Technical methods

Monoclonal antibodies to human factor VII: a one step immunoradiometric assay for VII:Ag

T TAKASE, EGD TUDDENHAM, S CHAND, ALISON H GOODALL *From the Haemophilia Centre and Haemostasis Unit, Academic Department of Haematology, The Royal Free Hospital and School of Medicine, London.*

SUMMARY Three mouse monoclonal antibodies (RFF-VII/1, RFF-VII/2, and RFF-VII/3) which bind specifically to different epitopes on human factor VII antigen were raised. Two of the antibodies, RFF-VII/1 and RFF-VII/2, bound strongly to factor VII antigen (VII:Ag), but only RFF-VII/1 and RFF-VII/3 were potent inhibitors of factor VII coagulation activity (VII:C). RFF-VII/1 and RFF-VII/2 were used in a one step, double monoclonal immunoradiometric assay for VII:Ag. This was highly reproducible and detected as little as 0.05 U/dl VII:Ag. Values for VII:Ag obtained for plasma samples from normal subjects ($n = 20$), patients with liver disease ($n = 20$), patients treated with warfarin ($n = 20$), and those congenitally deficient in factor VII ($n = 7$) correlated very well ($r = 0.96$) with data obtained in a radioimmunoassay using polyclonal rabbit antiserum to factor VII.

This simple and sensitive monoclonal antibody based assay offers a convenient method for the detection of VII:Ag in various disease states.

Factor VII is a vitamin K dependent zymogen of serine protease, present in small quantities (0.4 $\mu\text{g/ml}$) in plasma. It forms a complex with tissue factor and then activates factor X. It is a single polypeptide chain of 50 000 daltons that can, in a purified system, be activated by proteolytic cleavage of disulphide bonds by factor Xa, factor IXa, thrombin and factor XIIa.¹⁻⁵

Factor VII is reduced in congenital VII deficiency and reduced concentrations are seen in patients with liver disease⁶ and in those treated with warfarin.⁷ Increased concentrations of factor VII have been implicated as a prognostic indicator of cardiovascular disease.⁸

Due to the low circulating concentrations of VII:Ag in plasma it is difficult to assay by the Laurell technique (electroimmunoassay) using polyclonal antisera. Radioimmunoassay techniques (RIA) using rabbit antisera have been reported, with detection limits of 0.3 U/dl.^{5,7} The production of monoclonal antibodies to human factor VII has been described,^{9,10} and these reagents have been used to make immunodepleted plasma⁹ or to detect factor VII cross reactive material in factor VIII deficient patients.¹⁰

In this paper we describe the production of monoclonal antibodies to factor VII and their use in a sensitive (0.05 U/dl) one step, immunoradiometric assay (IRMA) for VII:Ag.

Material and methods

IMMUNISATION AND FUSION

Balb/c mice were immunised with purified factor VII prepared by the method described by Mikami and Tuddenham.⁵ Mice were injected intraperitoneally with 10 μg of factor VII in complete Freund's adjuvant and boosted similarly at two $\times$ four weekly intervals with a further 5 μg factor VII. Four weeks after the second boost one of the mice was injected intravenously with 5 μg factor VII in saline and the spleen was removed four days later.

Spleen cells were fused with P3-NS/1-Ag 4 mouse myeloma cells using PEG 1500, as described previously.¹¹ Mouse serum and hybridoma culture supernatants were screened for anti-factor VII activity in a solid phase capture assay similar to that described for detecting monoclonal antibodies to factor VIII.¹² Hybridoma supernatants were also screened for inhibitor activity. At the time of fusion the mouse had an inhibitor level of 930 BU/ml and an antibody titre of 1/100 000.

TEST SAMPLES

Blood samples from 20 normal subjects, 20 patients with liver disease, 20 treated with warfarin, and seven with congenital factor VII deficiency were collected with plastic syringes and transferred into plastic tubes

containing 1/10 volume of 3.15% sodium citrate. The plasma samples were aliquoted and stored in plastic tubes at -70°C until assay. Paired serum samples were separated by centrifugation at 3000 rpm ($2000 \times g$) after collection into glass tubes, without citrate, and incubation for three hours at 37°C .

For all assays the standard was a 20 donor pool of normal plasma, aliquoted, and stored at -70°C .

VII:C was assayed by a one stage method using human thromboplastin plus calcium. The substrate for these assays was obtained commercially (Boehringer Mannheim), or an immunodepleted normal plasma was used.¹³ VII:C inhibitory activity was measured by preincubation of NPP with the monoclonal antibodies for one hour at 37°C .

DOUBLE MONOCLONAL IMMUNORADIOMETRIC ASSAY FOR VII:Ag

Monoclonal antibodies were purified from ascitic fluid by aluminium hydroxide absorption, followed by rivanol and ammonium sulphate precipitation, and radiolabelled with Na^{125}I using chloramine T, as described previously.¹²

LP3 polystyrene tubes (Sarstedt, West Germany) were coated with RFF-VII/2 monoclonal antibody. A rivanol-ammonium sulphate preparation (500 μl) of RFF-VII/2 (50 $\mu\text{g}/\text{ml}$) in 0.1M bicarbonate buffer (pH 9.6) was added to each tube and incubated at 4°C overnight. The tubes were washed three times with 1 ml of 0.01M sodium phosphate buffered saline (pH 7.2) containing 1% bovine serum albumin (PBS-A). Then 200 μl of sample and 200 μl of ^{125}I -labelled RFF-VII/1 IgG (60 000 cpm), both diluted in PBS containing 4% BSA were added simultaneously and

incubated overnight at 20°C . The tubes were then washed three times with distilled water and counted.

A calibration curve was performed using serial dilutions (1/10–1/2560) of normal pooled plasma. Patients' plasma samples were assayed at three different dilutions (usually 1/80, 1/160, and 1/320).

RADIOIMMUNOASSAY (RIA) FOR VII:Ag

An RIA for VII:Ag using rabbit anti-FVII was performed using a slight modification of the methods described by Mikami and Tuddenham⁵ and Fair.⁷ 0.1 ml of ^{125}I labelled, purified factor VII (10 000 cpm), 0.1 ml of test samples, and 0.1 ml of a 1/4000 dilution of rabbit antihuman factor VII were incubated in polystyrene tubes at 4°C overnight. 0.1 ml of a 1/100 dilution of a goat antirabbit IgG antiserum (Sigma) was added for one hour at 20°C , and antigen-antibody complexes were precipitated for one hour with 0.4 ml of 10% (w/v) PEG 8000. The tubes were centrifuged at 4000 rpm for 30 minutes at 4°C , and the precipitates were washed once with 5% PEG 8000 and counted. All samples and reagents were diluted in 0.042M borate buffer (pH 8.3) containing 0.025M sodium chloride, 0.5% bovine serum albumin, 0.01M benzamidine, and 0.02M sodium azide.

Results

CHARACTERISTICS OF MONOCLONAL ANTIBODIES

Three monoclonal antibodies were selected and cloned from one fusion. All the antibodies were of the IgG₁ isotype. RFF-VII/1 and RFF-VII/2 bound strongly to factor VII:Ag in the screening assay, but only RFF-VII/1 was a strong inhibitor of factor VII activity

Fig 1 Inactivation of VII:C by monoclonal antibodies. RFF-VII/1 (○—○), RFF-VII/2 (●—●), RFF-VII/3 (●—●).

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(3180 BU/ml) (fig 1). Maximum inhibition was, however, only 80%. RFF-VII/2 showed only 10 BU/ml inhibitory activity in the form of ascites or purified antibody. RFF-VII/3 was of lower affinity than the two other antibodies but showed almost total inhibition of VII:C and had a moderately high inhibitor titre (280 BU/ml).

Various combinations and concentrations of the three anti-factor VII monoclonal antibodies were tested in a one step, simultaneous incubation assay system. Using antibody at 10 µg/ml on the solid phase and 60 000 cpm/0.2 ml of tracer it was found that the combination of RFF-VII/2 on the solid phase and ¹²⁵I-RFF-VII/1 as tracer gave the most sensitive standard curve with normal pooled plasma. All other combinations of antibodies were poor in comparison.

Following optimisation studies it was found that coating the tube with 50 µg/ml RFF-VII/2 and adding 60 000 cpm/0.2 ml ¹²⁵I-RFF-VII/1 gave the best standard curve over a wide range of plasma dilutions (1/4 to 1/2560). A typical curve is illustrated in fig 2. The assay permitted the detection of as little as 0.05 U/dl factor VII (standard defined as 100 U/dl of normal plasma). The reproducibility of this method was determined and the coefficients of variation (inter-assay) were found to be 2.8% at 1 U FVII/ml increasing to 10.8% at 0.05 U/ml. Intra-assay variation was between 1.9% at 1 U/ml and 13.3% at 0.05 U/ml (n = 5).

Concentrations of VII:Ag in 20 samples of normal plasma were measured in the monoclonal antibody IRMA and found to range between 66 and 138 U/dl (mean (SD) 100 (20) U/dl). Paired samples of serum from the normal donors contained higher concentrations of VII:Ag; between 81 and 164 U/dl (128 (26) U/dl) (p < 0.001) (fig 3).

Patients with liver disease had factor VII:Ag concentrations of between 15 and 68 U/dl and patients receiving warfarin had between 12 and 88 U/dl factor VII:Ag (table 1). All data for factor VII:Ag obtained in the monoclonal antibody-IRMA correlated well with those obtained for the same samples in the polyclonal RIA (p > 0.05) (r = 0.96) (fig 4).

Fig 2 Binding curve of double monoclonal VII:Ag assay.

Fig 3 VII:Ag in paired plasma and serum samples from 20 normal subjects (p = < 0.001).

Plasma from seven patients with severe congenital factor VII deficiency (factor VII:C < 0.3 U/dl) were assayed (table 2). Two, who were CRM negative by RIA, were also found to be negative in the monoclonal antibody-IRMA. Four samples from patients who were CRM positive by RIA contained detectable, albeit lower, values of VII:Ag when measured in the monoclonal antibody-IRMA. One sample, which was CRM negative by RIA, contained 3.2 U/dl VII:Ag by monoclonal antibody-IRMA.

Discussion

Factor VII is present in plasma in trace amounts and thus sensitive assays are required for detecting VII:Ag. The availability of large amounts of high affinity monoclonal antibodies enabled us to use them at relatively high concentrations to develop a sensitive two site assay. By using two non-competing monoclonal antibodies that are directed against different epitopes on the factor VII molecule, the assay can be carried out as a single incubation. The use of RFF-

Table 1 VII:Ag concentrations in plasma and serum samples detected in monoclonal antibody-IRMA or by RIA

Sample	No	Mean (SD) concentrations of VII:Ag (u/dl)	
		Monoclonal antibody-IRMA	RIA
Normal plasma	(n = 20)	100 (20)	103 (24)
Normal serum	(n = 20)	128 (26)	147 (31)
Liver disease	(n = 20)	43 (17)	39 (17)
Treated with warfarin	(n = 20)	45 (30)	48 (15)

Fig 4 Correlation of VII:Ag concentrations obtained in monoclonal antibody-IRMA and by RIA. Sample of plasma from normal subjects (●) patients with liver disease (○), patients treated with warfarin (▲) or patients with severe congenital factor VII deficiency (×) were measured in monoclonal antibody-IRMA (x axis) or by RIA (y axis).

VII/1 and RFF-VII/2 in a simultaneous one step IRMA gives an assay with a sensitivity that is comparable with that seen in RIA.

The detection of VII:Ag and VII:C is not only of importance in diagnosis and treatment of congenital bleeding disorders, but has also been used to monitor various disease states.^{5,7,8} Factor VII is synthesised in the liver and depends on vitamin K for carboxylation into its active form. Thus patients with liver disease and liver damage have reduced values of VII:Ag⁶ as do those treated with warfarin.^{5,7} In this study reduced concentration of VII:Ag (and VII:C activity¹³) were observed in all such patients.

Values of VII:Ag in patients with CRM positive severe, congenital factor VII deficiency (VII:C < 0.3 U/dl) were similar in the monoclonal antibody-IRMA and the RIA. The monoclonal antibody-IRMA, binding to only two epitopes on the factor VII molecule, is

likely to be a more sensitive detector of factor VII abnormalities than polyclonal antiserum. Cases 4–7 all showed lower concentrations of VII:Ag by monoclonal antibody-IRMA than by RIA presumably due to defects in the region of the epitopes detected by RFF-VII/1 or VII/2 or both. One patient (case 3) showed a low concentration of VII:Ag in the monoclonal antibody-IRMA while being CRM negative in the RIA. Abnormal forms of factor VII have been reported in normal plasma and in factor VII deficient patients.^{10,14–19} Broze *et al* reported a form of VII:Ag detectable with a monoclonal antibody which is 4500 Kd smaller than native factor VII and functionally inactive.¹⁰

All three monoclonal antibodies seem to bind to different determinants on the factor VII molecule. RFF-VII/3, while having the lowest affinity, is capable of inhibiting factor VII activity by more than 90%. RFF-VII/1, which has the highest affinity, gives only partial (80%) inhibition of VII:C activity but has a high inhibitory titre (>3000 BU/ml). Preliminary studies using purified proteins (data not shown) have indicated that RFF-VII/1 inhibits the activation of FVII to FVIIa whereas RFF-VII/2 preferentially inhibits FVIIa activity. This reactivity pattern was also shown in Western blotting studies with purified FVII and FVIIa. These studies suggested that all three monoclonal antibodies bind to conformational determinants on factor VII as they all failed to react with reduced factor VII.

The preferential reactivity of RFF-VII/2 with FVIIa is reflected in the increased concentrations of VII:Ag detected in serum compared with that seen in plasma. This was found in both the polyclonal RIA and the monoclonal antibody IRMA and agrees with our earlier findings.⁵ This is not surprising in that the purified factor VII used to immunise the mice would undoubtedly undergo some activation on injection, either intraperitoneally or intravenously.

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Table 2 VII:Ag concentrations in plasma samples from patients with severe, congenital FVII deficiency detected in the monoclonal antibody-IRMA or by RIA

Case No	IRMA	RIA
1	<0.05	<0.3
2	<0.05	<0.3
3	3.2	<0.3
4	0.9	4.5
5	3.8	12.8
6	19.8	31.0
7	21.4	48.0

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Requests for reprints to: Dr A H Goodall, Haemophilia Centre, Royal Free Hospital, London NW3 2QG, England.