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Monoclonal antibodies against human factor VIII molecule neutralize antihemophilic factor and ristocetin cofactor activities

(hybridomas/von Willebrand factor/inhibition of factor VIII coagulant activity)

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ABSTRACT **A series of monoclonal antibodies have been raised against a preparation of the factor VIII/von Willebrand factor molecule. Of the seven hybridomas showing specific activity against the factor VIII molecule in a solid-phase radioimmunoassay, three (F4.55, F4.77, and F4.264) have been shown to partially inhibit ristocetin-induced platelet aggregation and two (F4.115 and F4.415) inhibit the antihemophilic activity of the factor VIII molecule. An additional monoclonal antibody was directed against a contaminant of the factor VIII preparation and is an antifibrinogen antibody.**

The human factor VIII/von Willebrand protein (F.VIII/vWF) is a complex plasma glycoprotein associated with two different biological activities (1, 2): a procoagulant activity (VIII:C) and a von Willebrand activity (VIIIR:WF). The former is implicated in platelet adhesion to the vascular subendothelium and is measured by platelet aggregation in the presence of ristocetin (3). The procoagulant activity (antihemophilic factor) is measured by coagulation time correction of plasma from a severe hemophilic patient (4).

Little is known of the biochemistry of VIII:C. VIIIR:WF has been identified as a high molecular weight glycoprotein that circulates in the plasma as multimers of a single M_r 220,000 subunit. The high molecular weight polymers found vary in M_r from several hundred thousand to several million and can be considered the native form of the molecule in plasma (5). The relationship of biochemical structure of the F.VIII/vWF molecule to the biological activities remains controversial. It is still unclear whether the two activities are carried by two different sites on a single molecule (6) or by two or more distinct molecules, the intact molecule thus being a heteropolymer (2).

Biochemical studies have been hampered by the low concentration of F.VIII/vWF in the plasma (5–10 μg/ml) and by the extreme lability of the F.VIII/vWF-associated biological activities. This has rendered extremely difficult the recovery of the native molecule after purification. The F.VIII/vWF molecule has thus remained one of the less-well-characterized factors involved in the hemostatic process.

Attempts at an immunological characterization of the F.VIII/vWF molecule have been limited. Two basic types of antisera exist. The first class, heteroantisera identifying the factor VIII-related antigen (VIIIR:Ag) are capable of neutralizing VIII:C and can neutralize or precipitate VIIIR:WF. Precipitating antisera recognize a single protein from normal plasma, presumably responsible for VIII:C and VIIIR:WF biological activities (7). The second class, alloantisera, arise either spontaneously or after transfusion of hemophilic A patients and identify the VIIIC:Ag. These VIII:C-neutralizing antisera are not precipitating (8). As with other proteins, antigenic reactivity and biologic activity do not necessarily correspond.

Given the limitations of these existing antisera and the potential represented by immunochemical and immunological techniques for probing the F.VIII/vWF molecule, the isolation and use of monoclonal antibodies seemed likely to be fruitful. One such report has appeared recently (9). We report here the establishment of seven hybridoma cell lines from a fusion involving spleen cells from a mouse immunized with a factor VIII concentrate. The seven hybridoma cell lines secrete antibodies active against the F.VIII/vWF protein. Three of them partially inhibit ristocetin-induced platelet aggregation and may be directed against an antigenic site(s) involved in von Willebrand activity. Two others inhibit the coagulation process and are directed against the antihemophilic activity of factor VIII. An eighth hybridoma isolated from this fusion series has been shown to secrete a monoclonal antibody directed against fibrinogen, a contaminant of all preparations of F.VIII/vWF protein.

MATERIALS AND METHODS

Commercial Factor VIII Preparation. "Actif VIII high-purity concentrate batch 9C 24 004" was kindly provided by Mérieux Laboratories. It contained VIIIR:Ag at 128 units (U)/ml, VIII:C at 28 U/ml, and VIIIR:WF at 33 U/ml.

Highly Purified Factor VIII. This was prepared from the commercial preparation. Lyophilized concentrates dissolved (20 mg of protein per ml) in 0.15 M Tris/NaCl, pH 7.4, were chromatographed on a Sepharose CL-2B column in this buffer plus aprotinin at 10 U/ml. Fractions containing VIIIR:Ag were pooled and concentrated by dialysis against polyethylene glycol. Such preparations having an A_{280} of 0.2–0.3 contained VIIIR:Ag at 20–40 U/ml, VIIIR:WF at 20–40 U/ml, and VIII:C at 15–25 U/ml.

Semipurified Factor VIII Fractions. These were prepared either directly from human citrated plasma by chromatography on Sepharose CL-2B in 0.15 M Tris/NaCl, pH 7.4, or by such chromatography after initial treatment by adsorption on aluminium hydroxide gel, cryoprecipitation at −80°C, thawing at 4°C, centrifugation, and resuspension in the chromatography buffer. The void volume fractions were collected and concentrated. "Direct" preparations contained 4–8 U of VIIIR:Ag per ml, 2–4 U of VIII:C per ml, and 4–8 U of VIIIR:WF per ml.

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Abbreviations: F.VIII/vWF, factor VIII/von Willebrand factor molecule; VIIIR:WF, von Willebrand factor; VIII:C, antihemophilic factor; VIIIR:Ag, factor VIII-related antigen; VIIIC:Ag, factor VIII coagulant antigen; U, unit(s); P_i/NaCl, phosphate-buffered saline.

Cryoprecipitated preparations adjusted to 1 U of VIIIR:Ag per ml contained 0.1–0.2 U of VIII:C and 1 U of VIIIR:WF per ml.

The methods used to estimate VIII:C (10), VIIIR:Ag (7), and VIIIR:WF (11) contents are detailed elsewhere. The characterization of these preparations was carried out by polyacrylamide gel electrophoresis as described by Peacock and Dingman (12).

Immunization. (BALB/c × C57Bl/6)F_1 mice were injected intraperitoneally with a homogenate of the commercial preparation (250 μg of protein) in complete Freund's adjuvant (1:1), followed 3 weeks later by an intraperitoneal injection of 200 μg of protein of the same preparation without adjuvant and then 1 week later by a third injection of the same preparation. Spleens were removed 4 days later.

Cell Lines and Maintenance Culture Conditions. The mouse myeloma cell lines used for hybridoma isolation P3-X63-Ag8 (X63) and Sp-2/0-Ag14 (Sp-2/0) were kindly provided by G. Köhler (13–15) and were routinely cultivated on Dulbecco's modified Eagle's medium (GIBCO) containing 20% (vol/vol) fetal calf serum, 50 U of penicillin and 100 μg of streptomycin per ml, 1% 100 mM sodium pyruvate, 1% 200 mM L-glutamine, and 1% D-glucose (350 mg/ml). This medium is referred to as complete DME medium. The HeLa line (epitheloid carcinoma, ATCC CCL2) was grown in DME medium containing 10% fetal calf serum and replicated by use of trypsin/EDTA every 3–4 days. All cultures were grown in a humid 7.5% CO_2/92.5% air atmosphere at 37°C.

Hybridoma Isolation Protocol. The fusion protocol used was essentially that described by Köhler and Shulman (15). Sp-2/0 cells (2×10^7) were fused with 2×10^8 spleen cells by using polyethylene glycol (M_r 4000; British Drug House, Poole, England) and then distributed into Costar 96-well plates; each well contained 5×10^5 splenocytes as feeders. At 24 hr later, 1 ml of the medium in each well was replaced with 1 ml of HAT medium (complete DME medium plus 0.1 mM hypoxanthine, 0.4 μM aminopterine, and 16 μM thymidine). Media were half-changed every 2 days for approximately 2 weeks, at which time the wells were tested for antibody activity by radioimmunoassay. Positive hybrid cells were then cloned twice by limiting dilution.

Solid-Phase Radioimmunoassay. The method used is adapted from that of Heusser and Stocker (16). We used 96-well polyvinyl flexible microtiter plates (SMRC 96; Flow Laboratories) as the solid support for attachment of both intact cells and soluble protein suspensions.

Plastic coating of soluble proteins. Proteins used included the different factor VIII preparations described above and, as controls, a fibrinogen preparation (98% clottable), human fibronectin (Collaborative Research, Waltham, MA, batch 1147–1), and human immunoglobulins. Immunoglobulins were prepared in our laboratory and fibrinogen was prepared as described by Kazal *et al.* (17). To each well was added 50 μl of solution containing 0.5 mg of protein per ml, and the plates were incubated for 1 hr at 37°C. The plates were washed three times with phosphate-buffered saline (P_i/NaCl) and then incubated at 37°C for 1 hr with 2% bovine serum albumin in P_i/NaCl. Routinely, factor VIII preparation plates were used within 24 hr of preparation.

Attachment of HeLa cells to the plastic support. Plates were first preincubated with a poly(L-lysine) solution (50 μg/ml; 100 μl per well) for 45 min at 37°C. After three P_i/NaCl washes, 5×10^5 HeLa cells were added per well, the plates were centrifuged at 600 × *g* for 5 min, and the cells then were fixed with 0.25% glutaraldehyde in P_i/NaCl. The plates were saturated with P_i/NaCl/albumin/0.2% Na azide.

Radioimmunoassay. The plates were first washed with P_i/NaCl. They were then incubated with 50 μl of culture supernatant or ascitic fluid for 1 hr at 37°C. After three P_i/NaCl washes, 50 μl of a ^{125}I-labeled goat anti-mouse Ig antiserum (100,000–150,000 cpm/50 μl) was added and the plates were incubated for 1 hr at 37°C. ^{125}I labeling of this developing serum was carried out by using the Iodogen reaction (18). The plates were then rinsed three times with P_i/NaCl, and the radioactivity of the wells was measured by using an LKB gamma counter.

Negative radioimmunoassay controls included the activity of the supernatants on P_i/NaCl/albumin-coated plastic and the activity of X63 culture supernatants or X63 ascites on coated plates. In some tests, this was complemented by the use of irrelevant monoclonal ascites: BBF 39-27 (anti-Friend virus), donated by F. Plata, or Ab 1.41 (anti-HLA-DR), a gift of D. J. Charron (19). Binding of the X63, BBF 39-27, and Ab. 1.41 ascites was identical on all preparations used. The levels of nonspecific binding are indicated in *Results*. Binding greater than 3 times that of the nonspecific controls was considered to be positive. "Titer" refers to the greatest dilution at which a serum or supernatant remained positive.

The quantity of factor VIII attaching per well was estimated as 20 μg by using a ^{125}I-labeled factor VIII preparation.

Inhibition of VIIIR:WF Activity. Inhibition of VIIIR:WF activity was tested in a system of ristocetin-induced platelet aggregation. Platelet-rich plasma was adjusted in homologous platelet poor plasma to a concentration of 400,000 platelets per μl and 0.2 ml of it was incubated for 5 min at 37°C with 0.1 ml of buffer or ascitic fluids in an aggregometer cuvette, then agitated at 1100 rpm with 0.1 ml of a ristocetin solution (final concentration, 1 mg/ml). The maximum of the aggregation curve was calculated for the control plasma and inhibition was calculated as percentage of it.

Inhibition of the VIII:C Activity. Inhibition of VIII:C, as a screening test, was performed in a partial thromboplastin time system using plasma from a severe hemophilic as substrate. Ascitic fluid or buffer was incubated with equal volumes of a standard plasma pool (1 U of VIII:C per ml) for 1 hr at 37°C; 0.1 ml of this mixture was then added to 0.1 ml of hemophilic plasma, 0.1 ml of partial thromboplastin, and 0.1 ml of a kaolin suspension and incubated for 5 min at 37°C. The mixture was then recalcified (0.1 ml of 0.025 M calcium chloride) and the coagulation time was measured.

Titration of Anti-VIII:C Activity. Standard pool plasma (1 U of VIII:C per ml) was incubated with buffer or dilutions of ascitic fluid for 2 hr at 37°C. Residual VIII:C was estimated by using the partial thromboplastin time system. Ascitic fluid dilutions were tested until 100% VIII:C activity was recovered.

At least two unrelated ascitic fluids were always included as controls in addition to the culture medium itself.

Identification of the Ig Subclasses Secreted by the Various Hybridomas. Culture supernatants concentrated on polyethylene glycol 20,000 were tested in a double-immunodiffusion system with specific rabbit antisera to mouse Ig subclasses (Nordic). The latter were verified by using purified Ig standards (Bionetics).

Ascites Production. Pristane-primed (BALB/c × C57Bl/6)F_1 mice were injected with 10^7 hybridoma cells. Ascitic tumors were withdrawn and clarified by low-speed centrifugation.

RESULTS

Hybridoma Selection. Hybridomas arose in 100% of the seeded wells. Of the 96 studied by radioimmunoassay, 85 supernatants were positive: 32 on a semipurified factor VIII preparation, 5 on the commercial preparation (Mérieux Laboratories), and 48 on both. All were strictly negative on both HeLa cells and albumin-coated plastic plates. From among these 85

Table 1. Antibody titers of hybridoma-derived ascitic fluids in radioimmunoassays on different substrates

Hybridoma	Reciprocal of titer: On semipurified VIII	On highly purified VIII	On fibrinogen
F4.6	40	400	0
F4.55	640	>10,000	0
F4.77	0	5	0
F4.115	40	2,560	80
F4.118	2,560	10,000	0
F4.177	10,000	>10,000	>10,000
F4.264	Not done	640	0
F4.415	10,000	>10,000	0

Controls: X63 and BBF 39-27.

supernatants, 17 were selected for their strong activity in the radioimmunoassay and the corresponding cells were cloned. After cloning and recloning, eight hybridomas were retained for further study. Of the eight hybridomas, six were from independent wells (F4.6, F4.55, F4.77, F4.177, F4.264, and F4.415). Two clones, F4.115 and F4.118, arose from a single well.

Characterization of Positive Hybridomas. All the hybridomas other than F4.55 secreted immunoglobulin belonging to the IgG1 class (the exception secreted IgG2a). It is known that each of these hybridomas are associated with K light chain. The absence of the λ chain has not been verified.

All the ascites except F4.77 were positive on the semipurified factor VIII with titers varying from 1:40 to 1:10,000 (Table 1 and Fig. 1). On highly purified factor VIII preparation, all, including F4.77, were positive. Ascites F4.6, F4.55, F4.77, F4.115, and F4.118 all had increased titers on the purified preparation, suggesting that they were indeed directed against the factor VIII itself and not a minor contaminant. The ascites that gave the highest titers had titers greater than 1:10,000. Regardless of titer considerations, some of the ascites showed qualitatively different binding profiles—for example, in Fig. 1*A* for ascites F4.415 and F4.115.

Hybridomas were tested against human fibrinogen, human plasma fibronectin, and total human immunoglobulins. All were negative on fibronectin and immunoglobulin preparations. Against fibrinogen, six of the hybridomas were completely negative and two showed some activity: F4.115 had a low titer (see also Fig. 1*C*) and F4.177 showed binding to fibrinogen equivalent to that shown on factor VIII preparations. Because the titer of F4.115 was much lower on fibrinogen than on highly purified factor VIII preparation, it seems likely that the activity of this hybridoma was directed against the factor VIII molecule(s) itself, the low activity on the fibrinogen preparation being due to minor contamination by factor VIII molecules or to some crossreacting antigen. If the first hypothesis is correct, only a restricted range of factor VIII antigenic determinants can be contaminating such fibrinogen preparations because otherwise the other hybridomas would have reacted with the preparation. Hybridoma F4.177, on the other hand, potentially is an anti-fibrinogen antibody.

This interpretation of both F4.115 and F4.177 activity on fibrinogen preparations is supported by experiments with factor VIII preparations from normal human plasma and plasma of an afibrinogenemic patient (Fig. 2). Binding of the ascites fluid to factor VIII prepared from normal plasma was more or less equivalent to that observed on a semipurified factor VIII preparation obtained after cryoprecipitation. Binding on the factor VIII preparation obtained from the afibrinogenemic patient was quite different. F4.177 was clearly negative, indirect proof that F4.177 probably is directed against the fibrinogen molecule itself. All the other ascites, and in particular F4.115, were positive on the fibrinogen-free preparation and they could be separated into two groups: (*a*) F4.55, F4.264, and F4.415, having equivalent binding on fibrinogen-free and normal preparations; and (*b*) F4.6, F4.77, F4.115, and F4.118, having increased activity on the fibrinogen-free factor VIII preparation.

The interpretation of these results must consider the type of radioimmunoassay used, and several alternative explanations can be envisaged. One possible interpretation is that the absence of fibrinogen increases the accessibility of the factor VIII molecule in the preparation for the plastic matrix, thus allowing an increase in antigenic density of those antigens recognized by the hybridomas in the second group. According to this interpretation, antigenic sites (epitopes) recognized by the first hybridoma group would already be present in excess and their binding would not be modified by the absence of fibrinogen. Such epitopes might be more repetitively distributed on the

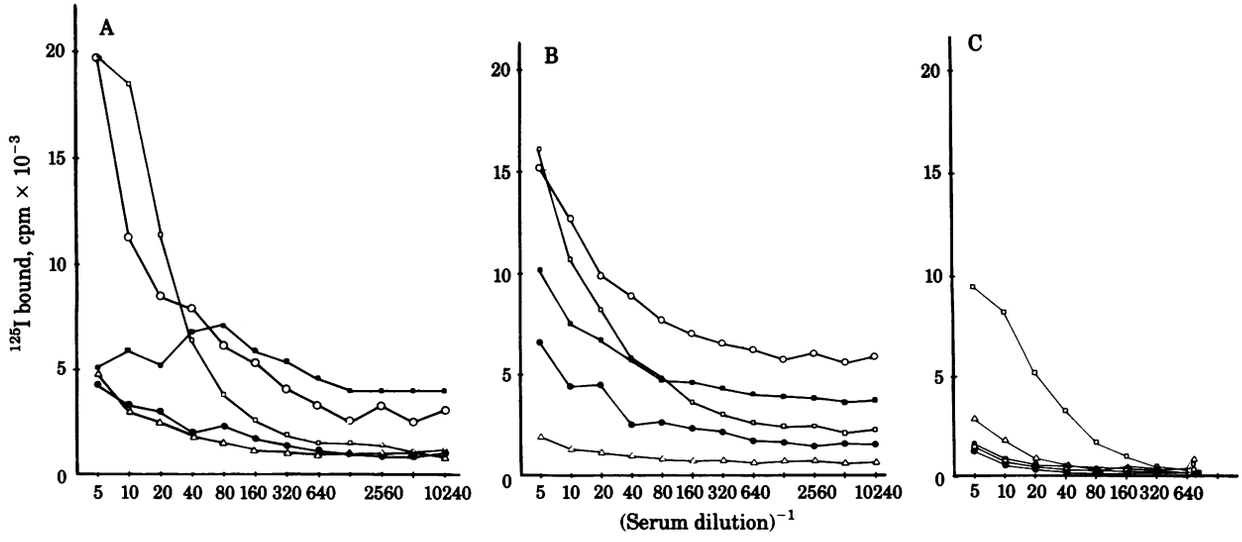

FIG. 1. Binding of ascitic fluids F4.55 (○), F4.77 (●), F4.115 (□), F4.415 (■), and X63 (△) on semipurified factor VIII (*A*), highly purified factor VIII (*B*), and fibrinogen (*C*).

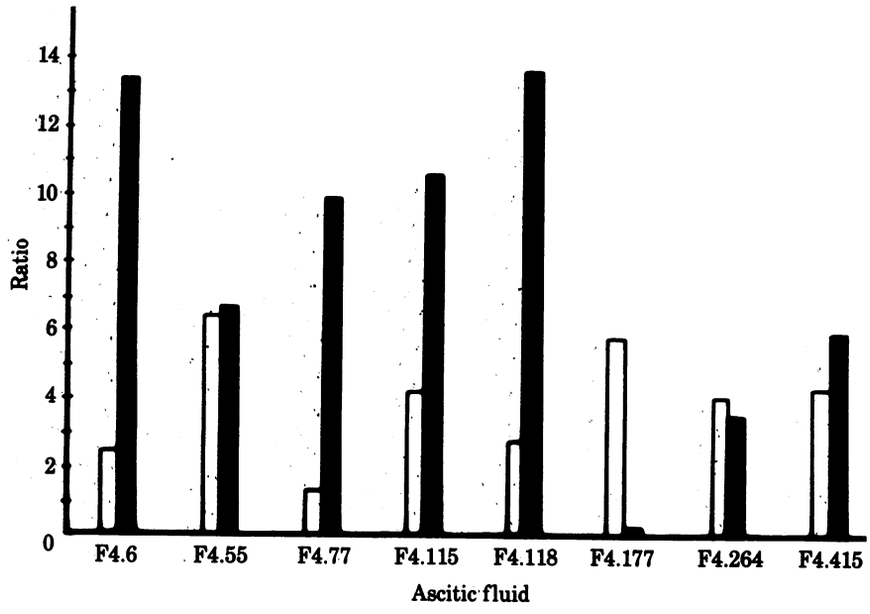

FIG. 2. Binding of the various ascites, expressed as the ratio cpm of sample/cpm of unrelated ascite X63 on preparations of factor VIII isolated from normal plasma (□) and afibrinogenemic plasma (■). The ascitic fluids of the various hybridomas were used at a dilution of 1:10. The binding of X63 at the same dilution was 4800 cpm on normal plasma factor VIII and 4300 cpm on afibrinogenemic plasma factor VIII.

factor VIII molecule. A second hypothesis would include possible partial masking of the factor VIII molecule, due to weak interactions between it and fibrinogen molecules.

Inhibition of Biological Activities. The seven ascitic fluids active against F.VIII/vWF were tested for biological activity. Three of the seven ascites were able to inhibit ristocetin cofactor activity (Table 2). Incubation of these ascites with normal platelet-rich plasma produced partial inhibition of ristocetin-induced platelet aggregation. Fig. 3 illustrates the differences seen between the control (unrelated ascites X63) and the F4.55 positive ascitic fluid and the dose–response effect. Inhibition of aggregation was 58% at the 1:10 dilution, 46% at 1:40, and 30% at 1:80. Inhibition by clone F4.264 occurred only for the 1:5 dilution; with clone F4.77, 50% inhibition was observed at the 1:5 dilution and persisted to the 1:40 dilution. The inhibition titers for aggregation seem somewhat lower than those noted in the radioimmunoassay, suggesting that possibly a large number of antigenic sites must be occupied before aggregation inhibition can be observed. Support for this idea comes from mixing experiments such as that shown in Fig. 3*B*. A 1:1:1 mixture of 1:5 dilutions of hybridoma ascites F4.55, F4.77, and F4.264, which individually never gave more than 50% aggregation, gave inhibition of 75% or greater. However, 100% aggregation inhibition has never been observed in such simple mixing experiments.

Inhibition of coagulation activity was shown only by clones F4.115 and F4.415 (Table 2). They showed dissimilar VIII:C neutralizing curves when neutralization was studied as a function of ascitic fluid concentration, and preliminary experiments suggest they may have different specific VIII:C neutralizing properties (Fig. 4).

Table 2. Inhibition of factor VIII biological activities by ascites of the different hybridomas

Hybridoma	Ristocetin cofactor activity	VIII:C activity
F4.6	0	0
F4.55	+	0
F4.77	+	0
F4.115	0	+
F4.118	0	0
F4.264	+	0
F4.415	0	+

+, Inhibition of activity; 0, no inhibition of activity. Controls were X63 and BBF 39-27.

DISCUSSION

A series of hybridomas have been raised against a preparation of factor VIII concentrate. Of the eight hybridomas studied, one was directed against fibrinogen contaminating the factor VIII preparations. The seven others appear to be specific for the F.VIII/vWF. Of these seven, all of which are active in factor VIII radioimmunoassays, three have been shown to inhibit ristocetin-induced platelet aggregation and another two, F4.115 and F4.415, inhibit the VIII:C activity.

Immunization with a partially purified commercial preparation potentially aggravates the problem of raising hybridomas against contaminants such as fibrinogen which are constant contaminants of even highly purified factor VIII preparations. It therefore was no surprise that we detected one hybridoma having antifibrinogen activity.

The development of a factor VIII radioimmunoassay has proved indispensable for hybridomas isolation. Its indispensability is linked not only to its practicality but also to the problems besetting factor VIII biological activity assays on initial culture

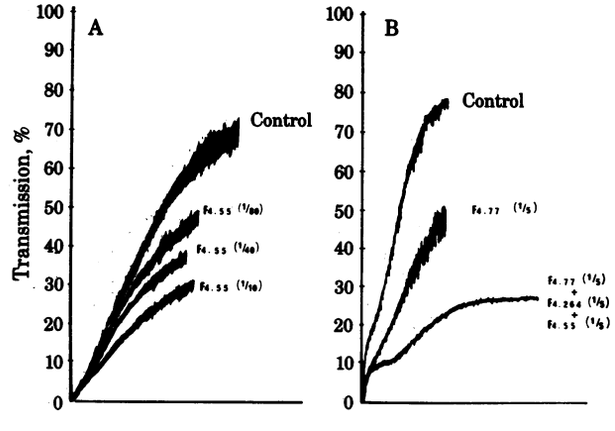

FIG. 3. Inhibition of ristocetin-induced platelet aggregation by different hybridomas. (*A*) Dose–response effect of the F4.55 hybridoma. (*B*) Synergistic effect of three hybridomas (F4.55, F4.77, and F4.264) active in this test.

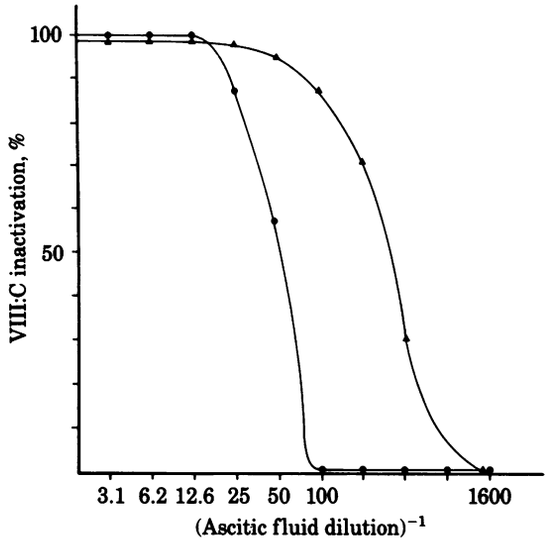

FIG. 4. Neutralization of VIII:C activity by monoclonal antibodies F4.415 (▲) and F4.115 (●). Incubation was for 2 hr at 37°C.

supernatants. The serum in the medium not only exercises an enhancing activity on platelet aggregation, thus potentially hiding antibody inhibition effects, but also shortens coagulation times.

The radioimmunoassay has its own limitations. Although we have been able to characterize the quantity of protein in a given factor VIII preparation attaching to the plastic, we are not sure whether there is a selective affinity for or against contaminating proteins in the preparation or whether there is a selective affinity for any particular multimer within the factor VIII multimeric series. We do have some indications, however, that fibrinogen adheres to the plastic with much greater avidity than does factor VIII, an observation supported by results with monoclonal F4.177 which is negative in a radioimmunoassay against a factor VIII preparation from an afibrinogenemic patient but is almost as effective against a factor VIII containing only several percent contaminating fibrinogen as against a >98% pure fibrinogen preparation. This result also emphasizes the importance of including radioimmunoassays on factor VIII purified from afibrinogenemic donors in any characterization.

The results obtained in radioimmunoassays with semipurified and highly purified factor VIII preparations (Table 1) have to be examined with this type of complexity in mind, and it is for this reason that the apparent relatively low binding of monoclonal antibodies F4.55 and F4.115 in radioimmunoassays with normal factor VIII preparation (Fig. 1*B*) cannot be taken automatically to indicate a low affinity for the factor VIII molecules and therefore their lack of suitability for further study.

As expected, against a molecule as large and as complex as the F.VIII/vWF, a series of hybridomas, of which at least several are certainly directed against different antigenic sites, have been isolated. Fig. 2 illustrates this effect and also demonstrates how, independent of their titer, the hybridomas divide into two different categories depending on whether their binding is similar on factor VIII preparations from normal and afibrinogenemic donors or whether their binding is markedly superior on the latter type of preparation. It is interesting to note that the hybridomas active in the biological activity tests do not fall into either of these two groups. It seems clear that the three hybridomas inhibitory against ristocetin-induced aggregation are directed against different antigenic sites because additivity in their inhibitory action is seen (Fig. 3*B*). There are no indications that F4.415 and F4.115 hybridomas recognize different antigenic sites but they clearly behave differently in the coagulation inhibition tests (Fig. 4).

Inactivation patterns such as those described above have been noted for hemophiliac antibodies against VIII:C (20), the different time course patterns of VIII:C inactivation depending on the patient considered. Inactivation patterns such as those described for hemophiliac antibodies therefore can be obtained with monoclonal antibodies from hybridoma cell lines.

The results of the biological activity tests, like those of the radioimmunoassay, have been expressed as a function of titer because normalization of these values for total proteins or for total immunoglobulin content of the ascites fluids does not provide a better basis for comparison of the specific activities of the hybridomas between themselves or with the classical allogeneic or autologous anti-VIIIR:WF or anti-VIII:C antisera.

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