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Research Article

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Inhibition of Human Factor VIIIa by Anti-A2 Subunit Antibodies

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Abstract

Human inhibitory alloantibodies and autoantibodies to Factor VIII (FVIII) are usually directed toward the A2 and/or C2 domains of the FVIII molecule. Anti-C2 antibodies block the binding of FVIII to phospholipid, but the mechanism of action of anti-A2 antibodies is not known. We investigated the properties of a patient autoantibody, RC, and a monoclonal antibody, 413, that bind to the region which contains the epitopes of all anti-A2 alloantibodies or autoantibodies studied to date. mAb 413 and RC were noncompetitive inhibitors of a model intrinsic Factor X activation complex (intrinsic FXase) consisting of Factor IXa, activated FVIII (FVIIIa), and synthetic phospholipid vesicles, since they decreased the V_{max} of intrinsic FXase by > 95% at saturating concentrations without altering the K_m. This indicates that RC and mAb 413 either block the binding of FVIIIa to FIXa or phospholipid or interfere with the catalytic function of fully assembled intrinsic FXase, but they do not inhibit the binding of the substrate Factor X. mAb 413 did not inhibit the increase in fluorescence anisotropy that results from the binding of Factor VIIIa to fluorescein-5-maleimidyl-D-phenylalanyl-prolyl-arginyl-FIXa (Fl-M-FPR-FIXa) on phospholipid vesicles in the absence of Factor X, indicating it does not inhibit assembly of intrinsic FXase. Addition of Factor X to Fl-M-FPR-FIXa, FVIIIa, and phospholipid vesicles produced a further increase in fluorescence anisotropy and a decrease in fluorescence intensity. This effect was blocked completely by mAb 413. We conclude that anti-A2 antibodies inhibit FVIIIa function by blocking the conversion of intrinsic FXase/FX complex to the transition state, rather than by interfering with formation of the ground state Michaelis complex. (*J. Clin. Invest.* 1994. 93:2497–2504.) Key words: Factor VIII • Factor IX • Factor X • inhibitor • kinetics

Introduction

Factor VIII (FVIII)[1] is a plasma glycoprotein consisting of a series of domains, A1-A2-B-A3-C1-C2, that are defined by internal sequence homology (1, 2). FVIII is activated by thrombin to form a 160-kD A1/A2/A3-C1-C2 heterotrimer (3). Thrombin-activated FVIII (FVIIIa) functions in blood coagulation by increasing the catalytic efficiency of Factor IXa toward the membrane-dependent, intrinsic pathway activation of Factor X (4).

Alloantibodies which inactivate FVIII (inhibitors) develop in ~ 20% of patients with hemophilia A in response to therapeutic infusions of FVIII. In addition, autoantibodies that inactivate FVIII rarely develop in individuals with previously normal FVIII levels. FVIII inhibitors present a serious clinical problem in which FVIII replacement therapy is frequently difficult or impossible. IgG from individual inhibitor patients usually includes antibodies that are directed against the amino-terminal half of the 40-kD A2 domain or the 20-kD C2 domain (5–7). Rarely, inhibitory antibodies with epitopes outside the A2 and C2 domains have also been identified (8–10).

Anti-C2 inhibitors prevent the binding of FVIII to phospholipid, and they have thus been proposed to act by interfering with membrane-dependent assembly of FVIIIa into the intrinsic FXase complex (11). This hypothesis is consistent with the observation that the C2 domain contains a binding site for phospholipid (12). In addition, the FVIII C2 domain is homologous to the C domains also found in human coagulation Factor V (13, 14) or the discoidins from *Dictyostelium discoideum* (1), both of which have been shown to have lipid-binding properties (15, 16).

The A2 domain is required for FVIIIa activity (17–19), but its function is not known. The minimal epitope for one human anti-A2 inhibitor has been localized to amino acid residues 345–536 of FVIII (20), which overlap the amino terminus of the A2 domain, defined herein as residues 373–740. Recombinant A2 domain polypeptides 336–606 (20) and 373–740 (10) were able to neutralize the FVIII inhibitory activity of a number of anti-A2 antibodies. Competition immunoradiometric assays in which several inhibitor IgG preparations were evaluated for inhibition of binding of a radiolabeled inhibitor IgG to recombinant A2 have indicated that, despite the large region that currently defines the A2 epitope, anti-A2 inhibitors appear to bind to the same or to closely spaced epitopes (7). The above results suggest that a common inhibitor epitope is located within residues 373–606 of the A2 domain. To evaluate the mechanism of inhibition of anti-A2 antibodies, we have investigated the effects of a human autoantibody (RC) and a mouse monoclonal antibody (413), both with anti-A2 specificity, on intrinsic FXase assembly and function.

Methods

Materials. Methoxycarbonyl-D-cyclohexylglycyl-glycyl-arginine-*p*-nitroanilide (Spectrozyme Xa) and murine mAb ESH 8 were purchased from American Diagnostica Inc. (Greenwich, CT). Small unilamellar phosphatidylcholine/phosphatidylserine (PCPS) (75/25 wt/wt) vesicles were prepared as described previously (21).

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1. *Abbreviations used in this paper:* Fl-M-FPR-FIXa, fluorescein-thioacetyle-maleimidyl-FPR-FIXa; FVIII, Factor VIII; FVIIIa, thrombin-activated FVIII; intrinsic FXase, complex of FIXa, FVIIIa, and PCPS; PCPS, phosphatidylcholine-phosphatidylserine vesicles.

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Protein isolation. Published procedures were used to isolate porcine Factors IXa, X, and Xa (22, 23) and porcine FVIIIa (3). Porcine fluorescein-5-maleimidyl-D-phenylalanyl-prolyl-arginyl-FIXa (Fl-M-FPR-FIXa) was prepared as described previously (24). These proteins were > 95% pure as judged by SDS-PAGE. Human FVIIIa was isolated by activating recombinant FVIII (4,000 U/mg) with thrombin, followed by CM-Sepharose chromatography, as described previously (25). The specific activity of the human FVIIIa preparation was 130,000 U/mg in a two-stage clotting assay (25). Human FVIIIa was stored at −80°C in small aliquots at a concentration of 2 μM in 0.6 M NaCl, 25 mM sodium acetate, 5 mM $CaCl_2$, and 0.01% Tween 20, pH 5.5. This procedure produces a heterotrimeric FVIIIa preparation that is stable for at least 24 h when thawed at 4°C. Human FIXa was purchased from Enzyme Research Laboratories (South Bend, IN). mAb 413 and RC IgG and Fab' fragments were purified as described previously (7).

Extinction coefficients and molecular masses. Extinction coefficients ($E^0_{1\,cm}$, $^{1\%}$) at 280 nm and molecular masses used were: porcine FVIIIa, 1.60, 160 kD (3); porcine FIXa 1.52, 45 kD (22); porcine FX 1.04, 57 kD (22); IgG 1.4, 150 kD (26). The extinction coefficient of human FVIIIa was assumed to be the same as that for porcine FVIIIa.

Inhibitor neutralization and competition binding assays. Specific details have been described previously (10). In the neutralization assay, a recombinant A2 domain polypeptide (amino acid residues 373–740) produced in Sf9 insect cells and partially purified was mixed in varying concentrations with inhibitor preparations. The inhibitors were diluted to an activity of ~ 5 Bethesda units/ml as defined by the Bethesda assay (27). After a 2-h incubation at 37°C, the residual inhibitor titer was determined using the Bethesda assay. The concentrations of inhibitor and FVIII were adjusted so that 90–95% of the added FVIII was inactivated in the absence of A2. The mAb 413 IgG was diluted into Tris-buffered saline with 1% BSA as a stabilizer.

The competition between mAb 413 and RC for binding to FVIII was studied by enzyme-linked immunoassay. FVIII (1 U/ml) was captured in Immulon 2 microtiter plate wells (Dynatech, Inc., Chantilly, VA) with immobilized mAb ESH 8, which binds to the FVIII light chain. The binding of mAb 413–biotin (0.83 ng/ml) to FVIII in the presence or absence of variable concentrations of RC IgG was detected by using steptavidin alkaline phosphatase and its chromogenic substrate *p*-nitrophenyl phosphate.

FVIIIa assay. A coupled chromogenic substrate assay for FVIIIa was used in which Factor X was activated at 25°C by an enzymatic complex consisting of FIXa, PCPS, and limiting amounts of FVIIIa in 0.15 M NaCl, 0.02 M Hepes, 5 mM $CaCl_2$, and 0.01% Tween 80, pH 7.4 (buffer A), in the presence of the chromogenic substrate for FXa, methoxycarbonyl-D-cyclohexylglycyl-glycyl-arginine-*p*-nitroanilide. The concentrations of reagents and the sequence of additions are described in the figure legends. Absorbance at 405 nm was measured at 3-s intervals for 1–10 min in a spectrophotometer (DU-65; Beckman Instruments, Inc., Fullerton, CA) at 25°C. Data collection was limited to < 10% conversion of FX to FXa and < 10% hydrolysis of the chromogenic substrate. In principal, if both enzymes, intrinsic FXase and FXa, obey the Michaelis-Menten equation, plots of A_{405} versus the square of time should be linear, from which the initial velocity of product (FXa) formation can be calculated, given the kinetic parameters, K_m and k_{cat}, the catalytic rate constant (obtained by dividing V_{max} by total enzyme concentration) for chromogenic substrate hydrolysis by FXa (28). These parameters were determined under the same conditions as the coupled assay using purified FXa. In practice, the slope of the plots decreased slightly, possibly secondary to decay of FVIIIa, but could be fit in all instances with excellent precision with a second degree polynomial (root mean squared deviation < 0.002 absorbance units). The initial velocity of FXa activation was estimated from the first derivative of the calculated second-order polynomial curve evaluated at zero time.

The steady state kinetic parameters, K_m and V_{max}, for the activation of porcine FX by intrinsic FXase in the presence and absence of inhibitor were calculated by nonweighted nonlinear least-squares fits to the Michaelis-Menten equation by using the Marquardt algorithm (29). Results are expressed as ±1 SD, assuming the error in the dependent variable is normally distributed and that there is no error in the independent variable.

Binding of FVIIIa to anti-A2 antibodies. The binding of FVIIIa (C) to antibody (I) is assumed to occur by the step-wise equilibria:

$$C + nI = nC \cdot I. \tag{1}$$

If it is assumed that antibody molecules bind independently to n equal sites on FVIIIa, then the concentration of FVIIIa–antibody complex ($C \cdot I$) is given by the quadratic equation:

$$C \cdot I = \frac{(nC_0 + K_i + I_0) - \sqrt{(nC_0 + K_i + I_0)^2 - 4nC_0I_0}}{2}, \tag{2}$$

where K_i is the microscopic dissociation constant, C_0 is the total concentration of FVIIIa, I_0 is the total concentration of antibody, and n is the number of antibody molecules bound per FVIIIa molecule. When the initial velocity, v, of FX activation is proportional to the concentration of FVIIIa, the fractional inhibition of FVIIIa (f), is given by:

$$f = \frac{C \cdot I}{C_0} = 1 - \frac{v}{v_0}, \tag{3}$$

where v_0 is the initial velocity in the absence of antibody. Values of f were determined from measurements of v and v_0 at fixed concentrations of FVIIIa, FIXa, FX, and PCPS with the concentration of antibody as the independent variable as described in the figure legends. Experimental values of $C \cdot I$ calculated from Eq. 3 were fit to Eq. 2 by nonweighted nonlinear least-squares analysis. The fitted parameters were n, K_i, and f_{sat}, the fractional inhibition at saturation. Calculation of the free concentration of antibody, I, for f versus log I plots was done using experimentally determined values of f from Eq. 3 and the fitted value of n according to:

$$I = I_0 - nC_0f. \tag{4}$$

Fluorescence measurements. Steady state fluorescence anisotropy measurements of Fl-M-FPR-FIXa were made in buffer A at 25°C by using spectrofluorometer (SLM-8000; SLM Aminco, Urbana, IL) in T-format with Glan-Thompson polarizers in the excitation (λ_{ex} 490 nm) and emission light paths as described previously (24). Fluorescence intensity measurements were calculated from the same readings using the relationship:

$$I = I_{\parallel} + 2I_{\perp}, \tag{5}$$

where I is the intensity and $I_{\parallel}$ and $I_{\perp}$ are vertical and horizontal components, respectively, of the emission intensity after correction for instrument factors as described (30). Corrected fluorescence emission spectra were obtained under the same conditions and were calculated by using software supplied by the manufacturer.

Electrophoresis. SDS-8% polyacrylamide gel electrophoresis was done using the Laemmli buffer system (31), and the protein bands were visualized by silver staining (32).

Results

Characterization of anti-A2 antibodies. In a previous study, eight human inhibitor IgG preparations with anti-A2 specificity inhibited the binding of the ^{125}I-Fab' fragment of inhibitor, CC, to recombinant A2 subunit (7). These results suggested that the inhibitors share an epitope or that they recognize closely spaced epitopes within the A2 domain. The analysis of FVIII inhibitors is complicated by the presence, in most inhibitor plasmas, of at least two distinct antibodies directed toward epitopes in the A2 and C2 domains and occasionally toward epitopes outside these domains. We have identified a human autoantibody inhibitor patient, RC, whose IgG bound only to the A2 domain using immunoblotting (33) and immunoprecipitation (7, 10) assays and which inhibited CC ^{125}I-Fab' binding

to FVIII (7). RC IgG, prepared from plasma by the caprylic acid method (7), has an inhibitor titer of 100 Bethesda units/mg IgG. We cannot strictly exclude the presence of other anti-FVIII antibodies. However, the ability of a recombinant A2 domain to completely neutralize RC inhibitor activity (Fig. 1) indicates that if other antibodies are present they are either not inhibitors of FVIII function or their concentration is too low for detection. Therefore, RC was selected as a prototype anti-A2 inhibitor.

A monoclonal antibody, 413, was found to bind the common A2 epitope since it also inhibited CC ^{125}I-Fab' binding to FVIII, and, conversely, CC inhibited mAb 413 binding to FVIII (7). Additionally, the ability of RC IgG to inhibit mAb 413–biotin binding to FVIII was evaluated as described in Methods and was found to produce 50% inhibition at 0.2 mg/ml (data not shown), providing further evidence that both antibody preparations share a common epitope. mAb 413 is a potent anticoagulant, with a titer of ~ 30,000 Bethesda units/mg IgG. The activities of mAb 413 and RC in the Bethesda assay are neutralized to 50% by approximately the same concentration of recombinant A2 fragment (Fig. 1). For unknown reasons, mAb 413 is incompletely neutralized (80%) at high concentrations of A2. mAb 413 is useful since it provides a defined, pure preparation of anti-A2 antibody. It is particularly suited for analysis of the assembly of intrinsic FXase by fluorescence measurements, which require relatively high concentrations (10–100 nM) of FVIIIa and correspondingly high concentrations of antibody for stoichiometric inhibition. The similar epitope specificity of RC and mAb 413 suggested that anti-A2 inhibitors may share a common inhibitory mechanism as well, which was tested in this study.

Inhibition of intrinsic FXase by mAb 413. Recently, a procedure has been developed to isolate human FVIIIa as an active heterotrimer (25). This preparation can be frozen in aliquots in pH 5.5 buffer and is stable for at least 24 h when thawed at 4°C. The use of purified FVIIIa avoids the potential confounding effects due to effects of antibody on FVIII activation or due to the presence of FVIII activation fragments. FVIIIa activity was assayed by its ability to promote Factor X activation by

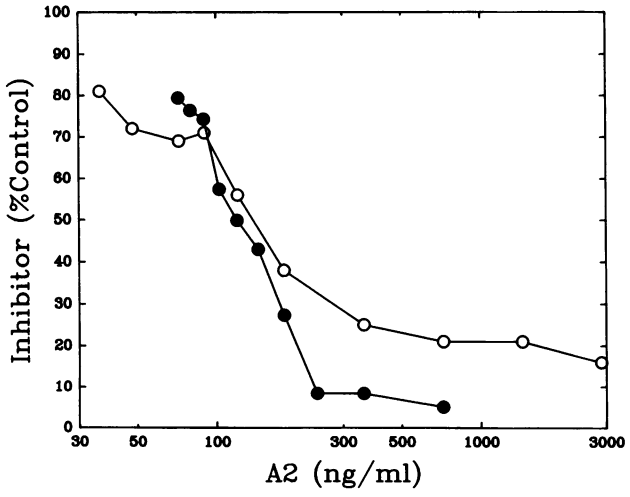

Figure 1. Neutralization of RC IgG and mAb 413 inhibitor activity by FVIIIa A2 subunit. RC (●) or mAb 413 (○) inhibitor IgG was incubated with the indicated concentrations of recombinant A2 subunit, and residual FVIII inhibitory activity was measured as described in Methods.

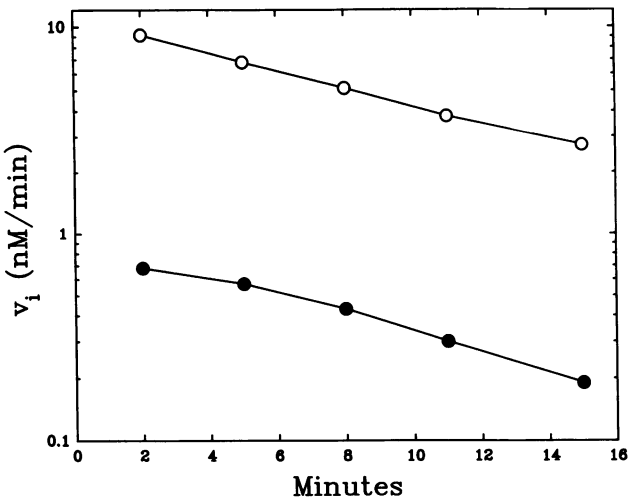

Figure 2. Inhibition of intrinsic FXase by mAb 413. Human FVIIIa was diluted to 0.2 nM in a mixture containing 5 nM human FIXa and 20 μM PCPS in buffer A, followed immediately by addition of 0.2 nM mAb 413 (●) or buffer control (○). At the times indicated, porcine FX and a chromogenic substrate for FXa, methoxycarbonyl-D-cyclohexyl-glycyl-glycyl-arginine-*p*-nitroanilide, were added to 100 nM and 0.6 mM, respectively, and the initial velocity (v_i) of FX activation was measured as described in Methods.

FIXa in the presence of PCPS vesicles in a defined, plasma-free system in the presence or absence of mAb 413 IgG (Fig. 2). mAb 413 or a buffer control was added to a mixture of Factor IXa, PCPS, and limiting FVIIIa, followed at various times by addition of porcine FX and chromogenic substrate for FXa. Under the conditions used, the initial velocity of FX activation is proportional to the concentration of FVIIIa in the sample. In the buffer control, FVIIIa activity decayed because of the dissociation of the A2 subunit which occurs after dilution of FVIIIa into pH 7.4 buffer (17–19). mAb 413, at a concentration selected to give partial inhibition of FVIIIa activity, caused a rapid initial drop in activity, but it had no effect on the spontaneous loss of activity. This indicates that the inhibitory effect of mAb 413 was complete by the time the first measurement was made. Additionally, this result indicates that mAb 413 does not inhibit FVIIIa by increasing the dissociation rate of the A2 subunit of FVIIIa, but acts instead by direct inhibition of intrinsic FXase complex. When porcine FVIIIa was substituted for human FVIIIa in this assay, mAb 413, at concentrations up to 1 μM, produced no decrease in FVIIIa activity (data not shown).

In contrast to its effect on FVIIIa, mAb 413 had no apparent effect on the activation of FVIII by thrombin. In this experiment, 0.1 μM human FVIII was incubated with 1 μM mAb 413 or buffer control in 0.15 M NaCl, 0.02 M Hepes, pH 7.4, for 20 min at room temperature, followed by addition of thrombin to 0.1 nM. SDS-PAGE analysis at various times up to 1 h showed no difference in the rate of appearance of the A1, A2, or A3-C1-C2 subunits compared with the buffer control (data not shown).

Effect of mAb 413 and RC antibodies on the steady state kinetics of FXa activation by intrinsic FXase. Woolf-Augustinsson-Hofstee transformations of the initial velocity of FX activation by intrinsic FXase in the presence of two fixed concentrations or in the absence of mAb 413 are shown in Fig. 3. The fitted parameters indicate that mAb 413 produces a signifi-

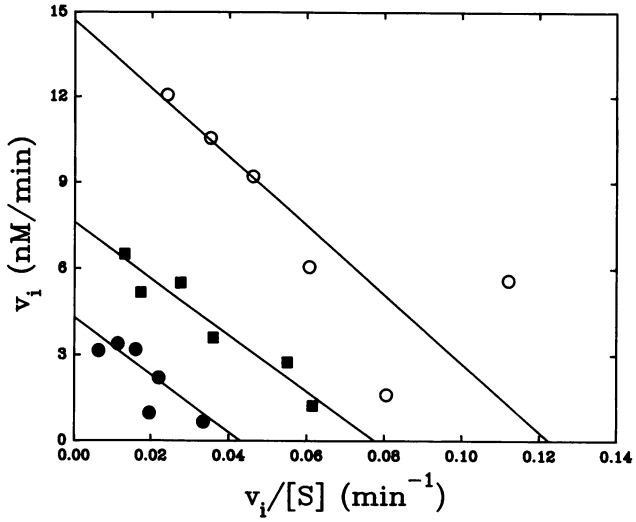

Figure 3. Steady state kinetics of the inhibition of intrinsic FXase by mAb 413. Human FVIIIa was diluted to 0.2 nM in a mixture containing 5 nM human FIXa and 20 μM PCPS in buffer A at 25°C, followed by addition of mAb to 0.1 nM (●), 0.05 nM (■), or buffer control (○). After 2 min, porcine FX at 20, 50, 100, 200, 300, or 500 nM and methoxycarbonyl-D-cyclohexylglycyl-glycyl-arginine-*p*-nitroanilide at 0.6 mM were added, and the initial velocity (v_i) of FX activation was measured as described in Methods. The data are plotted by using the Woolf-Augustinsson-Hofstee transformation. The regression lines are derived from nonlinear least-squares analysis of the Michaelis-Menten equation.

cant decrease in V_{max} but no change in K_m (Table I), which is indicated graphically by the approximately parallel regression lines. This pattern is indicative of noncompetitive inhibition of intrinsic FXase by mAb 413. Noncompetitive inhibition of intrinsic FXase by the Fab' fragment of mAb 413 and by RC IgG was also observed (Table I). These data indicate that the mechanism of action of naturally occurring human anti-A2 antibodies and mAb 413 is the same. The variation in the kinetic parameters at zero concentration of inhibitor shown in Table I is probably because of variation in the PCPS preparations used. For a given preparation there was no significant variation during the 2–3 h required to compare inhibitor with buffer control.

Noncompetitive inhibitors are usually studied under conditions in which the inhibitor concentration is in large excess. However, because of their high affinity for FVIIIa, mAb 413 and mAb 413 Fab' produce significant inhibition at nearly equal molar concentrations of FVIIIa and antibody. Thus, the free concentration of antibody is significantly less than the added concentration, which must be considered in the quantitative analysis of binding of antibody inferred from the kinetic data. The inhibition of FVIIIa activity as a function of antibody concentration was determined experimentally and fit to a quadratic function as described in Methods to determine the dissociation constant for inhibition, K_i, and the apparent stoichiometry for the binding of antibody to FVIIIa (Fig. 4). The binding of mAb 413 to FVIIIa is very tight, with a calculated K_i of 10 pM. The n value is slightly less than one, which is consistent with measurements of the binding of human FVIIIa to FIXa which indicate that a fraction of the human FVIIIa preparation is inactive (25). The calculated n value for binding of mAb 413 Fab' is sixfold greater than for mAb 413, suggesting that a significant fraction of the mAb 413 Fab' preparation is inactive. If this is the case, then the calculated K_i value is proportionately overestimated. Additionally, the fractional inhibition at saturating concentrations of mAb 413 or mAb 413 Fab', f_{sat}, calculated from the data indicates complete or near complete inhibition of intrinsic FXase when inhibitor is bound to FVIIIa.

Table I. Inhibition of Intrinsic FXase by Antibodies

Antibody	Concentration	K_m	V_{max}
	nM	*nM*	*nM/min*
mAb 413 IgG	0	120±26	14.7±1.1
	0.05	98±21	7.6±0.5
	0.1	100±36	4.3±0.5
mAb 413 Fab'	0	220±60	17.8±2.1
	0.4	220±34	12.0±0.84
RC IgG	0	65±20	14.0±1.1
	600	72±22	8.9±0.70

Effect of mAb 413 on assembly of intrinsic FXase. The usual interpretation of noncompetitive inhibition is that the inhibitor is bound to the Michaelis complex, rendering it inactive. However, for multicomponent enzymes like intrinsic FXase, noncompetitive inhibition could occur because of inhibition of the assembly of the enzyme complex, e.g., by blocking the binding of FVIIIa to FIXa or the binding of FVIIIa to PCPS.

The membrane-dependent binding of FVIIIa to a fluorescent, active site-blocked derivative of FIXa, Fl-M-FPR-FIXa, can be monitored by measuring the increase in fluorescence anisotropy of Fl-M-FPR-FIXa that occurs upon binding (24, 34). Therefore, the potential effects of mAb 413 on assembly of intrinsic FXase were assessed by using this assay. The presence of excess mAb 413 during the titration of Fl-M-FPR-FIXa with human FVIIIa did not significantly affect the fluorescence anisotropy (Fig. 5, *open and closed circles*). This indicates that

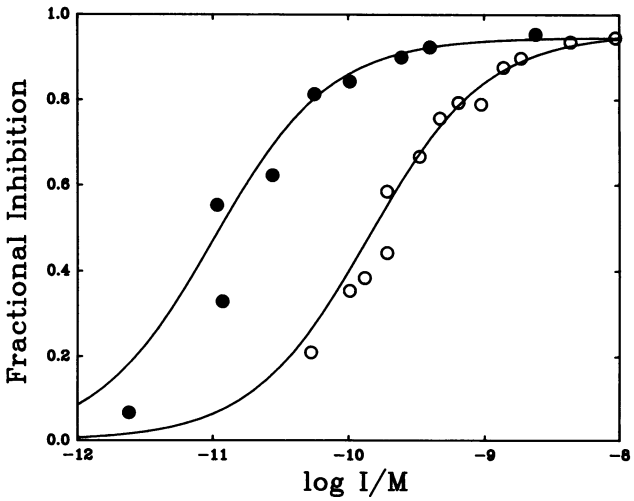

Figure 4. Titration of FVIIIa by mAb 413 and mAb 413 Fab'. The experimental conditions were as in Fig. 3, except that the concentration of porcine FX was fixed at 300 nM, and the concentrations of mAb 413 (●) or mAb 413 Fab' (○) were varied. The curves are calculated from Eq. 3 according to K_i, n, and f_{sat} of 10.1±3.5 pM, 0.58±0.04, and 0.95±0.02 for mAb 413 and 140±46 pM, 3.5±0.4, and 0.96±0.02 for mAb 413 Fab', respectively, as described in Methods.

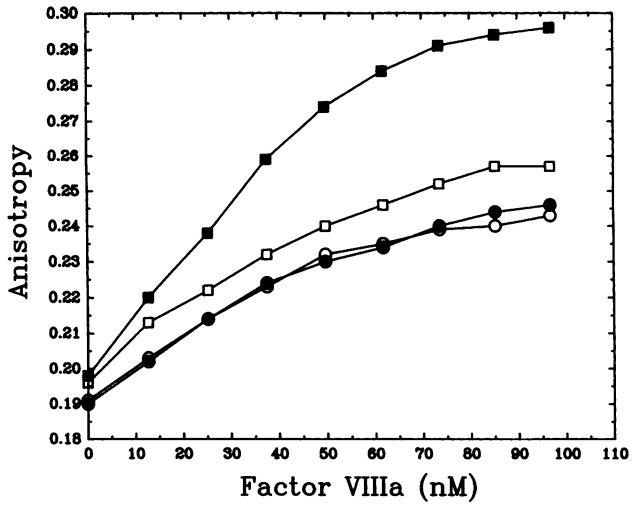

Figure 5. Human FVIIIa–dependent increase in fluorescence anisotropy of Fl-M-FPR-FIXa in the presence and absence of FX and mAb 413. Reaction mixtures (0.5 ml) contained 19 nM porcine Fl-M-FPR-FIXa and 100 μM PCPS, varying concentrations of human FVIIIa, and either no addition of FX or mAb 413 (●), 360 nM mAb 413 (○), 290 nM porcine FX (■), or 360 nM mAb 413 plus 290 nM porcine FX (□).

mAb 413 does not interfere with the binding of FVIIIa to Fl-M-FPR-FIXa or to the PCPS membrane and it thus appears to behave as a classical noncompetitive inhibitor.

Effect of FX on fluorescence properties of Fl-M-FPR-FIXa. During the course of these experiments, we found that FX produces a large increase in the fluorescence anisotropy of Fl-M-FPR-FIXa compared with FVIIIa/Fl-M-FPR-FIXa alone (Fig. 5, *closed squares*). The increment in anisotropy ($\Delta\Delta r$) due to FX was 0.056 at the highest concentration of FVIIIa. Fig. 5 also shows that this effect was FVIIIa dependent, since when the FVIIIa concentration was zero, only a slight increase ($\Delta r = 0.007$) in anisotropy was observed.

Additionally, FX produced a significant ($\approx$ 20%) decrease in the fluorescence intensity of Fl-M-FPR-FIXa in the same experiment (Fig. 6). The concentration of FX used, 290 nM, was saturating with respect to both the increase in fluorescence anisotropy and the decrease in fluorescence intensity (data not shown). In contrast, in the absence of FX, the addition of FVIIIa increased the fluorescence anisotropy of Fl-M-FPR-FIXa (Fig. 5), but it did not affect the fluorescence intensity (Fig. 6). Interestingly, mAb 413 blocked ~ 90% of the FX-dependent increase in fluorescence anisotropy ($\Delta\Delta r = 0.005$) (Fig. 5) and it completely blocked the FX-dependent decrease in intensity (Fig. 6). Titration curves essentially identical to those shown in Fig. 5 were obtained by substituting 360 nM mAb 413 Fab' for mAb 413 under otherwise identical conditions (data not shown). When FX was added at the completion of a titration of FVIIIa on Fl-M-FPR-FIXa instead of before the addition of FVIIIa, a similar increase in fluorescence anisotropy and decrease in intensity was observed. In contrast to the results in Fig. 5, in which mAb 413 blocked the increase in fluorescence anisotropy by $\approx$ 90%, addition of mAb 413 to preformed FX/FVIIIa/Fl-M-FPR-FIXa complex completely reversed the FX-dependent increase in anisotropy (data not shown). The reason for the small difference in anisotropy because of the order of addition of reagents is not known.

FX also produced an increase in fluorescence anisotropy of Fl-M-FPR-FIXa produced by porcine FVIIIa (Fig. 7) and a 20% decrease in fluorescence intensity (data not shown). Emission spectra in the presence and absence of FX did not show a wavelength shift in the emission maximum. However, mAb 413 did not block these FX-dependent fluorescence changes, consistent with its lack of inhibition of porcine FVIIIa in the functional assay. Titrations of Fl-M-FPR-FIXa with porcine FVIIIa can be fit to a quadratic function to calculate the apparent K_d and stoichiometry, n, for the binding of FVIIIa to

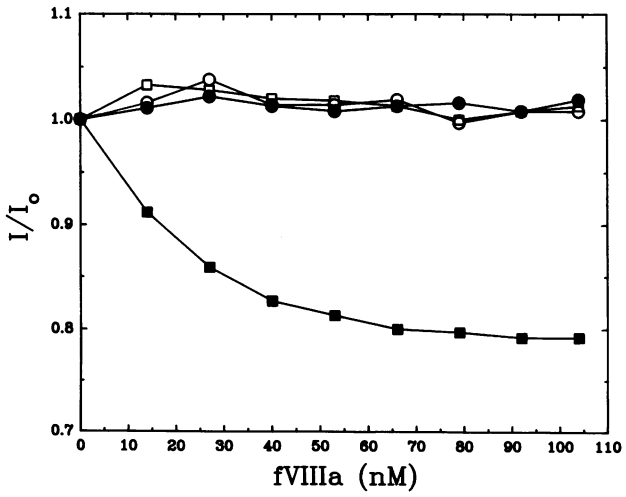

Figure 6. Human FVIIIa–dependent change in fluorescence intensity of Fl-M-FPR-FIXa in the presence and absence of FX and mAb 413. Conditions and symbols correspond to those in Fig. 5. The fluorescence intensity, I, was measured as a function of FVIIIa concentration and compared with the intensity at zero FVIIIa (I_0).

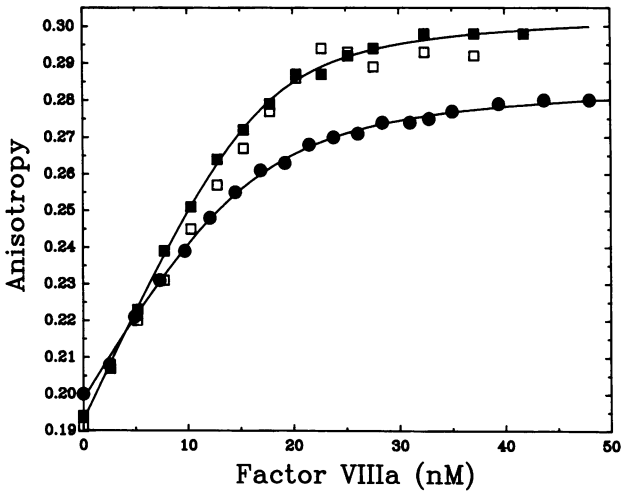

Figure 7. Binding of porcine FVIIIa to Fl-M-FPR-FIXa in the presence of FX. Conditions were as in Fig. 5, except that porcine FVIIIa was substituted for human FVIIIa. In addition to porcine Fl-M-FPR-FIXa and porcine FVIIIa, either 290 nM porcine FX (□), 360 nM mAb 413 plus 290 nM porcine FX (■), or no FX or mAb 413 (●) were present. The two curves are calculated from least-squares fits in the absence of mAb 413 and in the presence or absence of FX to a quadratic function described previously (24), assuming the binding of FVIIIa to n identical, noninteracting sites on Fl-M-FPR-FIXa. The fitted parameters for the dissociation constant (K_d), stoichiometry (n), and anisotropy at saturation (r_{sat}) are 1.3±0.3 nM, 0.89±0.03, and 0.304±0.002 in the presence of FX and 2.3±0.4 nM, 0.77±0.03, and 0.286±0.002 in the absence of FX, respectively.

Fl-M-FPR-FIXa (24). Fits in the presence and absence of FX are shown in Fig. 7. FX does not appear to affect the binding energy of FVIIIa to Fl-M-FPR-FIXa to a great extent because of the small differences observed in the calculated K_d and n values (Fig. 7, legend).

The observation that FX appears to bind Fl-M-FPR-FIXa in the presence of FVIIIa was unexpected since the active site of FIXa, including the specificity pocket, is presumably blocked by the arginyl chloromethylketone. This would preclude the interaction of the region of FX that interacts with the active site of FIXa during scissile bond cleavage. It has been noted previously that Fl-M-FPR-FIXa has no detectable activity toward FX in the presence of FVIIIa (24), but the sensitivity of the measurements was not established. Therefore, the activation of 300 nM FX by FIXa/FVIIIa/PCPS and by Fl-M-FPR-FIXa/FVIIIa/PCPS for 5 min were compared in the porcine system under the conditions described in Fig. 2, except that 0.3 nM FVIIIa was used. The initial velocity of FX activation by FIXa was 63.7 nM/min, whereas Fl-M-FPR-FIXa produced no detectable increase in absorbance. A conservative estimate of the sensitivity of the instrument is > 0.0015 A_{405}/min, which corresponds to > 0.04 nM FXa/min. This indicates that Fl-M-FPR-FIXa is > 99.9% inactive.

It is not possible to quantitatively analyze the binding of human FVIIIa to Fl-M-FPR-FIXa using the anisotropy measurements because the calculated stoichiometry values (FVIIIa molecules per Fl-M-FPR-FIXa molecule) of several human FVIIIa preparations tested are significantly greater than one, in the range of 1.5–2.0 (25). This indicates that part of the human FVIIIa preparation is inactive, precluding analysis of the binding affinity. However, the qualitative similarity between the human and porcine FVIIIa anisotropy measurements suggests that the predominant effect of FX is to alter the fluorescence properties of Fl-M-FPR-FIXa and not the energetics of the assembly of the intrinsic FXase complex. The effects of the anti-FVIII A2 antibodies used in this study indicate that they are bound to FVIIIa in the FX/FVIIIa/FIXa/PCPS complex and block the FVIIIa-dependent structural alteration of the complex induced by FX.

Discussion

In this study we investigated the mechanism of inhibition of A2 domain–specific antibodies that are directed against human FVIII. The similar epitope specificity (33) and the competitive binding of human and monoclonal inhibitors to the amino-terminal region of the A2 domain (residues 373–606) (7) suggested that they represent a class of antibodies with similar biological properties. Since many of them contain anti-FVIII antibodies which recognize other epitopes, however, we also used a monoclonal antibody, 413, which has the same properties, to investigate their mechanism of action. mAb 413 and a rare human autoantibody IgG, RC, with only anti-A2 inhibitor activity, were both found to inhibit the intrinsic FXase complex noncompetitively.

From the kinetic data alone, two explanations are consistent with noncompetitive inhibition. First, anti-A2 antibodies could block incorporation of FVIIIa into the FXase complex. Since FVIIIa promotes FX activation by increasing the k_{cat} of the reaction by several orders of magnitude but only slightly decreases the K_m (23, 35), inhibition of FVIIIa binding to FIXa would decrease the k_{cat}, producing noncompetitive inhibition. However, this mechanism appears to be excluded by the observation that the binding of FVIIIa to Fl-M-FPR-FIXa is not affected by mAb 413.

Alternatively, anti-A2 antibodies could bind to the entire Michaelis complex, consisting of membrane-bound FIXa, FVIIIa, FX, and PCPS, and could prevent turnover of the complex. This mechanism can be interpreted in terms of the following model of the assembly of intrinsic FXase in the absence of inhibitory antibodies, where E, C, and S refer to FIXa, FVIIIa, and FX, respectively (Scheme 1):

$$\begin{array}{lll} E + S \overset{K_{ES}}{\rightleftharpoons} & ES \overset{K_{ES}^{\ddagger}}{\rightleftharpoons} & ES^{\ddagger} \\ + & + & \\ C & C & \\ 1\downarrow K_{EC} & 1\downarrow K_{ESC} & \end{array} \qquad \text{(Scheme 1)}$$

$$EC + S \overset{K_{ECS}}{\rightleftharpoons} ESC \overset{K_{ECS}^{\ddagger}}{\rightleftharpoons} ESC^{\ddagger}.$$

This model corresponds to the conditions used in this study where phospholipid is present at saturating concentrations, E and C are membrane bound, and S can be either membrane bound or in solution. ES and ECS represent Michaelis complexes, whereas $ES^{\ddagger}$ and $ECS^{\ddagger}$ are transition state complexes for the formation of the FX-FIXa acyl-enzyme complex, which is the rate-limiting step in FX activation by intrinsic FXase (23). The transition state complexes are in equilibrium with the ground state, Michaelis complexes with formation of product being proportional to the concentration of transition state complexes (36). In the presence of anti-A2 antibodies, the equilibria involved are depicted by Scheme 2, where I denotes the inhibitor:

$$\begin{array}{lll} E + S \overset{K_{ES}}{\rightleftharpoons} & ES \overset{K_{ES}^{\ddagger}}{\rightleftharpoons} & ES^{\ddagger} \\ + & + & \\ CI & CI & \\ 1\downarrow K_{ECI} & 1\downarrow K_{ESCI} & \end{array} \qquad \text{(Scheme 2)}$$

$$ECI + S \overset{K_{ECSI}}{\rightleftharpoons} ESCI \overset{K_{ECSI}^{\ddagger}}{\rightleftharpoons} ESCI^{\ddagger}.$$

The binding of mAb 413 to FVIIIa is nearly stoichiometric at nanomolar concentrations since the association constant for the reaction is > 10^{11} M^{-1} (Fig. 4). Under these conditions, essentially all cofactor is present in CI or higher order complexes.

Previous kinetic analysis has indicated that in the presence and absence of FVIIIa, formation of the acyl enzyme is slow relative to the dissociation of the Michaelis complex (23). Thus, K_m^{-1} measured by steady state kinetic analysis approximates the association constants, K_{ECS} and K_{ES}, for Michaelis complex formation in the presence and absence of FVIIIa, respectively. Since mAb 413 and RC decrease the V_{max} but do not affect the K_m of intrinsic FXase, they do not appear to interfere with the substrate-binding steps shown in Scheme 2. Thus, since the anti-A2 inhibitory antibodies do not appear to inhibit substrate or cofactor binding to the enzyme, according to Scheme 2, they must block the progression of the ground state ESC complex to the transition state. Anti-A2 inhibitor effects are discussed in terms of the multiple equilibrium constants in Scheme 2 in more detail in the Appendix.

Fluorescence anisotropy and intensity measurements indicate that FX interacts with the active site-blocked Fl-M-FPR-FIXa/FVIIIa complex and that mAb 413 blocks this interaction. FX does not appear to affect the equilibrium binding parameters of FVIIIa to Fl-M-FPR-FIXa that lead to Michaelis complex formation (Fig. 7), which is consistent with previous

observations on the assembly of intrinsic FXase (24) and its homologous complex, prothrombinase (37). Instead, the data indicate that the FX/FVIIIa/Fl-M-FPR-FIXa complex has an altered structure compared with FVIIIa/Fl-M-FPR-FIXa alone, as judged by its increased fluorescence anisotropy and decreased fluorescence intensity at saturation. Whether this reflects a change in the environment of the fluorescent reporter group alone or a structural change in FIXa induced by the presence of both FVIIIa and FX that is propagated to the fluorescent reporter group is not known.

The observation that FX affects the fluorescence properties of Fl-M-FPR-FIXa in the presence of FVIIIa was surprising since one might predict that occupation of the active site of FIXa by the fluorescent tripeptidyl group would preclude the binding of FX, but that does not appear to be the case. How does mAb 413 block the effect of FX on the fluorescence properties of the Fl-M-FPR-FIXa/FVIIIa complex? We speculate that the Fl-M-FPR-FIXa/FVIIIa/FX complex has partial transition state character, corresponding to $ECS^{\ddagger}$ in Scheme 1, which is reflected by its fluorescence properties. By binding FVIIIa, the inhibitor blocks the ability of ECS to reach the transition state with the resultant change in probe fluorescence.

There are limitations to what we can say about the mechanism of action of anti-A2 inhibitors from the binding and kinetic data presented here. Scheme 2 indicates that it is not possible to determine which binary interaction is altered in the ESCI complex to inhibit its conversion to the transition state. Thus, it is not possible to determine whether anti-A2 antibodies inhibit a conformational change in FIXa toward the transition state induced by FVIIIa, or inhibit an interaction between FVIIIa and FX in the transition state. These considerations reflect our incomplete understanding of how FVIIIa and other protein cofactors in the hemostatic mechanism, including tissue factor, Factor Va, and thrombomodulin, increase the k_{cat} of their respective reactions. It is possible that FVIIIa induces a structural change in FIXa or FX or both to facilitate transition state formation. The A2 subunit could participate directly in this process or stabilize the FVIIIa molecule so that another structural region can interact with FIXa or FX.

A 10^4-fold lower concentration of mAb 413 compared with RC IgG is required to produce approximately equal inhibition in the intrinsic FXase assay (Table I). In contrast, the Bethesda titer of mAb 413 is only 300-fold higher than RC (30,000 vs 100 Bethesda units/mg IgG). In the intrinsic FXase assay, inhibitor activity can be interpreted at the molecular level, and for a homogeneous population of antibody such as mAb 413 the dissociation constant of the inhibitor for FVIIIa can be calculated (Fig. 4). In contrast, in the Bethesda assay, inhibitor activity is based on an arbitrarily defined prolongation of the fibrin clot time, so that it is not possible to make quantitative comparisons between the two assays. Most inhibitor plasmas are more complex than RC plasma, since the mechanism of inhibition additionally includes inhibition of FVIIIa binding to phospholipid due to anti-C2 antibodies, as well as noncompetitive inhibition of intrinsic FXase by anti-A2 antibodies. The relative contribution of anti-A2 and anti-C2 antibodies to the bleeding diathesis caused by inhibitors or to in vitro activity in the Bethesda assay is not known. It is possible that the Bethesda assay under- or overestimates the clinical importance of reaction of inhibitors to different FVIII epitopes. If so, the development of assays that measure epitope-specific anticoagulant activities of inhibitor plasmas may be more predictive in determining the response to therapy in inhibitor patients.

Appendix

Effect of anti-A2 antibodies on the equilibrium reactions during assembly and turnover of intrinsic FXase. FVIIIa produces a large increase in the k_{cat} of FX activation by FIXa and PCPS, ~ 2,000-fold under the conditions used in our laboratory (23). Thus, according to Scheme 1:

$$\frac{K_{ECS^{\ddagger}}}{K_{ES^{\ddagger}}} \approx 2{,}000.$$

In contrast the K_m is decreased by only about fourfold in the presence of FVIIIa, or:

$$\frac{K_{ECS}}{K_{ES}} \approx 4.$$

Since the inhibitor does not affect the K_m for the reaction, it follows that:

$$K_{ECSI} \approx K_{ECS},$$

and since the inhibitor does not affect the binding of FVIIIa to FIXa:

$$K_{ECI} \approx K_{EC}.$$

Because of the linkage relations:

$$K_{ECI}K_{ECSI} = K_{ES}K_{ESCI}$$

and

$$K_{EC}K_{ECS} = K_{ES}K_{ESC},$$

it follows that:

$$K_{ESCI} \approx K_{ESC}.$$

Thus, the inhibitor does not affect any of the ground state reactions involved in intrinsic FXase assembly and must affect the formation of the transition state:

$$K_{ECS^{\ddagger}} \gg K_{ECSI^{\ddagger}}.$$

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