2332) (20 μM; 20 h at 37 °C) and treatment of FVIII-containing normal plasma with varying concentrations of the reaction mixtures (diluted 1/2 and 1/6). FVIII inhibitory activity was computed as follows: 100 − 100 × (FVIII activity in presence of IgG/FVIII activity in absence of IgG). FVIII activity in absence of IgG was 0.54 IU/ml. Experimental procedures were as in Fig. 6. Values plotted are means of duplicates ± S.D.

That the irreversible E-FVIII binding activity in this study is a property of Ab variable domains is confirmed by observations that the antigen binding Fab fragments of IgG displayed this activity.

FX activation occurs via formation of the FVIIIa-FIXa complex on phospholipid surfaces (“tenase complex”). E-FVIII was a poor activator of FX compared with underivatized FVIII. A low level risk of thrombotic events is associated with overdose of bypass reagents such as recombinant Factor VII (9). The poor cofactor activity of E-FVIII may be a functionally useful property, as this diminishes the risk of a thrombotic effect. Possible reasons for the poor cofactor activity of E-FVIII are as follows: (a) structural perturbations because of introduction of phosphonate groups may render E-FVIII resistant to thrombin hydrolysis; (b) even if E-FVIIIa is produced by thrombin hydrolysis, it may not form the tenase complex; or (c) E-FVIII may inhibit serine proteases indiscriminately. Possibilities a and b are innocuous, in that they should not produce undesirable effects. The potential inhibition of serine proteases involved in the coagulation pathway, however, merits attention. High micromolar to millimolar concentrations of small molecule phosphonates are reported to bind covalently to the nucleophilic sites of serine proteases (17). E-FVIII did not interfere with FVIII-dependent hydrolysis of the peptidyl ester substrate catalyzed by FXa, indicating that it does not inhibit the enzymes necessary for this reaction (thrombin, FIXa, and FXa). Moreover, no prolongation of the time to fibrin clot formation was observed in the APTT test at an E-FVIII concentration that relieved the FVIII inhibitor activity of the Abs irreversibly. These observations indicate that E-FVIII can selectively inactivate Abs without functionally significant inhibition of serine protease clotting factors.

The phosphonate-containing E-FVIII is a first generation reagent. Future genetic and chemical manipulations may help develop improved E-FVIII analogs. As the selectivity of the reaction derives from noncovalent Ab binding, the conformation of E-FVIII should preferably be as close to native FVIII as feasible. As the covalent reactivity of E-FVIII is not rate-limiting, it may be useful to reduce the electrophilicity of E-FVIII to further minimize reactions with non-Ab serine proteases while maintaining sufficiently rapid covalent Ab bonding capability. Carbon-based electrophiles placed at defined positions within FVIII may offer sufficient electrophilicity to inactivate Abs directed to individual epitopes with minimal overall structural perturbation of FVIII. Electrophilic pyruvate analogs and dicarbonyl compounds react covalently with protein nucleophilic sites (38, 39), and suppressor tRNA technologies for incorporation of such compounds into the protein backbone are available (40). The half-life of FVIII in human circulation (t½) is ~12 h (41). Aggregation of proteins can influence their clearance in vivo. E-FVIII aggregation appears to involve a naturally occurring nucleophile that reacts with the phosphonate groups. Availability of an FVIII mutant with deficient nucleophilicity will help minimize the aggregation reaction. Site-directed mutagenesis has been applied previously to generate enzymes and Abs with deficient nucleophilic reactivity (20, 42). A significant proportion of inhibitory Abs to FVIII in HA patients is directed to the C2 domain (43). However, Ab diversity and the conformational character of the immunodominant epitopes pose significant challenges. This is illustrated by our observations using E-C2 and E-(2303–2332), a C2 peptide analog. E-C2 was consistently less reactive with HA IgG than E-FVIII. E-(2303–2332) displayed very limited reactivity. For these reasons, developing mixtures of large E-FVIII polypeptides or optimization of the full-length E-FVIII struc-
ture are preferred routes to clinical application of the covalent inactivation approach described here.

The FVIII inhibitory potencies of Abs in HA patients can vary over 3 log orders, and the titers can exceed 1000 Bethesda assay units/ml (44, 45). The magnitude of the titers depends on the concentration and affinity of Abs directed to the neutralizing FVIII epitopes. The irreversible reaction of E-FVIII offers the advantage of more potent Ab inactivation if certain conditions are met. For example, 84% of 1 nM Ab with $K_d = 10$ nM will be in free state after equilibrium is attained with 2 nM reversibly binding antigen (see supplemental Fig. S1). Assuming a covalent binding rate constant that does not limit the overall reaction rate (as observed in this study), only 6% of the Ab will exist in free state after incubation for 12 h with an irreversible binding antigen. Consumption of the Abs by the irreversible antigen will occur at superior levels as long as the Ab concentration does not exceed the $K_d$. With increasing Ab concentration or decreasing $K_d$ values, Ab consumption by the irreversible and reversible antigen will approach equivalence, and the potential advantage of the former antigen is lost. Even if the Abs are initially consumed at equivalent levels by E-FVIII and FVIII, the former reagent should provide more long lasting Ab inactivation in vivo. With reducing concentrations of free FVIII in blood because of metabolic clearance, a progressive reduction in the concentration of reversibly associated immune complexes will occur. The irreversibly associated E-FVIII immune complexes, on the other hand, are not subject to dissociation because of metabolic clearance of E-FVIII. With respect to catalytic Abs, a nonhydrolyzable antigen analog such as E-FVIII offers superior inactivation potency even when $[\text{Ab}] > K_d$ (see supplemental Fig. S1).

In addition to Abs to FVIII, other antigen-specific Abs exert harmful effects in various diseases, e.g. autoantibodies to the acetylcholine receptor in myasthenia gravis (46) and to platelet antigens in autoimmune thrombocytopenic purpura (47). Nonspecific immunosuppressive agents are used to control Ab production, but this is often accompanied by profound side effects and enhanced susceptibility to infection. As several electrophilic polypeptides are documented to bind their cognate Abs irreversibly (14, 15), it is reasonable to consider the generality of the irreversible Ab targeting approach in future studies. Consideration of the effect of electrophilic antigens on the cells responsible for Ab synthesis is also warranted. Antigen binding to the antigen receptor (immunoglobulins complexed to signal transducing proteins) drives maturation of B lymphocytes into Ab secreting cells. Saturation of the antigen receptor induces B cell apoptosis and immune tolerance (48, 49). Administration of very large amounts of FVIII on the order of grams induces immune tolerance to FVIII in a subpopulation of HA patients (10, 11). We reported covalent binding of an electrophilic hapten to nucleophilic B cell antigen receptors (23). Electrophilic antigen analogs are predicted to saturate cellular antigen receptors more readily than the reversibly binding antigen. In preliminary studies (50) we observed that prior treatment with E-FVIII attenuated Ab synthesis by B cells challenged with FVIII. Taken together, these considerations support further development of electrophilic antigen analogs for irreversible inactivation of pathogenic Abs.

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