[Chem. Pharm. Bull. 34(6)2550—2554(1986)]

Enzyme-Linked Immunosorbent Assay for von Willebrand Factor Antigen Using a Monoclonal Antibody

KAYO INOUE,*,[a] TOSHIO MAZDA,[a] TAKASHI TOBE[b] and MOTOWO TOMITA[b]

Biochemistry Laboratory, Japanese Red Cross Tokyo Metropolitan Blood Center,[a] Kyonan-cho, Musashino-shi, Tokyo 180, Japan and School of Pharmaceutical Sciences, Showa University,[b] Hatanodai, Shinagawa-ku, Tokyo 142, Japan

(Received December 2, 1985)

A monoclonal antibody (MAb) to von Willebrand factor antigen (vWF: Ag) was produced. In the present study, we used the MAb for an enzyme-linked immunosorbent assay (ELISA) to quantify vWF: Ag. The amount of vWF: Ag bound to a rabbit antibody coated on a plate was measured by applying sequentially MAb, a peroxidase-labeled antibody to murine immunoglobulin and enzyme substrate. The assay is quick and simple.

Cryoprecipitates were prepared with three thawing (slow-thaw, rapid-thaw and modified thaw-siphon) methods. ELISA for vWF: Ag and an assay for factor VIII clotting activity (VIII: C) were performed on cryoprecipitates and on cryosupernatants. The recoveries of vWF: Ag and VIII: C in the cryoprecipitate by the modified thaw-siphon method were the highest among the three methods. The ratio of VIII: C to the content of vWF: Ag was high in the cryosupernatant and low in the cryoprecipitate.

Keywords——von Willebrand factor; enzyme-linked immunosorbent assay (ELISA); monoclonal antibody; factor VIII; cryoprecipitate

Introduction

Factor VIII consists of two immunologically distinct proteins[1]; von Willebrand factor antigen (vWF: Ag) and factor VIII-procoagulant antigen (VIII: CAg). Factor VIII is deficient or abnormal in patients with hemophilia A and with von Willebrand's disease. Cryoprecipitate is prepared by cold-precipitation from the plasma, and is used for treatment of those patients. The supernatant of cryoprecipitate, called cryosupernatant, has other clinical uses.

The cryoprecipitate sometimes shows low activity of factor VIII. It has been reported that thawing methods in preparing the cryoprecipitate have a considerable effect on the recoveries of factor VIII clotting activity (VIII: C) and other plasma proteins.[2,3] To date, the assay of VIII: C is only performed as a quality control procedure during plasma fractionations such as preparation of cryoprecipitate. The measurement of vWF: Ag is also important; the ratio of VIII: C to the content of vWF: Ag is indicative of VIII: C inactivation during plasma fractionations.

The techniques commonly used for quantitating vWF: Ag are Laurell immunoelectrophoresis[4] and immunoradiometric assay,[5] both of which are time-consuming and inadequate for treating large number of samples. Recently, enzyme-linked immunosorbent assays (ELISA) for vWF: Ag have been reported; ELISA techniques are relatively rapid and simple.[6-9] Therefore, we produced monoclonal antibodies specific for vWF: Ag and developed an ELISA technique using one of the monoclonal antibodies to monitor the recovery of vWF: Ag on plasma fractionations. In particular, we have used the technique for

the evaluation of several thawing methods for cryoprecipitate production.

Materials and Methods

Reference and Test Samples——A plasma pool of 12 normal donor samples was used as a reference, representing 1.0 unit/ml of vWF : Ag and VIII : C. Cryoprecipitates were prepared by the following three thawing methods[3]; modified thaw-siphon, rapid-thaw or slow-thaw. Plasma of patients with severe von Willebrand's disease was obtained from George King Bio-Medical, Inc.

Assay of VIII : C——The activity of VIII : C was measured by an activated partial thromboplastin time assay (APTT),[10] with commercial coagulation factor VIII-deficient plasma of patients with hemophilia A (Dade) as a substrate.

Purification of Factor VIII——Partially purified factor VIII was isolated from cryoprecipitates. Cryoprecipitates were dissolved in 0.025 M imidazole–saline buffer, and applied to a Sepharose 4B column (70 × 2.6 cm). Fractions containing VIII : C, eluted at the void volume, were pooled and concentrated by ultrafiltration for immunization. Further, VIII : C fraction was obtained by gel chromatography of partially purified factor VIII in the presence of 0.25 M $CaCl_2$. The fractions showing VIII : C activity were pooled and concentrated. The concentrate was used as VIII : C fraction for monoclonal antibodies (MAb) screening (see below).

Preparation of Monoclonal Antibodies——BALB/c mice were immunized with partially purified factor VIII (50 μg). The MAb were produced by a modification of the method of Kohler and Milstein.[11] Briefly, 1×10^8 spleen cells obtained from an immunized mouse were fused with 5×10^8 NS-1 murine myeloma cells. After cell culture, the hybridoma cells producing specific antibody against factor VIII were detected by an ELISA technique described below and were cloned by the limiting dilution technique. The MAb were produced in mouse ascitic fluid and purified by ammonium sulfate precipitation and ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose. Proteins in each fraction were analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Screenings of MAb were done by an ELISA technique (Hybri–Clonal EIA screening kit, Kirkgaard & Perry Lab.) using polystyrene plates which had previously been coated with partially purified factor VIII, VIII : C fraction, fibrinogen (Kabi), albumin or commercial factor VIII (Conco-eight, Midori-Juji).

ELISA for vWF : Ag——EIA screening kit reagents for MAb assay were also used. The rabbit antibody against vWF : Ag was purified from rabbit antisera (Behring, Clotimmun AHG-associated protein). The antibody solution was diluted to 50 μg protein/ml in 0.05 M carbonate buffer, pH 9.6, and an aliquot of 50 μl was added to each well of a 96-well microplate (Falcon 3911, Microtest III assay plate). After incubation for 1 h at 37 °C, each well of the plate was filled with diluent/blocking solution (DBS; EIA kit reagent) and allowed to stand overnight at 37 °C. Plates were then tapped dry and stored at −40 °C until use. The assay procedures were as follows: (1) Reference plasma or samples, 50 μl/well, diluted serially with DBS, were incubated at 37 °C for various periods of time. After decanting, each well was washed 5 times with wash solution (EIA kit reagent). (2) Then 50 μl/well of diluted MAb (170 μg/ml) in DBS was allowed to react with the plate at 37 °C for 30 or 60 min and each well was washed as before. (3) The third antibody, peroxidase-labeled antibody (EIA kit reagent) (50 μl), was added to each well, incubated for 30 or 60 min at 37 °C and washed. (4) Color development was initiated by adding 50 μl of the enzyme substrate (EIA kit reagent) to each well. After 15 min at room temperature, the reaction was stopped by adding 50 μl of 10% SDS and the absorbance at 405 nm was measured.

Results

Properties of MAb

The monoclonal antibody screened by the described method was positive toward partially purified factor VIII and factor VIII concentrate, and negative toward VIII : CAg fraction, fibrinogen and albumin. The antibody did not inhibit VIII : C activity in APTT assay. The MAb used in the present study belonged to the immunoglobulin G (IgG) class as determined by SDS–polyacrylamide gel electrophoresis, but the subclass was not determined.

Partially purified factor VIII was applied to the affinity column of MAb-coupled Sepharose 4B. Fractions containing VIII : C were eluted from the affinity column in the presence of 0.25 M $CaCl_2$, but MAb screening of these fractions was negative. The following fractions, eluted with 0.2 M glycine–HCl, pH 2.5, reacted with MAb (data not shown). In addition, the rabbit antibody used as the first antibody inhibited VIII: C activity in the APTT assay. After partially purified factor VIII solution was incubated with the rabbit antibody, vWF : Ag was not detectable by ELISA in the solution, indicating that the MAb used for

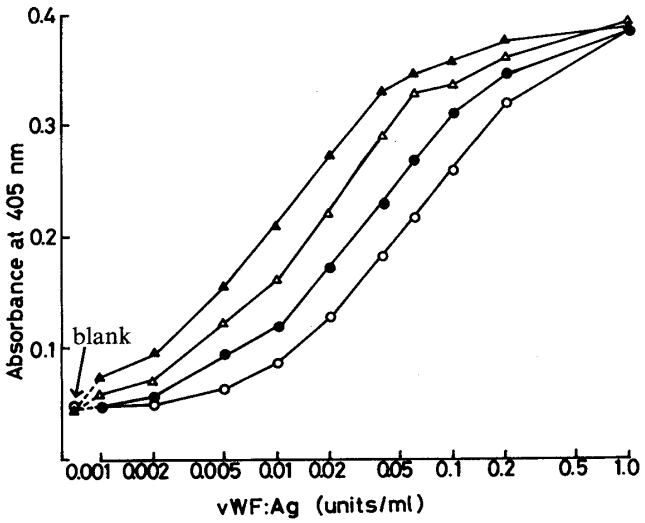

Fig. 1. Standard Curves for vWF : Ag Obtained with Various Incubation Periods of the Reference Plasma with the Plate

Blank solution also contained 50 μl/well DBS. The incubation period were 15 min (○), 30 min (●), 60 min (△), 90 min (▲).

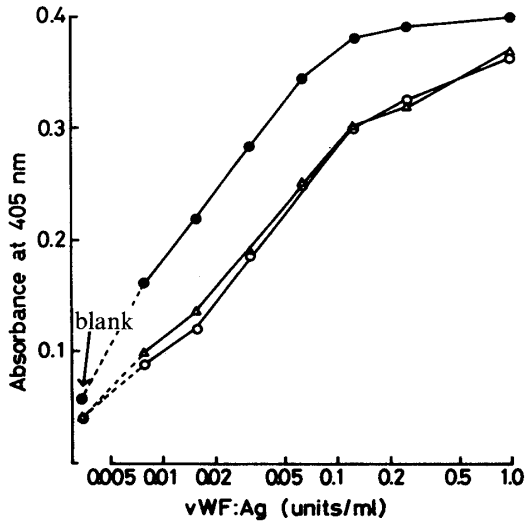

Fig. 2. Standard Curves for vWF : Ag

A 30-min incubation was performed for all three reaction steps (○). The incubation of MAb was for 60 min (△). The incubation of the peroxidase-labeled antibody was for 60 min (●). Blank tests contained 50 μl/well DBS.

ELISA was bound to vWF : Ag.

Assay Characteristics

Figure 1 shows standard curves for vWF : Ag in reference plasma, produced with various incubation periods of the reference plasma with plates, from 15 to 90 min. Absorbance values for negative controls were below 0.05 for all incubation periods. The standard curve obtained by 90-min incubation was linear from 0.005 to 0.04 units/ml of vWF : Ag with a minimal detectable concentration of 0.001 units/ml. For 15-min incubation, the linear range was between 0.02 and 0.2 units/ml and the minimal detectable concentration was 0.005 units/ml. The same range of absorbance values gave linear plots regardless of the incubation period tested.

A comparison of different incubation periods for the binding of MAb and the labeled antibody is shown in Fig. 2. No difference between a 30-min and a 60-min incubation for MAb was observed.

When the reaction with the labeled antibody was prolonged for up to 60 min, absorbance values for the negative controls and non-diluted plasma did not increase significantly. Although the vWF : Ag contents of some samples were measured by varying the incubation period for each reaction step, the same values of the vWF : Ag content were observed regardless of the incubation period (Table I). Thus, a 30-min incubation was used for all of the three reaction steps.

Sample dilutions had no effect on the vWF : Ag contents (Fig. 3). At the range of absorbance values in the linear portion of the standard curve, the dose–response curves for samples were also linear and parallel with the standard curve. The dose–response curve for factor VIII-deficient plasma was the same as the standard curve. For severe von Willebrand's disease plasma, absorbance values were not different from those of the negative control.

vWF : Ag in Cryoprecipitate

vWF : Ag assays by ELISA were performed on cryoprecipitate and cryosupernatant prepared by the three thawing methods described above. The results indicated that the modified thaw-siphon method gave a higher vWF : Ag content than the other two methods. Similar results were also obtained for VIII : C recovery (Table II). However, the differences in

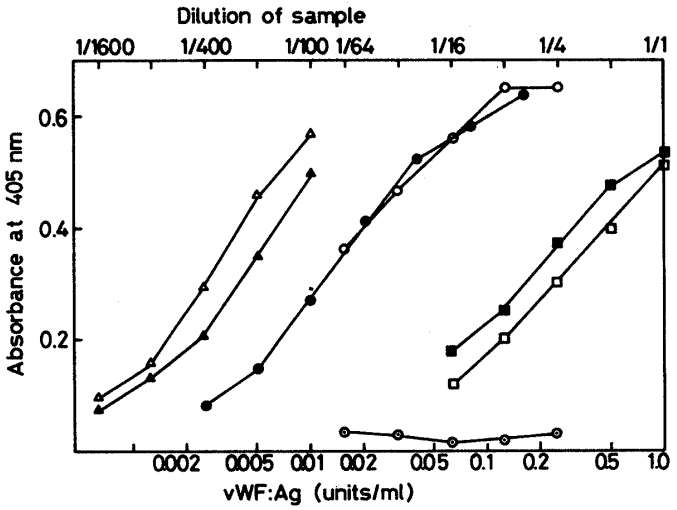

Fig. 3. Dose–Response Curves for Reference Plasma vWF:Ag (●), Cryoprecipitates (△, ▲), Cryosupernatants (□, ■), Factor VIII-Deficient Plasma (○) and Severe von Willebrand's Disease Plasma (⊙)

Dilution of test samples is indicated at the top.

TABLE I. Effect of Various Incubation Periods for the Three Reaction Steps on vWF:Ag Results

Time (min)			Cryosupernatant vWF:Ag (units/ml)[a]	
Sample	MAb	Labeled Ab[b]	Sample 1	Sample 2
30	30	30	0.10±0.01	0.13±0.01
90[c]	30	30	0.10±0.01	0.12±0.01
30	60[c]	30	0.10±0.00	0.13±0.01
30	30	60[c]	0.11±0.01	0.13±0.00

a) Mean±S.D. ($n=3$). *b*) Peroxidase-labeled antibody. *c*) Prolonged incubation time.

TABLE II. Effect of Different Thawing Methods for Cryoprecipitate Preparation on vWF:Ag and VIII:C

Thawing method	Modified thaw-siphon	Rapid-thaw	Slow-thaw
vWF:Ag (units/ml)[a]	7.93±0.41	7.10±0.47	6.10±0.71
VIII:C (units/ml)[a]	7.13±0.52	5.90±0.55	4.10±0.38

a) Mean±S.D. ($n=6$).

TABLE III. Ratio of VIII:C to vWF:Ag Content Produced by the Three Cryoprecipitate Preparation Methods

Thawing method	Modified thaw-siphon	Rapid-thaw	Slow-thaw
Cryprecipitate[a]	0.88±0.06 (6)	0.81±0.10 (6)	0.70±0.14 (6)
Cryosupernatant[a]	3.33±0.72 (3)	3.52±0.17 (3)	1.68±0.11 (3)
Plasma	1.17±0.15 (6)		

a) Mean±S.D. (number of samples).

vWF : Ag content between thawing methods were smaller than those of VIII : C. The ratios of VIII : C to vWF : Ag contents in cryoprecipitates were 0.88 for thaw-siphon and 0.70 for slow-thaw (Table III). The values were lower than that of 1.17 for plasma. On the other hand, the ratios were 1.68—3.52 for cryosupernatants, with VIII : C being much higher than vWF : Ag.

Discussion

The reaction period of samples with the antibody-coated plates affected color development in the ELISA for vWF : Ag. When incubation periods were prolonged for up to 90 min, the linear portion of the standard curve was shifted to lower concentrations of vWF : Ag and smaller amounts of vWF : Ag could be determined with better accuracy.

For our purpose of evaluating the thawing methods, the standard curve obtained with a 15-min incubation (0.02—0.2 units/ml) was acceptable, since the sensitivity of the assay was sufficient. However, an incubation period of 30 min was chosen for our routine assay, since it was convenient when several samples were simultaneously manipulated.

Using this system, vWF : Ag in cryoprecipitates and other samples could be assayed and a number of thawing methods in preparing cryoprecipitates were easily evaluated not only for vWF : Ag recoveries but also for the ratio of VIII : C to vWF : Ag content. It has been reported that the ratios of VIII : C to vWF : Ag content in the cryoprecipitate were about 0.5—0.8 by immunoradiometric assay[12] and 0.87.[13] These values are consistent with our data (0.70—0.88).

It has been reported that the properties of factor VIII in cryosupernatants are distinct from those of the factor in cryoprecipitates.[14,15] These reports suggested that smaller forms of factor VIII present in plasma remain in the cryosupernatant and correspond to VIII : CAg complexed with vWF multimer of relatively low molecular weight. We found that the ratios of VIII : C to vWF : Ag contents in cryoprecipitates were decreased when compared with plasma, while the ratios in cryosupernatants were high. These results suggested that vWF multimers in cryosupernatant were lower multimeric forms than those in cryoprecipitate, in accordance with previous reports. The ELISA system presented here requires less time and is technically simpler than other ELISA methods for vWF : Ag. Further studies are in progress in order to characterize the differential reactivities of the MAb with variable oligomeric forms of vWF.

Acknowledgment The authors wish to express their gratitude to Dr. W. L. Gyure of the Jersey City Medical Center for his help in preparing this manuscript.

References

1) L. W. Hoyer, *Blood*, **58**, 1 (1981).
2) E. P. Kang, *Vox Sang.*, **38**, 172 (1980).
3) K. Inoue and T. Mazda, *J. Jpn. Soc. Blood Transfusion*, **30**, 108 (1984).
4) T. S. Zimmerman, L. W. Hoyer, L. Dickson and T. S. Edgington, *J. Lab. Clin. Med.*, **86**, 152 (1975).
5) L. W. Hoyer, *Methods Enzymol.*, **84**, 51 (1982).
6) A. Bartlett, K. M. Dormandy, C. M. Hawkey, P. Stableforth and A. Voller, *Brit. Med. J.*, **1**, 994 (1976).
7) L. D. Yorde, C. V. Hussey, D. E. Yorde and E. A. Sasse, *Clin. Chem.*, **25**, 1924 (1979).
8) K. Ogata, H. Saito and O. D. Ratnoff, *Blood*, **61**, 27 (1983).
9) L. A. Bradley, E. L. Franco and H. M. Reisner, *Clin. Chem.*, **30**, 87 (1984).
10) R. D. Langdell, R. H. Wagner and K. M. Brinkhous, *J. Lab. Clin. Med.*, **41**, 637 (1953).
11) G. Kohler and C. Milstein, *Nature* (London), **256**, 495 (1975).
12) L. Thorell and B. Blomback, *Thromb. Res.*, **35**, 431 (1984).
13) C. Th. S. Sibinga, H. Welbergen and P. C. Das, *Lancet*, ii, 449 (1981).
14) H. J. Weiss and S. Kochwa, *Brit. J. Haematol.*, **18**, 89 (1970).
15) J. Over, B. N. Bouma, J. A. van Mourik, J. J. Sixma, R. Vlooswijk and I. Bakker-Woudenberg, *J. Lab. Clin. Med.*, **91**, 31 (1978).