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# PRODUCTION OF MONOCLONAL ANTIBODIES SPECIFIC TO HUMAN VON WILLEBRAND FACTOR

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## ABSTRACT

A monoclonal antibody specific for human von Willebrand factor, vW1E2, was produced after immunization of BALB/c mice with partially purified FVIII/von Willebrand factor. Testing of sera and screening of hybridomas was done by ELISA using rabbit anti-vWF serum as capture layer of vWF. The specificity of vW1E2 to vWFAg was determined by testing it against plasmas of normal individuals, hemophiliacs A and vWD patients. This specificity was corroborated by immunoprecipitation experiments in which vWFAg was precipitated from plasma by vW1E2 with the aid of rabbit anti- mouse Ig serum, as assessed by electroimmunoassay (EIA) and ELISA. An ELISA employing vW1E2 was evaluated as an assay for quantification of plasmatic vWFAg. Plasma samples from type I and type II vWD patients were analyzed as well as from hemophilic A patients and normal individuals. The levels of vWFAg obtained by ELISA were compared to those by EIA and a significant correlation was observed ($r = 0.735$; $df = 35$; $P < 0.001$).

## INTRODUCTION

Hemostasis refers to the system which maintains the integrity of the mammalian circulatory system after the occurrence of vascular lesions. Vital roles are played in this system by von Willebrand factor (vWF) which promotes the formation of the platelet thrombus in the injured blood vessels. vWF also circulates with blood coagulation factor VIII (FVIII) in the plasma as a FVIII/FvW complex that protects FVIII against proteolytic degradation and removal from circulation.

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vWF abnormalities characterize von Willebrand disease (vWD), the most common inherited bleeding disorder in humans, with an estimated prevalence as high as 1% (Rodeghiero *et al.*, 1987). Disorders of vWF are highly diverse. Some patients have reduced amounts of apparently normal vWF (type I vWD), while others have clearly abnormal vWF with aberrant structure and functions (type II vWD). Rare patients virtually or completely lack vWF (severe or type III vWD).

The measurement of vWF antigen (vWFAg) using heterologous polyclonal antibodies is used for differential diagnosis between hemophilia A and von Willebrand disease and in the detection of hemophilia A carriers. Although of value, heterologous anti-sera contain a heterogeneous mixture of antibodies for a diverse array of epitopes on vWF. For purpose of unequivocal specificity in immunochemical assays, as well as in isolation and characterization of its structure, monoclonal antibodies (Mabs) represent a significant advance.

Since the first report on Mabs against FVIII/FvW complex (Meyer *et al.*, 1980), several other monoclonal antibodies against one or another protein of this complex have been described (Goodall and Meyer, 1985).

This paper describes the production and characterization of a monoclonal antibody specific for human vWF. As far as we know, monoclonal antibodies specific for vWF have not yet been produced in Brazil.

## MATERIAL AND METHODS

### *Plasmas and antisera*

Reference plasma was constituted by a pool of citrated plasmas from 16 normal donors buffered to pH 7.3 with HEPES and lyophilized. Sample plasmas were obtained from patients with von Willebrand disease and hemophilia A and were maintained at -20°C. The patients were diagnosed at the Laboratório de Hemostasia, Departamento de Genética, Universidade Federal do Rio Grande do Sul using routine coagulation assays. Two rabbit polyclonal anti-human vWFAg sera were used for electroimmunoassay (EIA, Laurell "rocket" assay). One of them was a kind gift from Dr. T.S. Zimmerman (Scripps Clin. Res. Found., CA, USA), and the other was produced by us using the antigen described below.

### *Preparation of antigen*

FVIII/vWF commercial concentrate (LIPSA 200 U, P. Alegre, Brazil) was filtered through Sepharose 4B-200 as described (Yang *et al.*, 1977). Fractions were tested for the presence of vWFAg by electroimmunoassay (EIA) using an anti-vWFAg rabbit

antibody and tris-tricine buffer, pH 8.6 (Zimmermann *et al.*, 1975; Monthony *et al.*, 1978). Protein contents in the fractions were determined by the method of Bradford (1976). Fractions with peaks of vWFAg were pooled and lyophilized or concentrated by dialysis.

*Immunizations*

The working antigen dosage was determined by immunizing C57BL/6 mice with 2, 10, 20 or 50 µg of antigen in 0.1 ml volumes with equal volume of complete Freund's adjuvant. The mice received two intraperitonial injections 3 weeks apart. Serum was collected 7 days after each injection and tested by ELISA.

BALB/c mice, immunized as described above with 10 µg antigen, were used for the production of hybridomas. A further boost was given 3 days before the fusion procedure.

*Production of hybridomas*

Hybridomas were produced after standard techniques (Nardi and Onsten, 1988), with SP20/Ag-14 as myeloma partner. Hybridomas selected by growth in HAT medium (RPMI 1640 medium (Flow, USA) with hypoxanthine, aminopterin and thymidine) were tested by ELISA against FVIII/vWF antigen. Positive hybridomas were expanded, cloned and subcloned by limiting dilution. Mabs were obtained as culture supernatants or ascite fluid.

*ELISA*

An ELISA, in which the vWFAg from immunogen or plasma was retained by anti-vWFAg rabbit antibody (ATAB, USA) immobilized in the solid phase, was employed for testing the antisera and supernatants of the hybridomas. Plates were incubated overnight with anti-vWFAg rabbit sera diluted 1/500 in carbonate/bicarbonate buffer, pH 9.6. The plates were washed and incubated for 30 min at 37°C with blocking solution (5% non-fat dry milk in phosphate-buffered saline, PBS). After two further washings with washing solution (1% non-fat dry milk in PBS) and one with PBS, the plates were incubated for 1-2 h at room temperature (RT) with antigen or plasma diluted in PBS. After one washing with PBS and two with washing solution, the plates were incubated (1 h, RT) with mice sera diluted in the washing solution or hybridoma supernatant. The plates were washed and incubated with peroxidase-labelled anti-mouse immunoglobulin (2 h, RT). The plates were then washed with PBS and the enzyme substrate (3.4 mg o-phenylenediamine in 10 ml citrate/phosphate buffer, pH 5.0, with 3.5

μl $H_2O_2$ 30 vol) was added. The reaction was stopped after 5-10 min by addition of 12% $H_2SO_4$. Optical densities were read at 492 nm, or analysed visually. The quantification of vWFAg from plasma was performed in the same test but here anti-vWFAg Mab (vW1E2) was used for detection. Plasmas from type III vWD patients as well PBS buffer were used as negative controls. The level of vWFAg in the diluted sample was obtained as a percentage of diluted reference plasma (100% of vWFAg by definition). The $OD_{492}$ of negative control was subtracted from these values. A total of 12 plasma samples from type I vWD patients and 8 from type II were analysed, as well as 10 samples from normal individuals and 7 from hemophilic A patients.

### *Immunoprecipitation of vWF from plasma*

Reference plasma was incubated for 1 h at 37°C with hybridoma supernatant. Control reactions were done with PBS or SP20/Ag-14 supernatant. 100 μl of each mixture was incubated (1 h, 37°C) with 50 μl of either rabbit anti-mouse Ig serum, normal rabbit serum or PBS. The mixtures were centrifuged at 10.000 x g for 3 min. The supernatants were collected and tested for vWFAg by electroimmunoassay and ELISA, and for capacity of inhibiting FVIII coagulating activity. Sera and supernatants were mixed (9:1, v/v) with 3.8% sodium citrate before use.

## RESULTS

### *Purification and antigenicity of FVIII/vWF complex*

A chromatogram using Sepharose 4B-200, representative of purification results, is shown in Figure 1. Pooled antigenic fractions presented 49 units vWFAg/mg protein, as compared to fresh normal plasma (1 ml pooled fresh normal plasma contains 1U vWFAg and, on average, 0.014 units vWFAg/mg protein).

The partially purified antigen induced the production of antibodies in mice after the second injection, except when the dosage of 2 μg was used (Figure 2).

### *Production of hybridomas*

The best fusion experiment yielded 182 hybridomas from which only one was positive for vWFAg. This hybridoma, vW1E2, was cloned and subcloned and is stable in culture. The Mab was isotyped by gel diffusion as IgG1.

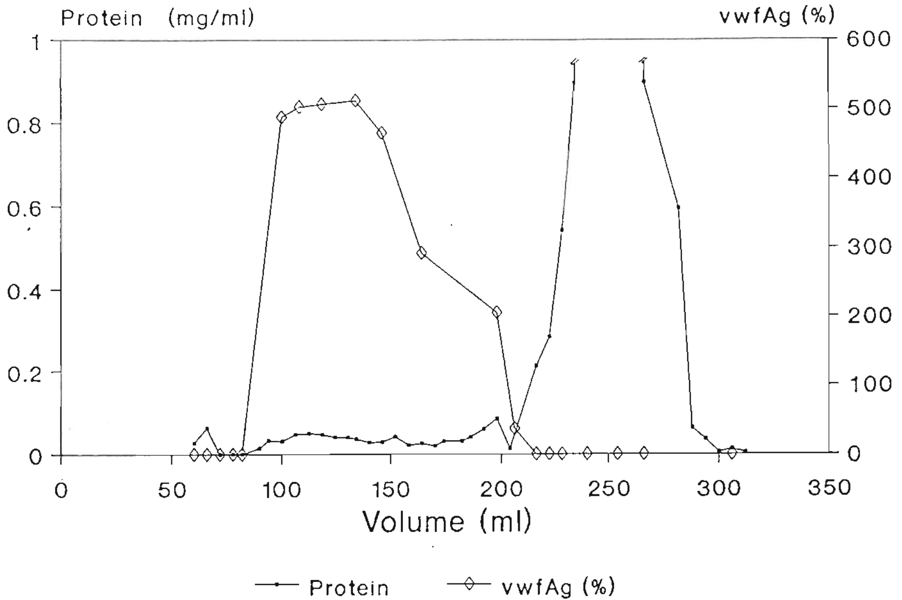


Figure 1 - Partial purification of FVIII/vWF complex on Sepharose 4B-200. Protein contents were determined by spectrophotometry. vWFAg concentration relative to reference plasma (100% vWFAg) was assayed by electroimmunoassay.

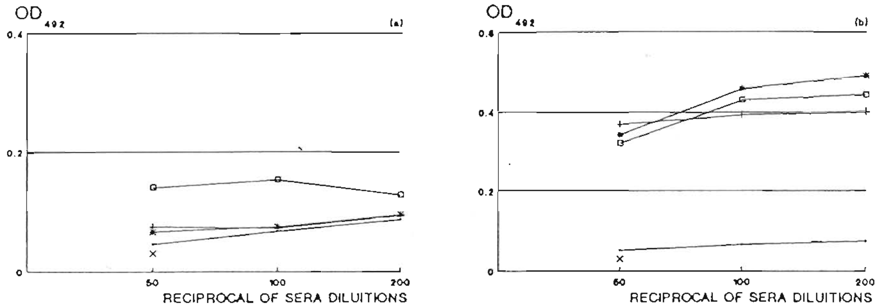


Figure 2 - Immunogenicity of partially purified human FVIII/vWF complex. C57Bl/6 mice received two injections, 3 weeks apart, of antigen in four different dosages: 2 µg (.), 10 µg (1), 20 µg (*) and 50 µg (□). Control mice (x) were not immunized. Each group was composed by 3 or 4 animals. Blood was collected 7 days after injection, and sera were analysed by ELISA. (A) primary response. (B) secondary response.

*Specificity of Mab vW1E2 and vWFAg quantification*

The use of plasma as antigen in ELISA required rabbit anti-vWFAg as a capture layer, due to the very low concentration of vWF in plasma (5-10 μg/ml). This layer however is not necessary when partially purified FVIII/vWF complex is employed as antigen (data not shown). A nonspecific reaction of immune mouse serum with the rabbit anti-vWFAg sera used as capture layer, as well as with normal rabbit sera, was observed. This reaction does not occur when normal mouse serum is tested.

Supernatants of vW1E2 cultures were tested by ELISA against plasma from normal individuals or from type III vWD or hemophilic A patients. In all type III vWD samples analysed, the levels of vWFAg detected were indistinguishable from those of PBS buffer.

Plasma samples from type I and type II vWD patients as well as hemophilic A and normal individuals, were analysed by ELISA employing vW1E2. In spite of some individual variability observed within each group, it is clear that whereas normal and hemophilic plasmas present normal levels of vWFAg, these levels were lower in vWD plasmas. The levels of vWFAg obtained by ELISA were compared to those by EIA and a significant correlation was observed ($r = 0.735$; $df = 35$; $P < 0.001$) (Table I).

Table I - Comparison of vWFAg measured by ELISA and by EIA in normal individuals, patients with hemophilia A and patients with von Willebrand's disease.

| | n | EIA (%) | | ELISA (%) | |
|---|---|---|---|---|---|
| | | Range | X ± s | Range | X ± s |
| Normals | 10 | 46-141 | 88.1 ± 25.0 | 77-181 | 117.7 ± 35.3 |
| Hemophilia A | 7 | 50-245 | 140.1 ± 59.4 | 66-197 | 117.7 ± 48.4 |
| DvW I | 12 | 0-45 | 12.5 ± 13.9 | 0-52 | 18.1 ± 18.1 |
| DvW II | 8 | 37-162 | 84.1 ± 38.7 | 7-68 | 30.6 ± 23.3 |
| Total | 37 | 0-245 | 72.5 ± 57.4 | 0-197 | 66.5 ± 56.6 |

## DISCUSSION

Qualitative and/or quantitative abnormalities of von Willebrand factor characterize von Willebrand disease. Together with hemophilia A, it constitutes more than 90% of the hereditary bleeding syndromes. Much of what is presently known about the structure of FVIII, vWF and the FVIII/vWF complex is derived from studies

employing monoclonal antibodies. Several murine Mabs directed to FVIII/vWF proteins have been described (reviewed by Goodall and Meyer, 1985). These highly specific antibodies have allowed the characterization of functional domains of FVIII and vWF and a better elucidation of their roles during hemostatic processes (Takahashi *et al.*, 1987; Mohri *et al.*, 1988; Ruggeri, 1990; Fujimura *et al.*, 1991). These reagents have been employed in other kinds of studies such as isolation of the complex or its individual components (Croissant *et al.*, 1986; Mejan *et al.*, 1988), studies of heterogeneity of hemophilia A and vWD by immunoradiometric or immunoenzymatic assays (Mazurier *et al.*, 1977; Bradley *et al.*, 1984; Goodall *et al.*, 1985b; Hall *et al.*, 1987); preparation of FVIII-deficient plasma (Hornsey *et al.*, 1988) and cloning of FVIII and vWF (Lynch *et al.*, 1985).

The Mab vW1E2 described in this paper was shown by ELISA to recognize specifically vWF present in partially purified samples as well as in plasma. The use of plasma as a source of antigen in ELISA demanded the presence of a capture layer, but on the other hand allowed the screening of hybridomas with native vWF. The nonspecific reactivity of immune serum with rabbit immune or normal sera could not be explained by this study, but it did not interfere with the results of the assay.

Binding of vW1E2 to plasma vWF was corroborated by the results from the immunoprecipitation experiments, in which the antibody was able to remove vWFAg from plasma (Figure 3). In the conditions employed in this study, however, vW1E2 could not remove FVIII procoagulant activity from plasma. This result can be explained in different ways. It is possible that FVIII and vWF do not precipitate together due to the dissociation of the non-covalent complex in the test conditions or, alternatively, that free vWF is preferentially removed. This point requires further studies to be elucidated.

Although a small number of vWD plasmas were assayed, vW1E2 was shown to be able to distinguish type III vWD patients from normal or hemophilia A individuals. Our results suggest that vW1E2 can also be used for the quantification of vWFAg in assays as relatively simple as ELISA. All individuals analyzed also presented results consistent with the diagnosis already established. Even patients presenting vWFAg levels within normal limits when tested by EIA, showed reduced vWFAg, consistent with vWD when tested by ELISA. Similar results were reported by Mazurier *et al.* (1977) and Mazurier (1980) and have allowed an increase in the sensitivity of differential diagnosis. An explanation for the higher sensitivity of ELISA may be that EIA results depend on the electrophoretic mobility of the protein, whereas ELISA results do not.

These results demonstrate the potential of ELISA as a quantification assay for vWFAg. This assay is easily carried out, highly sensitive and widely used as routine laboratorial assay. The production of anti-vWF monoclonal antibodies may stimulate the research of vWF and hemorrhagic associated diseases, which is still very incipient in Brazil. Furthermore, they allow the establishment of laboratorial diagnostic tests, avoiding the importation of costly equivalent reagents.

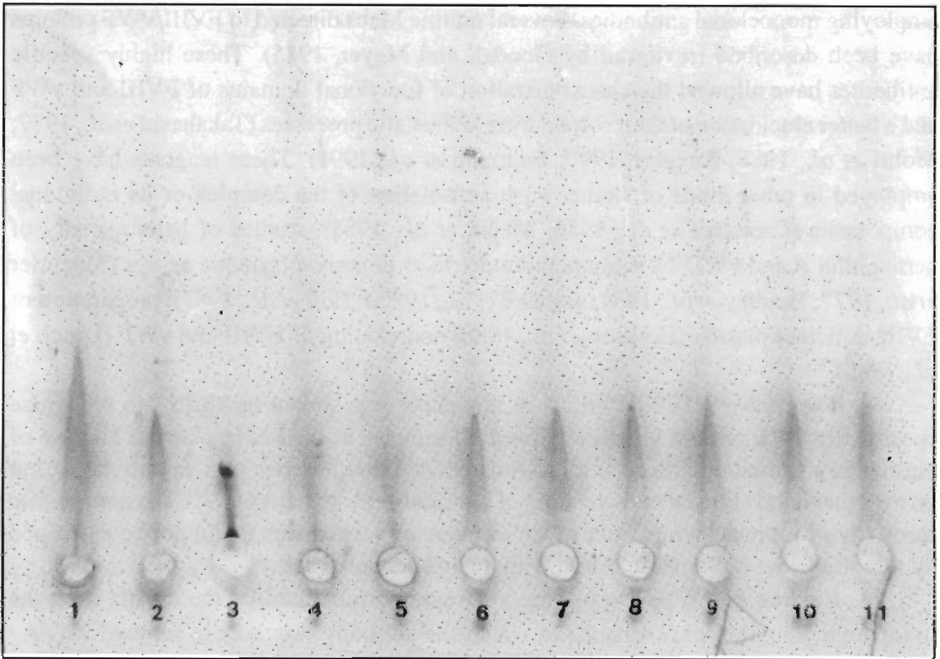


Figure 3 - Immunoprecipitation of vWF. Plasma was incubated with vW1E2 and then with rabbit anti-mouse Ig serum, or with control solutions, and centrifuged. The supernatant was submitted to electroimmunoassay. 1, 2, 3 - reference plasma diluted to 1/2, 1/4 and 1/8 respectively. Following lanes: reference plasma incubated with; 4, 5 - vW1E2 + rabbit anti-mouse Ig serum; 6, 7 = SP2/O-Ag14 supernatant + rabbit anti-mouse Ig serum; 8 = PBS; 9, 10 - vW1E2 + normal rabbit serum; 11 = PBS + normal rabbit serum.

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## RESUMO

Um anticorpo monoclonal específico para o fator von Willebrand (FvW) humano, o vW1E2, foi produzido imunizando-se camundongos da linhagem BALB/c com FVIII/FvW parcialmente purificado. A análise do anti-soro e a triagem dos hibridomas foram feitas por ELISA empregando anti-soro de coelho anti-FvW como anticorpo de captura do FvW plasmático. A especificidade do vW1E2 ao FvW foi demonstrada

testando-o contra plasmas de indivíduos normais, hemofílicos A e pacientes com DvW. Esta especificidade foi confirmada através de experimentos de imunoprecipitação nos quais o FvWAg era precipitado do plasma pelo vW1E2 com auxílio de anti-soro de coelho anti-Ig de camundongo, como demonstrado por imunoeletroforese (EIA) e ELISA. Foi então desenvolvido um ELISA, empregando o vW1E2, o qual foi avaliado como técnica de quantificação do FvWAg plasmático. Foram testados amostras de plasmas de pacientes com DvW tipo I e tipo II, assim como de indivíduos normais e hemofílicos A. Os níveis de FvWAg obtidos por ELISA foram comparados aos obtidos por EIA observando-se uma correlação significativa entre eles ($r = 0,735$; $GL = 35$; $P < 0,001$).

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