An Antibody Specific for Coagulation Factor IX Enhances the Activity of the Intrinsic Factor X-activating Complex*

Received for publication, May 28, 2004, and in revised form, July 6, 2004
Published, JBC Papers in Press, July 20, 2004, DOI 10.1074/jbc.M405966200

Randolf J. Kerschbaumers, Klaudia Riedrichi, Martina Krali, Katalin Varadisi, Friedrich Dorner§, Jan Rosings, and Friedrich Scheifflinger‡**

From the §Pre-Clinical Product Development, Baxter BioScience, Biomedical Research Center, A-2304 Orth/Donau, Austria, ¶Vascular Biology, Baxter BioScience, A-1220 Vienna, Austria, and Cardiovascular Research Institute Maastricht, Maastricht University, 6200MD Maastricht, The Netherlands

During hemostasis the zymogen factor X (FX) is converted into its enzymatically active form factor Xa by the intrinsic FX-activating complex. This complex consists of the protease factor IXa (FIXa) that assembles, together with its cofactor, factor VIIIa, on a phospholipid surface. We have studied the functional properties of a FIXa-specific monoclonal antibody, 224AE3, which has the potential to enhance intrinsic FX activation. Binding of the antibody to FIXa improved the catalytic properties of the intrinsic FX-activating complex in two ways: (i) factor VIIIa bound to the FIXa-antibody complex with a more than 18-fold higher affinity than to FIXa, and (ii) the turnover number ($k_{cat}$) of the enzyme complex increased 2- to 3-fold whereas the $K_m$ for FX remained unaffected. The ability of 224AE3 to increase the FXa-generation potential (called the “booster effect”) was confirmed in factor VIII (FVIII)-depleted plasma, which was supplemented with different amounts of recombinant FVIII. In the presence of antibody 224AE3 the coagulant activity was increased 2-fold at physiological FVIII concentration and up to 15-fold at low FVIII concentrations. The booster effect that we describe demonstrates the ability of antibodies to function as an additional cofactor in an enzymatic reaction and might open up a new principle for improving the treatment of hemophilia.

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** To whom correspondence should be addressed: Baxter BioScience, Biomedical Research Center, Uferstrasse 15, 2304 Orth/Donau, Austria. Tel.: 43-1-20100-3410; Fax: 43-1-20100-4679; E-mail: scheiﬂing@baxter.com.
§ The abbreviations used are: FX, factor X; Fxa, factor Xa; rFVIII, recombinant factor VIII; FU, fluorescence units.

The physiological importance of the intrinsic FX-activating complex is well defined because the severe bleeding disorders hemophilia A and hemophilia B are characterized by very low plasma levels (<5%) or by the absence of the pro-cofactor FVIII and the pro-enzyme FIX, respectively. Treatment of hemophilia is successfully achieved by supplementing the blood of patients with either human FVIII or human FIX. Both proteins can be derived from human plasma or produced as recombinant protein in mammalian cell culture (6, 7). Although progress in the production of coagulation factors to ensure their purity, efficacy, and viral safety has been made over the last 20 years, treatment of hemophilia is still beset with several difficulties. First, production of recombinant and plasma-derived, therapeutic factors is expensive, and products predominately reach only the industrialized world. Second, ~30% of patients with hemophilia A and 1.5–3% of patients with hemophilia B develop antibodies against FVIII or FIX that inhibit the respective coagulation factor and complicate treatment (8–10).

Because of the clinical relevance of the intrinsic FX-activating complex, FVIII and FIX are important targets of clinical and biochemical research. A major focus of the research on the intrinsic FX-activating complex has been to overcome some of the limitations in hemophilia treatment mentioned above and to improve the production yields, reduce immunogenicity, and increase the specific activity of FVIII and FIX (11). For these purposes genetically engineered FVIII-derivatives (e.g. B-domain-deleted FVIII (12, 13)) and FIX-derivatives (e.g. point-mutated FIX (14) and chimeric FIX (15)) have been produced. In this study a completely different approach toward improving the efficiency of FX activation by the FIXa-FVIIIa complex is described. We isolated mouse monoclonal antibodies specific for human FIXa applying hybridoma technology. When we studied the influence of these antibodies on the intrinsic FX-activating complex we discovered an antibody, named 224AE3, that binds to FIXa and enhances both the formation and the catalytic activity of the intrinsic FX-activating complex. We call this effect of the 224AE3 antibody the “booster effect.”

EXPERIMENTAL PROCEDURES

Reagents—Human thrombin and human FIX, Fx, Fxa, and FIXa were purchased from Enzyme Research Laboratories (South Bend, IN). Recombinant human coagulation FVIII (rFVIII) was prepared by Baxter BioSciences (Glendale, CA). Bovine serum albumin was purchased from Calbiochem. Human FVIII-depleted plasma was obtained from Baxter BioSciences (Vienna, Austria). Nonspecific mouse IgG was purchased from Sigma. Phospholipid vesicles (60% dioleoylphosphatidylcholine, 40% dioleoylphosphatidylserine) were prepared by Baxter BioSciences (Vienna, Austria) from synthetic phospholipids (Avanti Polar Lipids, Alabaster, AL). Pathromtin SL was purchased from Dade Behring (Vienna, Austria).
Anti-factor IX Antibody Enhances Intrinsic FX Activation

Behring (Deerfield, IL), and Ancrod was purchased from the National Institute for Biological Standards and Control (Potters Bar, UK). Chromogenic substrate Pefachrome FXA (Pef-5523) and the thrombin inhibitor N-α-(2-naphthylsulfonyl)glycyl-4-amidino-(o-,l-)-phenylalanine piperidide were purchased from Pentapharm (Basel, Switzerland). The fluorogenic substrate Z-Gly-Gly-Arg-AMC.HCl was purchased from Bachem (Bubendorf, Switzerland).

**Hybridoma Cell Lines**—Balb/c mice were immunized four times with human FIXa at intervals of 1 week. At each immunization 100 µg of antigen was applied using Al(OH)3 as adjuvant. Spleen cells were obtained 3 days after the last immunization, and hybridoma cell lines were generated according to standard procedures (16). Cell lines expressing antibodies specific for human FIXa (all of them also bound to non-activated human FIX) were subcloned four to six times to ensure that the cell line was monoclonal and stable.

**Production and Purification of Antibodies and Fab, F(ab')2, and Fab Fragments**—Monoclonal hybridoma cell lines were grown in RPMI 1640 medium supplemented with 5% fetal calf serum (Invitrogen) for 2–3 weeks. IgG were purified over protein G-Sepharose 4 Fast Flow (Amersham Biosciences) according to standard procedures (17). Fab fragments and F(ab')2 fragments were prepared from the purified antibody using the ImmunoPure Fab Preparation Kit and the ImmunoPure F(ab')2 Preparation Kit, respectively (Pierce) according to the manufacturer’s instructions.

**FXa Generation Assay**—Reactions were performed in polypropylene tubes (Micronic, Lelystad, The Netherlands) in a water bath at 37 °C as follows: 152 µl of reaction buffer containing 50 mM Tris, pH 7.4, 175 mM NaCl, 10 mM CaCl2, 0.5 mg/ml bovine serum albumin, and 13.4 µM phospholipid vesicles was prewarmed to 37 °C, and 25 µl of an antibody/FXa mixture, 50 µl of rFVIII, and 5 µl of thrombin were added. After incubation for 2 min to activate FVIII, 23 µl of FX was added to start FXa generation. The final reaction mixture contained 8 µM phospholipid vesicles, 6 mM CaCl2, 10 nM thrombin, and concentrations of FIXa, rFVIII, FX, and antibody as described under “Results.” After different time intervals, 20-µl aliquots from the reaction mixture were transferred to 230 µl of EDTA buffer containing 50 mM Tris, pH 8.3, 9 mM EDTA, and 428 mM NaCl to stop FXa formation. The amount of FXa generated was determined by mixing 210 µl of the diluted aliquots with 40 µl of a mixture containing 5 mM FXa-specific chromogenic substrate Pef-5523 and 6 µM thrombin inhibitor N-α-(2-naphthylsulfonyl)glycyl-4-amidino-(o-,l-)-phenylalanine piperidide in a 96-well microplate and measuring the rate of chromogenic substrate conversion at 405 nm (ΔA/min) at 37 °C in a microplate reader (EMIS-Reader, Labsystems, Helsinki, Finland). The FXa concentration was calculated for each point in time using a calibration curve made with known amounts of FXa, and control experiments ensured that the antibody did not influence the cleavage of the chromogenic substrate Pef-5523 by FXa.

**Thrombin Generation Assay**—FVIII-depleted plasma was defibrinated with 1 units/ml Ancrod, and fibrin was removed with a plastic stirring rod. 40 µl of defibrinated plasma, 10 µl of antibody, 10 µl of rFVIII, and 25 µl of Pathromtin SL were mixed in 96-well microplates (black flat bottom, Greiner, Kremsmuenster, Austria) and prewarmed at 37 °C. 15 µl of a substrate-CaCl2 mixture was added to each well to give final concentrations of 7.5 mM CaCl2 and 0.5 mM thrombin-specific fluorogenic substrate Z-Gly-Gly-Arg-AMC.HCl. The concentrations of rFVIII and antibody are described under “Results.” Fluorescence (excitation, 340 nm; emission, 440 nm) was read immediately after substrate-CaCl2 addition every 30 s for 30 min in a fluorescence reader at 37 °C (SPECTRAFluor Plus Reader, Tecan, Groedig, Austria). The resultant curve shows the fluorescence units (FU) as a function of time. The tangent at each time point (ΔFU/min) is proportional to the thrombin concentration present in the plasma mixture. Thus, the first derivative of the resultant curve divided by a calibration factor for fluorogenic substrate conversion by thrombin (ΔFU/min/nM thrombin) gives a thrombin generation curve, i.e. the thrombin concentration in plasma as a function of time. The calibration factor was determined from a standard curve made with known amounts of human α-thrombin.

**RESULTS**

**Features of Antibody 224AE3**—We isolated a series of monoclonal antibodies that specifically bound to human FIX as well as human FXa. In preliminary tests of a number of these antibodies in a FXa-generation assay we discovered (in addition to antibodies that inhibited or had no effect on FX activation) one monoclonal antibody, termed 224AE3, that was able to strongly increase the rate of FX activation, and we called this effect the booster effect. Western blotting and enzyme-linked immunosorbent assay experiments showed that monoclonal antibody 224AE3 specifically bound to the heavy chain (protease domain) of human FIX and FXa and did not cross-react with other coagulation factors such as prothrombin, factor VII, FVIII, FX, or their activated counterparts. Bicore experiments revealed that this antibody bound with a very high affinity to FIXa, which was immobilized on a sensor chip. The on-rate was 1.1 × 106 M−1 s−1 but the off-rate was too low to be determined properly. Affinity thus was estimated to be in the low picomolar range (Kd < 50 pM) (data not shown).

**The Effect of Antibody 224AE3 on FXa Generation**—In our

![FIG. 1. The effect of antibody 224AE3 on time courses of FXa generation at different FVIII concentrations.](image-url)
first set of experiments we investigated the booster effect in more detail by determining the rate of FXa generation at 33 pm FIIAs and different concentrations of FVIIAs. The experiments were done either without antibody (Fig. 1A), at equimolar FIIA and antibody concentrations (33 pm, Fig. 1B), or with a large molar excess of antibody (1000 pm, Fig. 1C). The FX concentration was kept at 100 nm, which is at least 3-fold above \( K_m \) (see below). FXA generation curves were linear during the initial phase of the activation reaction, indicating that FXA was formed at a constant rate. After ~3 min, the rates of FXA generation decreased, which probably reflects inactivation of FVIIAs through dissociation of the A2 domain (18). The rates of FXA formation (nm/min) were calculated from the slope of the linear part of FXA generation. Surprisingly, we found that the FXA formation rates with the antibody were higher than without antibody and that the booster effect of 224AE3 became more pronounced at low FVIII concentrations. At 2 nm FVIII, the FXA generation rate increased ~4-fold, whereas at 100 pm FVIII3 (concentrations of FIIA and antibody were 33 pm), the antibody increased the rate of FXA generation ~35-fold.

Plots of rates of FXA formation as a function of the FVIII concentration (Fig. 2) could be fitted to a single-site ligand-binding model (19), which enabled calculation of the FVIII concentration required to obtain half-maximum rates of FXA formation (Table I). This value corresponds to the apparent \( K_d \) (\( K_{d(app)} \)) for dissociation of the phospholipid-bound FIXA-FVIII complex. Binding of 224AE3 to FIXA led to an 18-fold acceleration of FXA generation (2.8-fold compared with the value obtained in the absence of antibody. Optimal enhancement of \( V_{max} \) seems to occur at a FIXA:antibody ratio at which most antibody molecules have bound 

| Parameter                      | Without antibody | 33 pm 224AE3 | 1000 pm 224AE3 |
|--------------------------------|------------------|--------------|---------------|
| FVIII-saturation               |                  |              |               |
| \( K_{d(app)} \) (nm)          | 2.4 ± 0.3        | 0.13 ± 0.02  | 0.17 ± 0.04   |
| \( V_{max} \) (nm/min)        | 2.9 ± 0.6        | 5.7 ± 0.7    | 2.6 ± 0.3     |
| Michaelis-Menten kinetics      |                  |              |               |
| \( k_{cat} \) (min\(^{-1}\))   | 76 ± 10          | 202 ± 40     | 73 ± 5        |
| \( K_m \) (nm)                 | 26 ± 3           | 31 ± 4       | 29 ± 5        |
| \( k_{cat}/K_m \) (min\(^{-1}\) nm\(^{-1}\)) | 2.9              | 6.5          | 2.5           |

Optimal enhancement of \( V_{max} \) seems to occur at a FIXA:antibody ratio at which most antibody molecules have bound two FIXA molecules. In contrast, the decrease of the \( K_{d(app)} \) appears to be independent from such a double saturation. To prove this, we prepared monovalent Fab and divalent F(ab\(^\prime\))\(_2\) fragments of antibody 224AE3. These fragments were used in FVIII-titration experiments, and at all antibody fragment concentrations tested both fragments were able to enhance the affinity of FIXA for FVIII to a similar extent as the antibody; the monomeric Fab fragment gave a \( K_{d(app)} \) of 0.25 nm, and the dimeric F(ab\(^\prime\))\(_2\) fragment gave a \( K_{d(app)} \) of 0.11 nm. But at saturating FVIII concentrations the F(ab\(^\prime\))\(_2\) fragment (but not the Fab fragment) increased the rate of FXA generation. As expected, the ~3.5-fold enhancement was observed at a molar FIXA:F(ab\(^\prime\))\(_2\) ratio where double saturation of the dimeric fragment with FIXA can be expected (Fig. 3B).
FX-activating complex and on the affinity of the complex for FX (Km), we performed a Michaelis-Menten kinetic analysis. Rates of FX activation were determined as a function of the FX concentration (i) at 33 pM FIXa, 100 nM FX, 8 μM phospholipid vesicles, 6 mM CaCl2, and 6 nM FVIII without antibody or 3 nM FVIII when different antibody or antibody fragment concentrations were added. The resultant FX formation rates were plotted as a function of the corresponding antibody concentrations (0–2.5 nM) and (B) the corresponding F(ab’2) or Fab concentrations (0–1.0 nM).

The Effect of Antibody 224AE3 in Thrombin Generation Assays—The experiments described so far show the booster effect of the procoagulant 224AE3 antibody on intrinsic FX activation in a well defined model system containing purified proteins. We used thrombin generation assays to test whether the antibody also has a beneficial effect in plasma. The thrombin generation assay probes the whole intrinsic coagulation cascade from contact activation to the formation of thrombin as well as the inactivation of activated coagulation factors by plasma protease inhibitors. All reactants were present in plasma at their physiological concentrations except FVIII, as FVIII-depleted plasma was used and rFVIII was added at known concentrations. The method applied was a modified version of the thrombin generation assay described by Hemker and Beguin (20). In our approach, all reactants, including a fluorogenic thrombin substrate, were present in the reaction mixture, and after equimolar concentrations of FIXa and antibody, but at high antibody concentrations the kcat returned to the value determined without antibody. The Km for FX (26 ± 3 nM) was not affected by the presence of antibody, and in all cases its value was below the physiological (plasma) FX concentration (136 nM). Michaelis-Menten kinetic analysis with the Fab and the F(ab’2) fragment of antibody 224AE3 showed that the F(ab’2) fragment, like the antibody, increased the kcat of the intrinsic FX-activating complex 3-fold (246 min−1) only when FIXa and the F(ab’2) fragment were present in the same concentration range and that the monomeric Fab fragment had no effect on the kcat (74 min−1).

FIG. 3. The effect of varying concentrations of antibody 224AE3 or its fragments on FXa generation. FXa generation assays were performed at 33 pM FIXa, 100 nM FX, 8 μM phospholipid vesicles, 6 mM CaCl2, and 6 nM FVIII without antibody or 3 nM FVIII when different antibody or antibody fragment concentrations were added. The graphs show the FX formation rates plotted as a function of (A) the corresponding antibody concentrations (0–2.5 nM) and (B) the corresponding F(ab’2) or Fab concentrations (0–1.0 nM).

FIG. 4. Michaelis-Menten kinetic analysis of FX activation by FIXa-FVIIIa and FIXa-FVIIIa-antibody complexes. FXa-generation assays were performed at 33 pM FIXa, 8 μM phospholipid vesicles, 6 mM CaCl2, 3 nM FVIII with antibody, or 6 nM FVIII without antibody and at different FX concentrations (0–100 nM). The resultant FX formation rates were plotted as a function of the FX concentration. Hyperbolas were obtained without antibody ( ), with 33 pM antibody ( ), and with 1000 pM antibody ( ). Data were fitted to the Michaelis-Menten equation, and the kcat and Km values obtained are summarized in Table I.
Anti-factor IX Antibody Enhances Intrinsic FX Activation

The booster effect became gradually more pronounced at lower FVIII concentrations. At a FVIII concentration of 10 milliunits/ml the peak time measured corresponded to ~60 milliunits/ml FVIII. Below 1% FVIII (the region of severe hemophilia A) the presence of antibody increased the coagulant activity up to 15-fold. Thrombin generation assays were repeated with nonspecific mouse IgG, which had no influence on thrombin generation (data not shown).

DISCUSSION

FVIIIa functions as a cofactor of FIXa, which depending on the assay conditions increases the rate of FXa formation ~20,000- to 200,000-fold (3, 4). The exact mechanism by which FVIIIa enhances the catalytic activity of FIXa toward FX is still unknown. It is generally believed that FVIIIa has three functions within the intrinsic FX-activating complex: (i) FVIIIa stabilizes a conformation of FIXa that has strongly increased protease activity toward FX, (ii) FVIIIa acts as a receptor for FIXa on activated platelets (2), which in vivo provide the procoagulant phospholipid surface (21), and (iii) more recent data indicate that FVIIIa directs the cleavage sites in FX toward the active site of FIXa (22). We identified a murine monoclonal antibody, 224AE3, that specifically binds to human FIXa and that enhances the catalytic activity of the FVIIIa-FIXa-Ca$$^{2+}$$-phospholipid complex. Kinetic analysis revealed that the antibody had two different modes of action: first it increased the affinity of FIXa for FVIIIa $$\sim$$ 18-fold, and second it caused a 2.5-fold increase of the $$k_{cat}$$ of FX activation. Overall, at low FIXa and FVIIIa concentrations (FVIIIa and FIXa in picomolar range) the antibody enhanced the rate of activation of FX to FXa more than 30-fold.

Plasma assays confirmed the results obtained in the model systems and verified the potential of what we called the booster effect of the antibody 224AE3. Assays in FVIII-depleted plasma, which was supplemented with known amounts of rFVIII, showed that the procoagulant activity was enhanced up to 15-fold in the presence of the antibody. Again, the booster effect became more pronounced at low FVIII concentrations. The kinetic data indicate that at high FVIII concentrations the booster effect of the antibody is primarily caused by a 2.5-fold increase in the $$k_{cat}$$ of FX activation, which is in line with the 2-fold enhancement of FVIII activity at physiologic FVIII concentrations. At lower FVIII concentrations the enhanced affinity of FIXa for FVIIIa becomes more important because lower FVIII concentrations are required to saturate FIXa bound to 224AE3 than to saturate FIXa without antibody.

The FXa-generation experiments show that our antibody does not influence the loss of FVIII activity observed during FX activation in model systems. In the presence as well as absence of antibody, the rate of FXa generation decreases after ~3 min. Thus, the antibody does not prolong FXa generation, but it increases the initial rate of FX activation. Inactivation of FVIII in this assay is probably by dissociation of the A2 domain (18). We also concluded from the thrombin generation curves that 224AE3 only amplifies the activity of the intrinsic FX-activating complex and does not influence other reactions of the intrinsic coagulation cascade. The shapes of the curves are the same with and without antibody and show a thrombin burst and inhibition of the generated thrombin after the peak has been reached. Thus, neither the activation of coagulation factors other than FX nor their inhibition or inactivation seems to be affected by 224AE3.

Antibodies are known to be versatile molecules that have properties beyond their classic immunological function and have been shown to be able to modify the biochemical and biological properties of their target proteins. For instance, antibodies with catalytic properties have been described that...

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**Fig. 6.** Effect of antibody 224AE3 on the coagulant activity (determined in FVIII-depleted plasma supplemented with different FVIII concentrations). Thrombin generation assays were performed as described under “Experimental Procedures” in FVIII-depleted plasma containing 60 nM 224AE3 and supplemented with different FVIII concentrations. The result...
serve as enzymes. Numerous inhibitory antibodies have been identified that block enzymes, neutralize cytokines, or act as receptor antagonists. However, antibodies that have agonistic properties are relatively rare but have been generated for some receptors (23–26). The antibody 224AE3, which was isolated by conventional hybridoma technology, shows a new feature as it functions as an additional cofactor in an enzyme reaction within the coagulation cascade. The molecular mechanism of action of this antibody is unknown, but our data suggest that this antibody stabilizes a conformation of the protease FIXa, which has an increased affinity for its cofactor FVIIIa. The enhanced FIXa-FVIIIa interaction (effect on $K_{d \text{app}}$) is obtained when FIXa is saturated with antibody or its Fab or $\text{F(ab')}_2$ fragment and does not alter when the antibody is present in large molar excess. However, the increase of the turnover number ($k_{\text{cat}}$) is lost at high antibody or $\text{F(ab')}_2$ fragment concentrations and is not observed in experiments with the monomeric Fab fragment. This indicates that the effect of the antibody on the $k_{\text{cat}}$ requires a double saturation of the antibody (2 moles of FIXa per mole of antibody), and thus the cross linkage of two FX-activating complexes.

Although the practical relevance of antibodies that enhance intrinsic FIXa generation and that promote coagulation and clot formation has to be proven, the booster effect we describe might open up a new principle for improving treatment of hemophilia. A human antibody, an antibody derivative, or an antibody mimetic that shows the same features as 224AE3 could be developed and used as a specific enhancer of coagulation in patients with hemophilia.

Acknowledgment—We thank Elise Langdon-Neuner for expert editing.

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J. Biol. Chem. 2004, 279:40445-40450.
doi: 10.1074/jbc.M405966200 originally published online July 20, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405966200

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