A Monoclonal Antibody to VIII:C Produced by a Mouse Hybridoma

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Spleen cells of a BALB/c mouse immunized with factor VIII procoagulant activity (VIII:C) (isolated by affinity chromatography) were fused with mouse myeloma cells (P3 × 63 Ag8). After the fusion, 12/32 wells produced an inhibitor to VIII:C. Cells from one well (1B3) were subcloned four times in order to isolate the hybridoma that produces the anti-VIII:C antibody. Injection of hybridoma cells in pristane pretreated BALB/c mice results in anti-VIII:C titers of 5000–10,000 Bethesda U/ml. Analysis of the produced immunoglobulin demonstrated heavy chains of IgG1 (produced by the myeloma cell line) and IgG2a subclass. The 1B3 antibody neutralizes VIII:C in LMW FVIII, cryosupernatant, cryoprecipitate, and normal plasma. It was found that binding of the IgG to VIII:C results in a delay in its activation and not in an inhibition of its cofactor activity. The antibody removes VIII:C from pooled normal plasma when coupled to Sepharose; when coupled to plastic tubes, it binds VIIIICAG from isolated VIII:C, purified FVIII, and pooled normal plasma; it does not bind VIIIIR:AG, fibrinogen, or serum VIIIICAG. The 1B3 antibody can be used successfully in an IRMA for VIIIICAG.

MATERIALS AND METHODS

VIII:C (or LMW FVIII) was isolated in a 3-step procedure consisting of cryoprecipitation, gel-chromatography on Biogel A 15M, and affinity chromatography on anti-HMW FVIII Sepharose-4B.1 VIII:C, eluting from the affinity column in the presence of 0.25 M CaCl2, was collected either in 30 times diluted mouse serum (nonimmune BALB/c mice; the serum was heated 30 min at 56°C) or in 0.1% bovine serum albumin (BSA). Fractions containing VIII:C were dialyzed against 0.01 M Tris-HCl (pH 7.1), 0.015 M CaCl2 for 2 hr when used for immunization or in neutralization studies.

Human factor IXa, factor X, and factor VIII were isolated as previously described.12

Immune Mouse Spleen Cells

Female BALB/c mice (12–14 wk) were immunized by intraperitoneal injection of 0.5–2.5 U VIII:C on day 1 (mixed with Freund incomplete adjuvant), and on days 24, 56 and 65 (mixed with Freund incomplete adjuvant), followed by an intravenous injection of 0.8 U VIII:C on day 79. Three days later, mice were sacrificed. A spleen cell suspension was prepared in 10 ml HEPES 1640 medium. After removal of cellular debris, white cells were counted, centrifugated at 200 g (5 min), and resuspended in HEPES 1640 medium.
Preparation of Mouse Anti-VIII:C-Producing Hybridoma

Immune mouse spleen cells (10^8) were fused with P3 X 63 Ag8 myeloma cells (3 X 10^5) in the presence of 1 ml 50% PEG 1000. Fusion was stopped by dilution after 1 min. Cells were seeded in 36 wells of a Limbro tissue culture dish (Limbro Division, Flow Laboratories Inc, Hamden, Conn.) and grown on selective medium (HAT medium). After 13 days, supernatants were tested for the presence of anti-VIII:C activity. Clonation of anti-VIII:C-producing hybridomas was performed by growing the cells in 0.25% soft agar (starting three times with 1000 cells/well and once with 50 cells/well); after sufficient growth of the cells, individual clones were isolated using a micropipet and an inverse phase contrast microscope. These clones were expanded separately in 30% conditioned RPMI 1640 medium and assayed for the production of anti-VIII:C. Cells from anti-VIII:C producing hybridomas were stored in liquid nitrogen.

Assay of Anti-VIII:C Activity

Supernatants of cultured hybrid cells were centrifuged and tested for the presence of anti-VIII:C activity by a modified inhibitor assay: pooled normal plasma was diluted 5 times in Michaelis buffer; 200 µl of the diluted plasma was incubated with 200 µl supernatant (2 hr, 37°C), after which the mixture was assayed for residual VIII:C; to this purpose, 0.1 ml undiluted sample was added to a mixture of 0.1 ml VIII:C-deficient plasma (plasma of a patient with severe hemophilia A) and 0.1 ml of a phospholipid/kaolin suspension, which had been preincubated for 25 min at 37°C. After a further 5-min incubation (37°C), coagulation was started by the addition of 0.1 ml 0.033 M CaCl2. Clotting times, more than 10 sec higher than that of a control (culture medium without antibodies), were considered as a positive indication for the presence of anti-VIII:C antibodies. The potency of the anti-VIII:C antibodies was expressed in Bethesda units.

Production of Monoclonal Antibodies in Mouse Ascites

BALB/c mice were treated with either complete Freund adjuvant or pristane to induce ascites. About 7 days later, 10^6 hybridoma cells were injected intraperitoneally. Mice were observed daily; ascites was punctured from day 7 till 14 (sometimes more than once); whenever a solid tumor developed, tumor cells were cultured in vitro.

Purification of Anti-VIII:C Antibodies From Ascites

Ascites was treated at 56°C for 30 min, after which any precipitate was removed by centrifugation (5 min, 12,000 rpm). Supernatant was dialyzed against 0.01 M sodium phosphate (pH 7.3), 0.15 M NaCl (PBS), and passed through a protein A Sepharose column (3 ml gel/ml ascites). The column was washed with 3 volumes of PBS before IgG was eluted in a linear KCNS gradient (2 x 50 ml; 0-2 M KCNS). Anti-VIII:C activity eluted at the very start of the gradient. No anti-VIII:C activity was found either at high KCNS concentrations or after subsequent elution with 0.1 M glycine (pH 2.5). Anti-VIII:C preparations (400-1000 U/ml) were dialyzed against 0.01 M sodium citrate (pH 7.7), 0.15 M NaCl, and stored at 4°C in the presence of 0.02% sodium azide.

Subclass specificity of immunoglobulin produced by mouse hybridomas was analyzed by two methods. (1) A modified double antibody sandwich ELISA technique: wells of a microtiter plate were coated with goat anti-mouse IgG (Miles, Slough, U.K.); to these wells were added subsequently 50 µl culture supernatant containing hybridoma antibody, rabbit anti-mouse IgG subclass antibody, peroxidase-labeled sheep anti-rabbit IgG, and 5-amino benzoic acid. (2) By analysis of the material obtained by radioimmunoprecipitation with subclass specific antibodies.

Radioimmunoprecipitation

Protein from 50 µl culture supernatant was iodinated using the chloramine-T technique.14 After treatment with Dowex 1 X2-100 ion exchange resin and overnight dialysis, the radioiodinated protein was isolated using a protein A Sepharose column and assayed for the presence of anti-VIII:C. Activity was measured as displaced iodide, which was determined by a modified method described by Weiss et al.18 VIII:C was measured as described by Veltkamp et al.19 using plasma from a patient with severe hemophilia A as substrate plasma. One unit of activity or antigen is defined as the amount of activity or antigen present in 1 ml of pooled normal plasma. Factor Xa was measured by means of its amidolytic activity towards S2337 under conditions described previously.16

Two-dimensional immunoelectrophoresis of VIIIIR:AG was performed as described earlier using a specific rabbit antiserum against isolated HMW FVIII.3 Immunodiffusion with specific antisera (rabbit anti-mouse IgG subclass; Miles, Slough, U.K.) was performed using agarose 2.5% (agarose 1.2%, 2.5% agarose) as described by Ouchterlony.16

Purified monoclonal anti-VIII:C antibodies were immobilized (1) by coupling to CNBr-activated Sepharose-4B (about 10 ug/mg protein/g dry weight) according to the instructions of the manufacturer (Pharmacia, Uppsala, Sweden), and (2) by coating of plastic tubes with purified antibody (40 µg/ml) in 0.1 M NaHCO3 (pH 8.6). These tubes were used in an immunoradiometric assay (IRMA) for FVIII:Ag.

Human anti-VIII:C IgG was coated to plastic tubes as described earlier.10 125I-fibrinogen (specific activity 8 x 10^6 cmp/mg protein) was kindly provided by Dr. W. Noyens (Gaubius Institute TNO, Health Research Organisation, Leiden).

A two site immunoradiometric assay (IRMA) for FVIII:Ag was developed using the monoclonal hybridoma anti-VIII:C in the first phase and a 125I-labeled human anti-VIII:C in the second phase. Experimental details are identical to those described earlier.10 A reference curve was constructed from serial dilutions of pooled normal plasma.

Blood was collected in 1/10 volume of 0.11 M sodium citrate. Pooled normal plasma was prepared from the platelet-free plasmas (30 min, 20,000 g, 4°C) of 32 healthy volunteers (14 women, 18 men; average age 26.7 yr) and stored at -70°C. Pooled normal serum was prepared from the sera (supernatant of blood stored in glass for 2 hr at 37°C and overnight at 4°C) of 30 healthy volunteers.
RESULTS

Production and Selection of Mouse Hybridomas Producing Anti-VIII:C

After fusion of mouse myeloma cells with splenocytes of a mouse immunized with isolated human VIII:C, the fusion products were seeded in 36 wells; 14 days later, the supernatants of 12 wells were found to contain significant amounts of anti-VIII:C activity. This study details the properties of anti-VIII:C produced by a hybridoma isolated from one of these wells (1B3). The 1B3 hybridoma was cloned by growing the cells in 0.25% soft agar (1000 cells/well). All wells produced anti-VIII:C activity; cells from one of these wells were expanded and again distributed over different wells on soft agar. This procedure was repeated 3 times; at the fourth cycle the initial number of cells was reduced to 50/well; under these circumstances it was possible to isolate individual clones from one of the positive wells. One of the anti-VIII:C-producing clones, which on statistical grounds contains only one type of hybridoma, has been used for in vitro and in vivo production of anti-VIII:C antibodies.

For the detection of antibodies in cell culture supernatants, a modified inhibitor assay was used; the specificity of the assay was checked by replacing the VIII:C-deficient plasma by FIX-deficient plasma: a result was considered to be positive only when no anti-factor-IX activity could be demonstrated. Anti-VIII:C titers in supernatants were maximally about 1 U/ml; however, titers were difficult to estimate due to the peculiar properties of this antibody (see below).

When the hybridoma cells were cultured in the ascites of BALB/c mice, anti-VIII:C titers of 5000–10,000 U/ml were obtained; this titer equals the titer found in the serum of the immunized mouse on the day of the fusion.

IgG Subclass of Anti-VIII:C Produced by Hybridoma 1B3

Culture supernatants of hybridomas producing anti-VIII:C activity were analyzed with respect to the presence of mouse IgG of the different subclasses (IgG1, IgG2a, IgG2b, IgG3). By means of a modified double antibody sandwich ELISA technique and the analysis of the radiolabeled supernatant protein precipitated with subclass-specific antisera, the presence of an immunoglobulin containing both IgG1 and IgG2b chains was demonstrated.

The monoclonal antibody (isolated from ascites by protein A Sepharose gradient elution) was found to be a homogeneous IgG preparation as analyzed by SDS-polyacrylamide gel electrophoresis (after reduction) and contains two components when analyzed by isoelectric focusing: pI 6.98 and 7.09 (see Fig. 1).

Anti-VIII:C Activity of the Hybridoma Antibody (1B3)

Table 1 shows that the time necessary to reach equilibrium binding of purified monoclonal antibody

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<th>Incubation Time (min)</th>
<th>Residual VIII:C (U/ml)</th>
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<tr>
<td></td>
<td>- Anti-VIII:C</td>
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<tr>
<td>15</td>
<td>0.38</td>
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<td>30</td>
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One volume pooled normal plasma was incubated with one volume monoclonal antibody (1B3) (500 U/ml) or with one volume of buffer; after different time intervals, samples were diluted and assayed for residual VIII:C.

![Fig. 1. Isoelectric focusing pattern of isolated 1B3 immunoglobulin.](image1)

![Fig. 2. Dose-response curves for mouse anti-VIII:C serum, human anti-VIII:C serum, and monoclonal antibody. One volume of pooled normal plasma was incubated with 1 volume antiserum (dilution) for 2 hr at 37°C. Residual VIII:C was measured and expressed in U/ml; (●●●●) human anti-VIII:C; (□□□□) mouse anti-VIII:C; (x–x) monoclonal antibody (1B3).](image2)
to plasma VIII:C is less than 15 min. This is considerably less then generally found for human anti-VIII:C antibodies. In Fig. 2, the dose–response curve for the monoclonal antibody has been compared with those for a human antibody and for the mouse anti-VIII:C serum (at the time of the fusion). It is obvious that the slope of the dose–response curve is much weaker in the case of the monoclonal antibody. Furthermore, it should be noted that even an undiluted IgG preparation does not inhibit plasma VIII:C completely.

In Table 2, the action of purified monoclonal antibody on VIII:C in plasma, cryoprecipitate, cryosupernatant, and isolated LMW FVIII was compared. In all cases substantial but incomplete inhibition of the factor VIII procoagulant activity was obtained. In the LMW FVIII preparation, no VIIIR:AG could be detected by a specific electroimmunoassay.

When purified monoclonal antibodies coupled to Sepharose were incubated with pooled normal plasma, the VIII:C in the supernatant decreased significantly without affecting the VIIIR:AG concentration (see Table 3).

The isolated VIII:C (used for the immunization of the mouse, from which the spleen cells were removed for the fusion experiment) was not always free from trace amounts of fibrinogen. Because all inhibitory effects of the antibody reported so far depend on the results of coagulation assays, it was checked if the antibody could bind fibrinogen. The experiment of Table 4 shows that the same amount of 125I-fibrinogen is bound to tubes coated with the monoclonal hybridoma anti-VIII:C as to tubes coated with an isolated human anti-VIII:C; in both cases, the presence or absence of fibrinogen in the incubation mixture does not influence the amount of radioactivity bound to the tubes.

Incubation of pooled normal plasma with undiluted monoclonal anti-VIII:C had no effect on the ristocetin cofactor activity nor on the VIIIIR:AG precipitation arc in a two-dimensional immunoelectrophoresis.

The Effect of the Monoclonal Anti-VIII:C on the Intrinsic Factor X Activator

The activation of factor X by factor IXa in the presence of phospholipids and Ca²⁺ is stimulated enormously by the addition of activated factor VIII. When the reaction is started in the presence of nonactivated factor VIII, a sigmoidal time course is obtained for the Xa formation (see Fig. 3), which is due to activation of FVIII by small amounts of Xa formed during the initial phase of the reaction. The lag time in the generation of factor Xa is independent of the actual VIII:C concentration, but depends mainly on the activation state of the VIII:C (Mertens and Bertina, unpublished observations). When excess of a human anti-VIII:C was added to the factor VIII preparation prior to its addition to the reaction mixture, a curve was obtained similar to that in Fig. 3 in the absence of added FVIII. However, when the FVIII was preincubated with an excess of the monoclonal hybridoma anti-VIII:C, the lag in the time course of FXa formation was almost doubled. This increase in lag time strongly indicates that the inhibitory effect of the monoclonal antibody on VIII:C is primarily due to an effect on the activation of VIII:C. An alternative
Fig. 3. The effect of 1B3 antibodies on the activation of factor X by factor IXa and factor VIII. Purified FX (1 U/ml) was incubated 5 min at 37°C in the presence of 0.2 mM phospholipid and 10 mM CaCl₂ in a buffer containing 50 mM Tris-HCl (pH 7.5) and 100 mM NaCl. 5 sec after the addition of 0.15 mg/ml ovalbumin (x—x), 0.5 U/ml FVIII plus 0.13 mg/ml ovalbumin (O—O), or 0.5 U/ml FVIII plus 0.13 mg/ml monoclonal anti-VIII:C (Q—Q), Xa formation was started by the addition of 0.5 μg/ml IXa. At different time intervals, samples were drawn and analyzed for the presence of FXa. The FVII added to the reaction mixture had been previously incubated for 30 min at 37°C either with ovalbumin or with monoclonal anti-VIII:C. Under these circumstances 80% of the VII:C activity was inhibited in a coagulation assay. The experiment was carried out in cooperation with Dr. Mertens.

The Use of Monoclonal Anti-VIII:C in an Immunoradiometric Assay

The monoclonal hybridoma anti-VIII:C can be used as a first antibody in an IRMA for VIII:CAG (see Fig. 4). The lower limit for detection of VIII:CAG in this way is about 0.01 U/ml. Replacement of the VIII:C of pooled normal plasma by that of LMW FVIII or isolated FVIII complex resulted in parallel dose–response curves in the IRMA. Figure 5 shows that no significant amounts of FVIII CAG were present in the plasma of a hemophilia A patient, previously shown to be A⁻ with a different IRMA technique. Surprisingly, it was found that also in pooled normal serum, no VIII CAG could be detected; this finding sharply contrasts with IRMA results obtained with human antibodies, where VIII CAG levels up to 0.7 U/ml have been reported.

The fusion of mouse myeloma cells with spleen cells of a mouse immunized with isolated human VIII:C was rather successful: after the fusion, 12/36 wells contained anti-VIII:C activity. When the fusion was performed with spleen cells of a mouse immunized for only 1 mo, no anti-VIII:C-producing hybridomas could be detected among the fusion products. In the present study we described the isolation of only one of the hybridomas (1B3) and the characterization of the anti-VIII:C immunoglobulin that it produces.

From the well 1B3, the anti-VIII:C-producing hybridoma was obtained after four subcloning cycles. At that stage the cell culture could be considered as monoclonal on statistical grounds. In vivo culture of these cells in the ascites of BALB/c mice resulted in very high antibody titers after about 2 wk. Analysis of the isolated IgG by isoelectric focusing revealed two components with a pI of 6.98 and 7.09. However, Köhler and Milstein reported that also the IgG, produced by the P3 × 63 myeloma cells consists of two forms when analyzed by isoelectric focusing. Analysis of the products of in vitro and in vivo cell culture revealed the presence of both IgG₁ and IgG₂ chains. The combined results indicate that the 1B3 hybridoma produces an anti-VIII:C immunoglobulin, consisting of IgG₁ and IgG₂ chains. So it might be that the 1B3 monoclonal antibody will have only one binding site for factor VIII (on the IgG₂ chain).

The study of the specificity of the 1B3 antibody showed that (1) it neutralizes VIII:C in isolated VIII:C preparations, in cryosupernatant, in cryoprecipitate, and in pooled normal plasma; (2) when coupled to Sepharose it removes VIII:C from pooled normal plasma; (3) when coated to plastic tubes it binds VIII:C from isolated VIII:C, purified FVIII, and pooled normal plasma. On the other hand, the antibody does not bind fibrinogen or VIIIR:AG. The latter observation is important because it has been shown previously that anti-HMW FVIII (rabbit) can inhibit the VIII:C of pooled normal plasma.

In Fig. 2 it is shown that VIII:C neutralization by the mouse anti-VIII:C serum is different and more complete than with the 1B3 antibody. This finding implies that the mouse antiserum contains more classes of anti-VIII:C antibodies that bind to different antigenic determinants on the VIII:C molecule.

The effect of the 1B3 antibody on the activation of FX in the presence of FIXa, FVIII, phospholipids, and Ca²⁺ (Fig. 3) strongly indicates that binding of VIII:C to the antibody only results in a delay in the activation of FVIII; in a coagulation assay, such a delay will cause a prolonged clotting time that can be interpreted
Fig. 4. IRMA of VIII:C using mouse anti-VIII:C (1B3) in the first phase and specific labeled human anti-VIII:C IgG in the second phase. (O-O) Purified FVIII (VIII:C 8 U/ml); (●-●) LMW FVIII (VIII:C 8 U/ml); (x-x) pooled normal plasma.

Fig. 5. IRMA of VIII:C using mouse anti-VIII:C (1B3) in the first phase and specific labeled human anti-VIII:C IgG in the second phase. (x-x) Pooled normal plasma; (●-●) plasma of a hemophilia A patient; (O-O) pooled normal serum.
as a decrease in VIII:C concentration (neutralization of the VIII:C); this also explains why VIII:C activity never could be neutralized completely, even in the presence of undiluted antibody preparations.

The finding that serum VIII CAG does not bind the 1B3 antibody suggests that proteolytic degradation of the molecule (activation and/or inactivation) is accompanied by a structural change resulting in a sharp decrease in affinity for binding to the anti-

VIII:C antibody. This finding is compatible with the abovementioned hypothesis that the effect of the 1B3 interferes with the activation of VIII:C by thrombin or FXa.

In the future the use of monoclonal antibodies will prove to be particularly useful in the study of heterogeneity of hemophilia A and in the elucidation of the mechanism by which VIII:C exerts its coagulant activity.

REFERENCES

2. Zimmerman TS, Ratnoff OD, Powell AE: Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand’s disease. With observations on combined deficiencies of antihemophilic factor and proaccelerin (factor V) and on an acquired circulating anticoagulant against antihemophilic factor. J Clin Invest 60:244, 1971
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