The anti-factor VIII (fVIII) C2 domain monoclonal antibody ESH8 inhibits fVIII activity only when fVIII is bound to von Willebrand factor (vWF). However, ESH8 binds with similar affinity to fVIII and fVIII-vWF complex, and it does not affect the kinetics of thrombin cleavage at positions 372 and 740 within the fVIII heavy chain and at 1689 within the light chain. The latter is required for fVIII release from vWF. We showed that ESH8 reduced the initial rate of thrombin-activated fVIII (fVIIIa) release from vWF by 4.3-fold compared to that in the absence of antibody. The complex of vWF:fVIII:ESH8 was activated, and the rate constant determined for fVIIIa dissociation from vWF was 4 x 10^{-3} s^{-1}. We constructed a mathematical model incorporating the measured rates for fVIIIa release from vWF and for inactivation of heterotrimeric fVIIIa due to the spontaneous loss of the A2 subunit and found that the decreased release rate is sufficient to explain our experimentally observed inhibition of fVIII activity by ESH8. We hypothesize that the slowed rate of fVIIIa release from vWF in the presence of ESH8 allows time for inactivation of unstable fVIIIa prior to its participation in the formation of the factor Xase complex. The relevance of these findings is illustrated by our observation that reduction of fVIIIa release from vWF represents an additional mechanism of fVIII inhibition by an anti-C2 domain antibody (epitope 2218–2307) from a hemophilia A patient. This rare antibody binds to a more amino-terminal epitope than other human anti-C2 inhibitors, resulting in its lack of inhibition of fVIII binding to vWF but not to phospholipid. These two fVIII ligands therefore bind to C2 sites which do not overlap completely.

The plasma glycoprotein factor VIII (fVIII) functions as a cofactor for the factor Xase enzyme complex of the intrinsic pathway of blood coagulation (1), and it is decreased or absent in patients with hemophilia A. Formation of a complex between fVIII and von Willebrand factor (vWF) is crucial to the stability of fVIII in the circulation, since patients with severe von Willebrand’s disease, who have a complete deletion of the vWF gene or mutations which reduce binding between fVIII and vWF, have a secondary deficiency of fVIII (2). Binding to vWF prevents fVIII from binding to phospholipid vesicles or platelets and factor IXa, functions required for its procoagulant activity.

fVIII internal protein sequence homology has led to the designation of six domains arranged in the order A1-A2-B-A3-C1-C2 (3). The heavy chain (HCh) consists of the A1, A2, and B domains, whereas the light chain (LCh) consists of the A3, C1, and C2 domains. Thrombin, the principal physiological activator of fVIII, proteolytically cleaves the fVIII protein at Arg^{772} and Arg^{1689} in the HCh and at Arg^{1689} in the LCh (4) to release the acidic peptide 1649–1689. Cleavage of the LCh is believed to be responsible for dissociation of activated fVIII (fVIIIa) from vWF (5), and it is required for maximal generation of fVIIIa activity along with the two HCh cleavages (6, 7).

The fVIII LCh contains sites for binding to vWF (5, 8) and phosphatidylserine (PS) (9). Binding sites for vWF and PS were localized to the C2 domain residues 2303–2332 (10–12). This results in a mutually exclusive binding of these ligands to fVIII (11). The acidic region of the LCh (amino acid residues 1649–1689) is also important for binding to vWF, since several monoclonal antibodies with epitopes within residues 1670–1689 (13–15) and deletion of part of this region (16) inhibits fVIII binding to vWF. The exact function of these residues, however, is not clear, since their direct binding to vWF has not been demonstrated.

Allotype antibodies which inactivate fVIII (inhibitors) develop in about 25% of hemophilia A patients following fVIII infusions. Epitopes for one of the major types of inhibitor are located in the C2 domain of fVIII (17, 18). These antibodies act by blocking the binding of fVIII to phospholipid (9, 19) and to vWF(10, 20). The inhibitory effects result from a partial overlap of the common core of the epitope (residues 2248–2312) for 11 C2 domain inhibitors (19–21) with the PS and vWF binding sites (10, 11). The monoclonal antibody (mAb) ESH8 recognizes a C2 domain epitope within residues 2248–2285. It does not inhibit fVIII binding to phospholipid (10, 19) and vWF (10), but it has a high fVIII inhibitor titer as determined in the Bethesda assay (19). These results suggest the existence of a second mechanism of inhibition of fVIII coagulant activity by anti-C2 antibodies, which we have investigated in this study.

**EXPERIMENTAL PROCEDURES**

Monoclonal and Human Antibodies—Purified IgG from mAb ESH8 was obtained from American Diagnostica. Fab’ fragments of mAb ESH8 were prepared by digestion of IgG with immobilized papain beads,
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was preincubated with 60 nmWt, which resulted in a >98% complex formation. The respective complexes in the presence or absence of mAb ESHS at 37 °C were activated by addition of 5 μM thrombin. Samples (250 μl) were taken from the mixture, and activation was terminated by addition of 5 μM p-phenylalanine-L-prolyl-L-arginyl chloromethyl ketone (PPACK, Calbiochem). Twenty-five-μl aliquots of each sample were analyzed by SDS-PAGE under nonreducing conditions (see below). Aliquots (100 μl) from the same samples were added to triplicate microtiter wells (Immulon 1, Dynatech) coated with 2.5 μg/ml anti-μWf mAb 2.2.9. Preliminary kinetic studies of the binding of 125I-VIII-Wt and 125I-LCh-vWf complexes to immobilized mAb 2.2.9 demonstrated that binding reaches equilibrium within 3 min at 37 °C. Following incubation for 4 min at 37 °C, wells were washed with Tris-buffered saline, 0.01% Tween 20, and bound radioactivities (binding values) were determined. Negative controls were mAb 2.2.9-coated wells containing all components except μWf, and these values were subtracted from average values of triplicates of all other wells. The values of these controls were ≤5% of the maximal binding values. The percentage of 125I or LCh bound to μWf (bound%) was calculated as (binding values of thrombin-activated 125I-VIII or 125I-LCh/binding values of unactivated 125I-VIII or 125I-LCh) × 100. The percentage of the released fVIIIαs or activated LCh was calculated as 100 − bound%.

Kinetic Measurements Using Biosensor Technology—The kinetics of ESH8-VIIIαs interaction with μWf and LCh or A3-C1-C2 with mAb ESH8 was studied by surface plasmon resonance using the IAsys biosensor (Fisons Instrs. Lab. Sys., UK) which measures protein binding and subsequent ligand dissociation in real time (34). fVIII (50 μg/ml) in 10 mM sodium acetate, pH 5.0, was covalently coupled to the activated carboxymethylated-coated biosensor chip at a coupling density 5 μg/cm². Incubation of μWf was immobilized to the carboxymethylated chip at 18 ng/mm² (35). The carboxymethylated chip and reagents for its activation, N-ethyl-N'-dimethylaminopropylcarbodimide hydrochloride and N-hydroxysuccinimide, and deactivation, ethanolamine, were purchased from Fisons. Ligand binding was measured in 200 μl of HBS, 5 mM CaCl₂, 0.01% Tween 20 at 37 °C. Disassociation was initiated by substitution with the same buffer (200 μl) lacking ligand. To regenerate the chip, complete dissociation of ESH8-VIIIαs bound to immobilized fVIII was achieved by addition of 20 mM Tris, 2 M NaCl, 0.01% Tween 20, pH 7.2, for 2 min. Complexes between ESH8 and LCh or A3-C1-C2 were dissociated by addition of 0.1 M glycine, pH 2.5, for 3 min.

Calculation of the Kinetic Parameters from Biosensor Kinetic Data—The surface plasmon resonance signal observed, R, is proportional to the formation of a complex between immobilized component and added ligand. It was shown (36) that dR/dt = k₉[R]max – (k₅C + k₆R), where [R]max is the capacity of the immobilized ligand surface expressed in response units (Arc seconds) and C is the concentration of ESH8-VIIIαs, LCh, or A3-C1-C2 in solution. A linear fit of dR/dt versus R yields an apparent first order association rate constant, k₉ = k₅C + k₆. The second order association constant (k₉) was determined from the linear fit of k₉ versus C. The values of the rate constants (k₅) for dissociation of ESH8-VIIIαs from μWf and LCh or A3-C1-C2 from ESH8 were determined by fitting the dissociation kinetic curves to an equation dR/dt = −k₉R. All the above fitting procedures were performed using the fast fit 1.0b computer program (Fisons).

Affinity Measurements: Binding of mAb ESH8 to μWf or μWf-VIII Complex—μWf was bound to 3.5 μg/ml mAb 413 immobilized on microtiter plates. Bound μWf was incubated with increasing concentrations of μWf (40 μl), and the affinity of ESH8 for immobilized VIII or VIIIα-VIII complex was determined by homologous displacement of 125I-ESH8 (0.05 μM) with 0.05–100 μl unlabeled ESH8, followed by washing and determination of bound radioactivity. Each well contained 100 μl, except at the step following mAb 413 immobilization, where 200 μl of Tris-buffered saline, 2% bovine serum albumin were added for blocking, and all incubation steps were at 37 °C for 1 h with shaking. After each step plates were washed four times with 200 μl Tris-buffered saline, 0.1% Tween 20.
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RESULTS

The mAb ESH8 Inhibition of fVIII Activity Requires the Presence of vWF—We found in preliminary experiments that mAb ESH8 IgG23 inhibited fVIII activity in a one-stage clotting assay, but it did not have any effect on fVIII activity in a chromogenic assay utilizing purified proteins and phospholipid. The chromogenic factor Xase assay, which will be referred to hereafter as the factor Xase assay, measures the ability of fVIIIa to act as a cofactor for factor IXa in the activation of factor X (24). Since fVIIIa activity cannot be directly measured in this assay, we determine the initial rate of factor X activation in the presence of mAb ESH8 and 413 is expressed as a percentage of that when no mAb was added.

In human plasma used as the source of fVIII in the one-stage clotting assay, fVIII is bound to vWF. We thus hypothesized that lack of inhibition of fVIII activity in the factor Xase assay may be due to the absence of vWF. This possibility was tested by incubation of increasing concentrations of mAb ESH8 IgG or Fab’ with fVIII in the presence or absence of a 6-fold excess of vWF, which was followed by activation of fVIII by thrombin and determination of fVIII activity in the factor Xase assay. mAb ESH8 inhibited factor Xase activity >90% only in the presence of vWF (Fig. 1). Similar molar concentrations of mAb ESH8 IgG (6 nM) and Fab’ (5 nM) reduced factor Xase activity by 50%, demonstrating that whole IgG is not required for inhibition. The molar ratio of IgG to fVIII required for 50% inhibition was 0.6.

It would seem likely from the results of the previous experiment that mAb ESH8 does not inhibit the activity of fVIIIa in the presence of vWF, since fVIIIa is not bound to vWF. To confirm this, the fVIII-vWF complex was maximally activated, which was followed by addition of varying concentrations of mAb ESH8 or anti-A2 mAb 413 and incubation for 15 min at 37°C. The residual fVIIIa activity was measured in the factor Xase assay. mAb ESH8 did not inhibit fVIIIa, whereas mAb 413 completely inhibited fVIIIa (Fig. 1), as expected (37).

mAb ESH8 Does Not Inhibit Thrombin Cleavage of fVIII—Inhibition of fVIII activity by mAb ESH8 may result from prevention of the thrombin cleavage at position 1689, which is required for fVIIIa dissociation from vWF (5) and its subsequent binding to phospholipid. Therefore, we studied the kinetics of fVIII-vWF cleavage by thrombin in the presence or absence of mAb ESH8. The percentage of 125I-fVIII cleavage at positions 372, 740, or 1689 in the samples taken from the reaction at varying times was determined by SDS-PAGE and densitometric analysis (32) of the autoradiograms shown in Fig. 2. The results demonstrate that fVIII cleavage at position 1689 of the LCh was not affected by mAb ESH8 nor were cleavages at residues 372 and 740 in the fVIII HCh. Repeating the experiment with unlabeled fVIII, followed by silver staining, gave similar results (not shown), demonstrating that radiolabeling of fVIII did not alter its activation properties.

mAb ESH8 Binds to fVIII with Equal Affinity in the Presence or Absence of vWF, but with Lower Affinity to fVIIa—The lack of fVIII inhibition by mAb ESH8 in the absence of vWF suggested that fVIII binding to vWF may be required for formation of a high affinity binding site for ESH8. To test this possibility, the affinity of ESH8 for fVIII or the fVIII-vWF complex was determined by the homologous ligand displacement assay described in our previous studies (11). FVIII or fVIII-vWF complex was immobilized to microtiter wells by anti-A2 mAb 413, and binding of 125I-mAb ESH8 to fVIII was determined in the presence of increasing concentrations of unlabeled ESH8, Fig. 3. The dissociation constants (Kd) were determined from the best fit of the data to a single class of binding sites by the LIGAND program (38). The similar Kd values derived for mAb ESH8 binding to fVIII (0.42 ± 0.14 nM) and fVIII-vWF complex (0.44 ± 0.07 nM) demonstrate that vWF is not required for high affinity interactions.
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affinity binding of ESH8 to fVIII. In order to prove that immobilization of fVIII did not affect its affinity for mAb ESH8, mAb ESH8 binding to fVIII was also determined by homologous displacement of 125I-labeled fVIII by unlabeled fVIII in solution using protein G-Sepharose beads for subsequent capture of the 125I-fVIII-ESH8 complex. A similar K\text{d} value of 0.68 ± 0.17 nM was determined.\(^3\)

The lack of inhibition of fVIIIa by mAb ESH8 observed in Fig. 1 may be due to loss of the high affinity binding site for mAb ESH8 upon fVIII activation. mAb ESH8 was thus immobilized on a biosensor chip, and LCh or A3-C1-C2 association and subsequent dissociation from ESH8 was measured in an IAsys biosensor instrument. The surface plasmon resonance phenomenon, which measures the change in refractive index due to association of a fluid phase ligand with an immobilized ligand or due to the dissociation of a formed complex (34) generates a signal proportional to the amount of protein bound.

Second order association rate constants (k\text{on}) and first order dissociation rate constants (k\text{off}) were derived from association and dissociation curves obtained at LCh or A3-C1-C2 concentrations of 5–50 nM. Representative curves are shown in Fig. 4. The k\text{on} values for LCh and A3-C1-C2 interaction with ESH8 were (7.7 ± 0.7) \times 10^4 M^{-1} s^{-1} and (5.46 ± 0.74) \times 10^4 M^{-1} s^{-1}, and the k\text{off} values were (3.91 ± 0.27) \times 10^{-5} s^{-1} and (1.53 ± 0.05) \times 10^{-4} s^{-1}, respectively. The k\text{off} values presented are the mean ± standard deviation of k\text{off} values determined for each ligand from five independent dissociation experiments. The K\text{d} values were calculated as k\text{on}/k\text{off}. Therefore, the K\text{d} value for ESH8 binding to LCh was 0.5 ± 0.06 nm, and that for binding to A3-C1-C2 was 2.8 ± 0.4 nm. Thus, cleavage of the LCh by thrombin reduces its affinity for ESH8 by 5.6-fold. The same method was used to determine that ESH8 binds with similar affinity to fVIII (K\text{d} = 0.56 nm) (data not shown) and to LCh (K\text{d} = 0.5 nm), demonstrating that HCh is not involved in ESH8 binding to fVIII. Therefore ESH8 affinities for A3-C1-C2 and fVIIIa are also expected to be similar. The predicted 5.6-fold reduction of the affinity of ESH8 for fVIIIa is not sufficient to prevent ESH8 binding to fVIIIa under the conditions used in Fig. 1, where the concentration of fVIIIa (10 nM) and the highest concentration of ESH8 (100 nM) significantly exceeded the K\text{d} (2.8 nm). Under these conditions, mAb ESH8 remains bound to fVIII after its activation by thrombin.

**mAb ESH8 Inhibits the Release of fVIIIa from vWF upon Thrombin Activation—**Preliminary studies of thrombin activation of the fVIII-vWF or ESH8-fVIII-vWF complexes, immobilized on polystyrene microspheres coated with anti-fVIII HCh mAb 1.56, demonstrated that ESH8 inhibits the kinetics of fVIIIa release from vWF by 2–3-fold.\(^4\)

To determine whether ESH8 also inhibits release of fVIIIa from vWF in the fluid phase, 125I-labeled fVIII and vWF were incubated in solution in the presence or absence of mAb ESH8 prior to activation of the complexes by thrombin and determination of fVIIIa release from vWF by the solid phase radioimmunoassay described under “Experimental Procedures.” Dose-dependent inhibition of fVIIIa release from vWF by mAb ESH8 but not by anti-A2 mAb 413 is shown in Fig. 5A. The difference between fVIIIa released in the presence or absence of mAb ESH8 was maximal (4-fold) after incubation with 0.1 nM thrombin for 6 min, the optimal time required for activation of fVIII. The percentages of fVIIIa released from vWF in the presence and absence of mAb ESH8 were 18.6 ± 3.5% and 76 ± 5%, respectively. In a control experiment it was shown that 125I-fVIII or 125I-LCh binding to vWF was linearly dependent on the concentration of the 0.5 to 20 nM. The concentration of mAb ESH8 required to reduce the release of fVIIIa by 50% compared to that in the absence of ESH8 was 6 nM. The maximal effect of ESH8 on fVIIIa release was observed at 25 nM.

This experiment was repeated with unlabeled fVIII using biotinylated anti-A2 mAb 8 for detection of bound fVIII after immobilization of thrombin-activated fVIII-vWF or ESH8-fVIII-vWF complex (abbreviated below as ESH8/vFIII-vWF), and similar results were obtained. Radiolabeling of fVIII thus did not affect its release from vWF upon thrombin activation. Our data suggest that the inhibitory effect of ESH8 on fVIII coagulant activity may be partially or completely due to the inhibitory effect of ESH8 on fVIIIa release from vWF.

**A Human Inhibitor Antibody also Inhibits fVIIIa Release from vWF but Not Its Thrombin Cleavage—**An epitope of a human VIII inhibitor antibody, previously designated as case 4 IgG (21), was localized to the C2 domain region 2218–2307, which overlaps the epitope of mAb ESH8. To determine if inhibition of fVIII activity by case 4 total plasma IgG resulted only from the presence of anti-C2 antibodies, an inhibitor neu-

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3 D. Scandella, unpublished data.

4 G. E. Gilbert, unpublished results.
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Procedures. Symbols used are: ESH8/125I-fVIIIa/vWf (●), no antibody; and fractional cleavage at residue 1689 in the presence (▲) or absence (△) of mAb ESH8 IgG. At the time indicated by the arrow, thrombin was inhibited in the reaction ▲, unlabeled ESH8/fVIIIa (100 nM) was added and samples were taken as above. ESH8/fVIIIa complex was prepared by incubation of fVIII (1 μM) with mAb ESH8 (2.5 μM) for 30 min at room temperature prior to activation by thrombin (340 nM) for 1 min at 37°C followed by inhibition of thrombin with 34 μM of PPACK. B, 125I-LCh (10 nM) preincubated with vWF (60 nM) in the presence or absence of mAb ESH8 (100 nM) was activated by thrombin (5 nM), and samples were taken as in A. Symbols used are: ▲, fVIIIa release from vWF in the presence (●) or absence (○) of mAb ESH8; fractional cleavage at residue 1689 in the presence (▲) or absence (△) of mAb ESH8 IgG.

In the absence of ESH8, fVIIIa release from vWF was 95% after 30 min. By contrast, in the presence of ESH8 the percent of fVIIIa released from vWF approached a plateau at 42%. We propose that this could be due to the following binding equilibrium: ESH8/125I-fVIIIa/vWF ⇌ ESH8/125I-fVIIIa + vWF (equilibrium 1). If this is true, unlabeled ESH8/fVIIIa will compete for the binding site.

mAb ESH8 Reduces the Rate of Dissociation of Arg1689-cleaved fVIII from vWF—Since the above results demonstrated that the rate of the cleavage at position 1689, which is known to be critical for fVIIIa release from vWF, was not affected by the presence of ESH8, but fVIIIa release was, we proposed that fVIIIa release in the presence of ESH8 may be delayed compared to the cleavage at position 1689. To test this, the kinetics of fVIIIa release and the kinetics of thrombin cleavage of fVIIIa-vWF complexes was studied in parallel experiments. The fractional cleavages of fVIII at positions 1689, 372, and 740 in the samples taken up to 30 min were determined using SDS-PAGE analysis and scanning densitometry as described above. Analysis of the three cleavages in the presence and absence of mAb ESH8 showed no difference in the cleavage at position 1689 (Fig. 6A), or at positions 372 and 740 (data not shown). The initial rate of fVIIIa release, determined from the initial linear part of the curve, was reduced 4.3-fold in the presence of ESH8 compared to that in its absence (Fig. 6A). ESH8 Fab gave the same results (data not shown), demonstrating that whole IgG is not required for the inhibition of fVIIIa release. The effect of case 4 (13 Bethesda units/ml) on the time course of fVIIIa release was also studied (Fig. 6A). The initial rate of fVIIIa release was inhibited 1.8-fold by case 4 IgG. When the same Bethesda titer of ESH8 was used in this assay (data not shown), reduction of the initial rate of fVIIIa release was 2-fold greater than that in the presence of case 4. In a parallel experiment, it was shown that, at the concentration used in this assay, case 4 had no effect on fVIII binding to vWF. A similar concentration of normal human IgG did not have any effect in the assay (data not shown).

The presence of antibody was determined as described under “Experimental Procedures.” Symbols used are: A, mAb ESH8 (●); mAb 413 (○); B, case 4 IgG (●); normal human IgG (○).

Fig. 5. Effect of mAb ESH8 and case 4 IgG on fVIIIa release from vWF. A, 125I-fVIII (10 nM) and vWF (100 nM) were preincubated with increasing concentrations of mAbs ESH8 or 413. B, 125I-fVIII (2 nM) and vWF (12 nM) were preincubated with increasing concentrations of case 4 IgG or normal human IgG. In A and B, activation by thrombin was performed as in Fig. 3. The percentage of fVIIIa released in the presence of antibody was determined as described under “Experimental Procedures.” Symbols are as follows: A, mAb ESH8 (●); mAb 413 (○); B, case 4 IgG (●); normal human IgG (○).

The initial rate of fVIIIa release was 2-fold greater than that in the presence of case 4 IgG. In A and B, activation by thrombin was performed as in Fig. 3. The percentage of fVIIIa released in the presence of antibody was determined as described under “Experimental Procedures.” Symbols are as follows: A, mAb ESH8 (●); mAb 413 (○); B, case 4 IgG (●); normal human IgG (○).

By contrast to the other 11 described C2 domain inhibitor antibodies (19–21), case 4 IgG does not inhibit fVIII binding to vWF (19) as we have shown for mAb ESH8. This suggests that its mechanism of fVIII inhibition may be similar to that of ESH8. We therefore tested the effect of case 4 on fVIIIa release from vWF. The fVIIIa release upon activation of the fVIII-vWF complex formed in the presence of increasing concentrations of case 4 IgG is shown in Fig. 5B. Since the available amount of case 4 IgG was limited, the concentration of fVIII in the assay was reduced 5-fold in order to increase the antibody/fVIII ratio. Case 4 but not normal human IgG inhibited fVIIIa release from vWF in a dose-dependent fashion, but at the highest possible concentration of case 4 IgG (580 μg/ml) inhibition of fVIIIa release was only 2-fold. SDS-PAGE analysis of the samples taken at varying times up to 30 min during activation of fVIII-vWF complexes formed in the presence or absence of case 4 IgG demonstrated that none of the three cleavages of fVIII at positions 372, 740, or 1689 were inhibited by case 4 (data not shown).

While the presence of antibody was determined as described under “Experimental Procedures.” Symbols used are: A, mAb ESH8 (●); mAb 413 (○); B, case 4 IgG (●); normal human IgG (○).

Since the above results demonstrated that none of the three cleavages of fVIII at positions 372, 740, or 1689 were inhibited by case 4 (data not shown), it was hypothesized that the initial rate of fVIIIa release in the presence of ESH8 may be delayed compared to that in its absence (Fig. 6A). ESH8 Fab gave the same results (data not shown), demonstrating that whole IgG is not required for the inhibition of fVIIIa release. The effect of case 4 (13 Bethesda units/ml) on the time course of fVIIIa release was also studied (Fig. 6A). The initial rate of fVIIIa release was inhibited 1.8-fold by case 4 IgG. When the same Bethesda titer of ESH8 was used in this assay (data not shown), reduction of the initial rate of fVIIIa release was 2-fold greater than that in the presence of case 4. In a parallel experiment, it was shown that, at the concentration used in this assay, case 4 had no effect on fVIII binding to vWF. A similar concentration of normal human IgG did not have any effect in the assay (data not shown).

In the absence of ESH8, fVIIIa release from vWF was >95% after 30 min. By contrast, in the presence of ESH8 the percent of fVIIIa released from vWF approached a plateau at 42%. We propose that this could be due to the following binding equilibrium: ESH8/125I-fVIIIa/vWF ⇌ ESH8/125I-fVIIIa + vWF (equilibrium 1). If this is true, unlabeled ESH8/fVIIIa will compete for the binding site.
with ESH8/125I-fVIIIa for binding to vWF, and therefore the release of 125I-fVIIIa will increase. As shown in Fig. 6A (arrow), addition of unlabeled ESH8-fVIIIa did indeed lead to a final increase of 125I-fVIIIa release from vWF from 42 to 83%, indicating that ESH8-fVIIIa is able to bind vWF. By contrast, fVIII, for which thrombin activation was followed by addition of ESH8 or fVIIIa in the absence of ESH8, did not have any effect in the assay. These data indicate that the ESH8-fVIIIa complex binds to vWF with higher affinity than fVIIIa alone.

To determine if equilibrium 1 is solely responsible for incomplete (42%) release of fVIIIa-ESH8 from vWF, the $K_r$ for ESH8-fVIIIa binding to vWF was determined by the biosensor technique. ESH8-fVIIIa binding to immobilized vWF and subsequent dissociation of the formed complex was measured and representative data are shown in Fig. 7. We assumed stable association between ESH8 and fVIIIa in our experiments, since the ESH8-A3-C1-C2 complex does not significantly dissociate in HBS (35). We postulated that the potential spontaneous dissociation of the fVIII A2 subunit (39) from the ESH8-fVIIIa complex (320 kDa) does not significantly affect kinetic measurements employing plasmon resonance detection since the loss of A2 only reduces the molecular weight of the complex by 13%. The association rate constant $k_{on} = (1.11 \pm 0.08) \times 10^5 \ M^{-1} \ s^{-1}$ and the dissociation rate constant $k_{off} = 4 \times 10^{-3} \pm 8 \times 10^{-4} \ s^{-1}$ were used to calculate a $K_r$ value of 36 $\pm$ 7 nM for ESH8-fVIIIa binding to vWF. The $K_r$ was further used to calculate that the concentration of ESH8-fVIIIa released from vWF under the conditions used in Fig. 6A was 4 nM. This is 40% of the total present fVIIIa (10 nM) and corresponds to the experimentally observed release of fVIIIa (42%) in Fig. 6A. In a control experiment, addition of 90 nM thrombin did not inhibit binding of 90 nM ESH8-fVIIIa to vWF (data not shown), which demonstrates that this binding was not due to uncleaved fVIII present in ESH8-fVIIIa. In contrast, fVIIIa to which ESH8 was added after activation (Fig. 7, curve 6) did not bind to vWF, which demonstrates that ESH8 has to be bound to fVIII during the activation step to maintain fVIII affinity for vWF. This result explains the above observation that fVIII activation followed by addition of ESH8 did not have any effect on equilibrium 1. We demonstrated, therefore, that the dissociation of fVIIIa from the vWF in the presence of mAb ESH8 is slower than in its absence and it is incomplete due to equilibrium 1.

Reduction of Activated LCh Release from vWF by ESH8—Since fVIII binds to vWF through its LCh (5, 8), it is expected that reduction of fVIIIa release is due to changes caused by ESH8 in the LCh of fVIII. If this is true, the effect of ESH8 on release of fVIIIa or thrombin cleaved LCh will be similar. To confirm this, the release of fVIIIa or activated LCh from vWF in the presence of mAb ESH8 was compared. Since activation of the LCh-vWF complex is significantly slower than that of the fVIII-vWF complex (32), the concentration of thrombin for activating the former at a similar rate was increased 50-fold. Results are shown in Fig. 6B. The reduction of the initial rate of the activated LCh release in the presence of ESH8 (5-fold), was similar to that determined above for fVIIIa (4.3-fold). Similarly to that observed for fVIIIa (Fig. 6A), the percent of activated LCh released from vWF in the presence of ESH8 approaches plateau at 43%, suggesting that similarly to that demonstrated for ESH8-fVIIIa, ESH8-cleaved LCh complex is also able to bind to vWF.

Effect of mAb ESH8 on Kinetics of Activation of fVIII-vWF Complex—In order to find a direct link between the inhibitory effect of ESH8 on the kinetics of fVIIIa release from vWF and inhibition of fVIII activity in the functional Xase assay, we studied the kinetics of fVIIIa generation upon activation of the fVIII-vWF complex in the presence and absence of ESH8. The effect of ESH8 on fVIII activation in the presence of vWF is shown in Fig. 8. In this experiment we calculated the concentration of fVIIIa using a calibration plot of initial velocity of factor X activation versus fVIIIa concentration (24). The initial rate of activation of fVIII-vWF complex formed in the absence of mAb ESH8 (0.2 nM fVIIIa/min) is approximately 10 times greater than that in the presence of ESH8. Inactivation of fVIIIa, which is responsible for the bell-shaped curve of the time dependence of the fVIIIa concentration, involves fVIIIa dissociation into the A2 subunit and A1/A3-C1-C2 heterodimer (40, 41). The peak concentration of fVIIIa achieved in the presence of ESH8 was 8-fold lower than that in its absence. In a similar experiment we found that ESH8 did not have any effect on the activation rate of fVIII (10 nM) in the absence vWF, as expected (data not shown). In the absence of vWF, the time course of fVIII activation was similar to that of fVIII-vWF complex.
Inhibition of FVIII Release from von Willebrand Factor

The possibility that reduction of FVIIIa release (Fig. 6A) from
vWF by mAb ESH8 is solely responsible for the 8-fold reduction of
FVIIIa concentration (Fig. 8) during activation of FVIII-vWF complex
in the presence of ESH8 was tested by mathematical simulation of the process of FVIII activation using the experimental data of the time course of FVIIIa release in the presence
and absence of mAb ESH8. The model was based on the following
previous observations (7, 32): 1) cleavage at residue 1689 and release of FVIIIA from vWF is required for FVIII activation,
2) released FVIIIA must be cleaved at residue 372 to become fully active, and 3) cleavage at position 740 is fast relative to that at position 372, therefore, it was not taken into consideration as a rate-limiting step. In addition, we assumed that the rate of FVIIIa inactivation due to dissociation of A2 from A1/A3-C1-C2 dimer is unaltered by bound ESH8.

The following equations were used to simulate the time
course of generation of functionally active cofactor (FVIIIa)
upon thrombin activation of FVIII-vWF and ESH8:FVIII-vWF complexes. The concentration of released activated FVIII (Ract)
during time interval Δt was calculated using Equation 1:

\[ R_{act}(\Delta t) = FVIIIa(t) + Δt \times FVIIIa(t + \Delta t) - R_{act}(t) \times FVIIIa(t) \]  

where FVIIIa(t) + Δt(1) and FVIIIa(t) are the fractional release of FVIIIa from vWF and the fractional cleavage at residue 372 at a given time t, respectively. In the absence or presence of mAb ESH8, vrel(1) and v372(1) were obtained as the continuous functions of time in the time interval 0–1800 s by fitting data of FVIIIa release (Fig. 6A) or fractional cleavage at residue 372 (data not shown) versus time to the exponential formula: \[ 1 - \exp(-kt) \]. Parameters a and k for each fit were determined using the Sigmaplot 1.02 computer program (Jandel Scientific). Taking into consideration inactivation of FVIIIa, its concentration at the time t + Δt is given by the Equation 2:

\[ FVIIIa(t + \Delta t) = FVIIIa(t) + R_{act}(\Delta t) - k_d FVIIIa(t) \times \Delta t \]  

where \( k_d \) is the dissociation rate constant for FVIIIa dissociation from vWF. The model uses the values of FVIIIa concentration calculated for the time t using Equations 1 and 2 to calculate those for the time t + Δt. The process was continually repeated to calculate the concentration of FVIIIa for each interval of time (Δt) using the Excel 4.0 program (Microsoft, Inc.). In order to avoid artificial results due to insufficiently small time intervals of (Δt), the model was subjected to repeated analyses at decreasing values of Δt until a convergent result was obtained. The calculated concentrations of FVIIIa (Fig. 8, lines) and those determined experimentally (Fig. 8, points) differ by ≤ 15%, which indicates that the effect of ESH8 on FVIIIa release is sufficient to explain inhibition of FVIII coagulant activity by mAb ESH8.

**DISCUSSION**

Our examination of the effect of mAb ESH8 on the ability of activated FVIII to act as a cofactor for factor IXa in the activation of factor X (24) demonstrated that ESH8 was inhibitory in this assay only in the presence of vWF; however, vWF was not required for high affinity ESH8 binding to FVIII. FVIIIa release from vWF after thrombin activation is an ultimate requirement for FVIIIa to exert its cofactor activity (7), since bound vWF prevents FVIII from binding to a phospholipid surface (42–44) and to factor IXa (45), which are both required for assembly of the factor Xase complex. Our studies have demonstrated that ESH8 inhibits FVIIIa release from vWF upon thrombin cleavage of the FVIII-vWF complex. The simplest explanation for the mechanism of this effect is that ESH8 inhibits thrombin cleavage at Arg1689, which is critical for FVIII release from vWF (8). We found, however, that ESH8 does not inhibit any of the three thrombin cleavages at positions Arg1689, Arg372, and Arg5008.

In the absence of ESH8, the fractional cleavage at Arg1689 within FVIII or isolated LCh was similar to the fraction of FVIIIa or activated LCh released from vWF. In our experiments, which require an immobilization step for determination of FVIIIa release, we were not able to isolate the FVIIIa-vWF complex and to determine the rate constant for FVIIIa dissociation from vWF. By contrast, in the presence of ESH8 we isolated the ESH8:FVIIIa-vWF complex and determined the rate constant of 4 × 10⁻³ s⁻¹ for FVIIIa dissociation from vWF. Although these data do not allow us to quantitatively compare dissociation rates of hypothetic FVIIIa-vWF complex and the experimentally demonstrated ESH8:FVIIIa-vWF complex, they suggest that in the absence of ESH8 dissociation of FVIIIa from vWF is a faster process that in the presence of ESH8. We also demonstrated that dissociation of the ESH8:FVIIIa-vWF complex is a reversible process due to ability of the ESH8:FVIIIa complex to bind to vWF. The final release of FVIIIa in the absence of ESH8 was complete (> 95%) whereas in the presence of ESH8 it was < 50% due to the equilibrium ESH8:FVIIIa-vWF = vWF + ESH8+FVIIIa. Our data, therefore, suggest that inhibition of FVIIIa release by ESH8 results from both the slowed and the incomplete dissociation of ESH8:FVIIIa-vWF complex, suggesting that these factors also contribute to inhibition of FVIII coagulant activity by ESH8.

We demonstrated that ESH8:FVIIIa is able to bind vWF only if ESH8 is bound to FVIII during the thrombin activation step; therefore, ESH8 binding to FVIIIa does not restore its affinity for vWF. It would then be expected, that addition of ESH8 following activation of the FVIII-vWF complex will not have any effect on the release of FVIIIa from vWF. This is consistent with the observed lack of inhibition of FVIIIa activity by ESH8 in the factor Xase assay when it is added after thrombin activation of the FVIII-vWF complex (Fig. 1).

We used experimental data of the time course of FVIIIa release from vWF in the presence and absence of mAb ESH8 to mathematically simulate the time course of FVIII activation by thrombin. The similarity of calculated FVIIIa concentrations and those determined experimentally in the factor Xase assay is consistent with the hypothesis that inhibition of FVIII coagulant activity by mAb ESH8 is caused only by its effect on FVIIIa release from vWF after thrombin cleavage. The slowed rate of release allows time for FVIIIa inactivation via dissociation of the A2 subunit from the active heterotrimeric cofactor A1/A2/A3-C1-C2 prior to its release from vWF and participation in the assembly of the factor Xase complex. This explanation implies that both the released FVIIIa and FVIIIa in the ESH8:FVIIIa-vWF complex inactivates at a similar rate due to dissociation of the A2 subunit.

FVIIIa release from vWF includes two steps: cleavage at position 1689 within the LCh of FVIII and dissociation of thrombin cleaved FVIII from vWF. In this model, ESH8 affects only the latter step, which results in a slowed and incomplete FVIIIa dissociation from vWF. The lower affinity of ESH8 for A3-C1-C2 than that for intact LCh, suggests that thrombin cleavage may produce a conformational change in the C2 domain of the LCh. We hypothesize that ESH8 bound through the FVIII activation step prevents this change, which is required for rapid release of vWF from its C2 domain binding site. Our hypothesis implies that ESH8 interferes with a step which normally occurs during FVIII activation, but our data do not allow us to exclude the possibility that ESH8 induces a novel nonphysiological change.
in the conformation of the LCh which results in its inhibitory effect.

Inhibition of fVIIIa release from vWF also represents an additional, novel mechanism of fVIII inhibition by a rare human inhibitor antibody, case 4 (21). The case 4 epitope, C2 domain residues 2218–2307, does not include amino acids 2308–2312 from the common core of the epitope, residues 2248–2312, of 11 previously characterized anti-C2 human inhibitors (19, 21). Case 4 like ESH8 does not inhibit fVIII binding to PS. Thus, the properties of case 4 are unique among monoclonal and human inhibitor antibodies that bind to C2. We conclude from our results that the vWF and PS binding sites, which are both localized to residues 2293–2332, do not completely overlap and that the PS site appears to be situated more amino-terminally. In addition, the vWF binding site and the site determining inhibition of fVIIIa release from vWF do not overlap significantly. The case 4 characteristics could be explained by a single type of antibody, but we cannot exclude the possibility that two different antibodies with slightly offset epitopes which cannot be distinguished by immunoblotting (21) inhibit fVIII/PS binding and fVIIIa release from vWF.

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