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von Willebrand's Disease: Use of Collagen Binding Assay Provides Potential Improvement to Laboratory Monitoring of Desmopressin (DDAVP) Therapy

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This report describes studies investigating the use of a collagen binding assay to improve the laboratory monitoring of desmopressin (DDAVP) therapy in patients with von Willebrand's disease (vWD). We evaluated the response of seven patients with vWD (four type I, three type IIA) to DDAVP, administered using a standard protocol, by assessing levels of von Willebrand factor (vWF) and factor VIII, as well as performing skin bleeding times (SBT) prior to, and at sequential time points following, DDAVP administration. The study employed the following assays: von Willebrand factor antigen assay (vWF:Ag; determined by ELISA); a novel functionally based collagen binding assay (CBA; determined by ELISA); ristocetin cofactor assay (RCof; determined by platelet aggregometry); von Willebrand factor multimer analysis (using SDS-agarose gels); factor VIII coagulant (FVIIIC; determined by clotting assay); and factor VIII antigen (FVIIICAG; determined by ELISA). All patients showed an initial incremental increase in vWF/FVIII levels using all assays above, and some showed some correction in SBT. Although the absolute levels of vWF/FVIII antigen or activity varied between patients, the CBA was found to provide consistently the greatest proportional incremental increases (i.e., -fold) compared to baseline (pre-DDAVP) levels. Accordingly, we consistently observed an increase in the CBA to vWF:Ag ratio for all patients evaluated. This supplements previous findings that have suggested a unique ability of our CBA procedure to bind preferentially to higher molecular weight (i.e., more functionally active) forms of vWF. We therefore propose that the use of the above test combination (e.g., vWF:Ag plus CBA) may provide the basis for more accurate estimation of a patient's functional responsiveness to DDAVP therapy in future studies.

Key words: von Willebrand's disease, von Willebrand factor, desmopressin, DDAVP, collagen binding assay

INTRODUCTION

Von Willebrand factor (vWF) has two primary roles in haemostasis. First, it permits adhesion of platelets to sites of vascular damage, and, second, it acts to stabilise factor VIII within blood [1–4]. Patients suffering defects in, or reduced level of, vWF are diagnosed as having von Willebrand's disease (vWD) and are typed according to classification protocols involving both clinical observations and laboratory analysis of patient plasma [1,2]. Indeed, vWD is now reported to be the most common inherited bleeding disorder [see, e.g., 4–6]. Laboratory investigation usually involves a battery of tests, including determination of (skin) bleeding times, vWF antigen (vWF:Ag) levels, functional activities such as factor VIII coagulant (FVIIIC) and ristocetin cofactor (RCof), and vWF multimetric analysis [1,2].

Generally, type I vWD patients suffer from a quantitative reduction in all vWF multimers present, with concurrently decreased levels of vWF:Ag and RCof activity (typically in similar proportions). Alternatively, type II vWD is characterised by qualitative abnormalities of

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vWF multimers. Although at least four subtypes have been characterised, these patients generally exhibit a prolongation in their bleeding time, normal to slightly reduced levels of both factor VIII and vWF:Ag, reduced RCof activity, and an absence of intermediate to large vWF multimers. Classification of the patient's vWD is important not only because the biological activity of vWF is related to its multimer profile, but also since subsequent clinical management may differ substantially on this basis [5–8]. In order to improve and simplify our laboratory's diagnostic procedure for vWD, we developed a novel functional collagen-based ELISA assay for vWF. Based on previous observations that vWF binds to collagen, with preference for high-molecular-weight forms, this assay is capable of sensitive discrimination between plasma derived from type II vWD patients, and that derived from either type I vWD patients, or from normal individuals [9,10]. The current study extends the findings of these reports, by analysing, in parallel, changes that occur in both von Willebrand factor and factor VIII in patients administered desmopressin (DDAVP) and utilising our improved laboratory procedure. This study is thus the first to report on the use of this CBA in such treatment procedures and suggests that this laboratory test, in association with other standard procedures (such as the vWF:Ag assay), may more properly reflect true functional changes to vWF following DDAVP than do currently existing laboratory protocols.

MATERIALS AND METHODS

Patient Samples

These were derived from patients previously assessed as having von Willebrand's disease type I or type IIA, but whose DDAVP responsiveness had not been established. All assays were performed using plasma isolated from venous blood collected into 0.012 M sodium citrate (9 parts blood, 1 part citrate), and centrifuged (1,500 *g*; 15 min) to remove cellular components, according to standard procedures. In addition to patient samples, a large number of individual normal plasma samples were also obtained from healthy volunteers and were utilised for control purposes and to provide a large pool (PNP; >60 individuals) for assay calibration. All plasma samples collected were snap frozen (at −70°C) to permit multiple analysis by various assays.

DDAVP (Desmopressin) Infusion and Blood Collection/Testing Protocol

This was a standard protocol for administration of desmopressin (0.3 μg desmopressin/kg i.v.; administered over a 30 min period). After we obtained informed consent, blood samples were drawn prior to DDAVP administration and at selected time intervals following completed administration, and specifically at plus 30 min, and then +1 hr, +2 hr, +4 hr, and +6 hr. Tests performed were as stated below, plus monitoring of routine coagulation parameters (prothrombin time, PT; activated partial thromboplastin time, APTT). A blood sample was also collected into EDTA at these times to permit monitoring of routine haematology parameters (platelet counts, white cell counts, red cell counts, haemoglobin, haematocrit, etc). Skin bleeding time (SBT) estimations were performed prior to DDAVP administration, and repeated at time plus 2 hr following administration.

Laboratory Protocol for Assessment of DDAVP Therapy

Assays for von Willebrand factor (vWF) antigen levels (vWF:Ag), ristocetin cofactor (RCof) functional activity, a functional collagen binding assay (CBA), and assays for factor VIII coagulant (FVIIIC), and factor VIII antigen (FVIIICAG) were as described below and elsewhere [9,10]. To permit comparative evaluation, and to allow derivation of assay to assay ratios (e.g., CBA to vWF:Ag ratio), the lower limit of sensitivity for each assay was arbitrarily set at 0.1%. Skin bleeding times were performed using Simplate II devices according to manufacturer's instructions (AKZO/Organon Teknika, Australia), and multimer analysis was performed as indicated below.

ELISA assay for vWF:Ag. This assay, a sandwich, indirect 96-well plate procedure, has been described in detail elsewhere [9]. Test plasma values for vWF:Ag were calculated with reference to PNP.

Ristocetin cofactor assay. This assay has also been described in detail elsewhere [9] and utilises platelet aggregometry.

Collagen binding assay for vWF. This assay, together with a detailed analysis of assay variables, has been outlined previously [9] and is based on modifications to the assay originally described by Brown and Bosak [11].

Factor VIII coagulant levels. These were assessed using a standard clotting based semiautomated procedure, utilising mixing tests with factor VIII deficient plasma (AKZO/Organon Teknika, Australia), and the ACL-300R automated coagulation analyser (Coulter, Australia). Factor VIII coagulant activity was calculated with respect to a commercial standard plasma (Verify plasma; AKZO/Organon Teknika, Australia); PNP was used as a secondary reference plasma source.

Factor VIII antigen assay. This was performed as an ELISA procedure, which utilised reagents similar to those previously described for vWF:Ag and CBA [9,10]. Specific modifications were as follows: Fresh 96-well plates were coated (overnight, 4°C) with antisera to factor VIII (immunoglobulin fraction derived and purified from a patient with factor VIII inhibitor; diluted according to previously established titre experiments). Plates were

then washed three times using BSA ELISA buffer [9], blocked using 5% BSA (1 hr, 22°C), and rewashed twice. Patient plasma was then introduced (at final 1:10 dilution in BSA ELISA buffer containing 0.1% sodium azide), and plates were left overnight (22°C) and rewashed five times. Biotinilated antisera (obtained from previously biotinilated immunoglobulin fraction derived and purified from patient above) were then introduced (using previously established dilution) in BSA ELISA buffer containing 0.1% sodium azide, and plates were again left overnight (22°C). Plates were rewashed six times, and wells were then incubated with a suitable dilution (typically 1:1,000) of commercially prepared streptavidin-HRP (Dakopatts, Denmark; 2 hr, 22°C). Plates were rewashed six times and colour visualised as per the vWF:Ag ELISA [9]. A calibration curve utilising PNP was set up with each plate to extend from 0% to 400%, with respect to PNP at final dilution 1:10 being equivalent to 100% of the normal level of FVIII CAG [see 9].

Multimer analysis. This was performed as previously described [12,13].

RESULTS

General Observations

A total of seven vWD patients were evaluated in this study (four type I, three type IIA). All patients tolerated DDAVP well, and all showed an initial incremental increase in vWF and FVIII levels using all relevant assays, although the sensitivity in the respective assays to detect such changes varied, both between patients and between assays. Some patients also showed some correction in their skin bleeding times following DDAVP infusion (see below). No patient showed any consistent change in any routine haematology (noncoagulation) parameter (e.g., white cell count, red cell count, platelet count, haemoglobin, haematocrit, etc.; data not shown). Monitoring of routine coagulation tests showed a small, but consistent, fall in the APTT for all patients following DDAVP infusion, whereas the PT did not change appreciably for any patient (data not shown).

Changes in vWF Parameters Following DDAVP

All patients evaluated in the current study showed an initial incremental increase in the levels of vWF in patient plasma following DDAVP, as detected by each of the four vWF assays employed (see below and Figs. 1, 2). However, patients differed markedly in respect to the magnitude of response on both a patient to patient and on an assay to assay basis. In this regard, then, type I vWD patients tended to show greatest absolute release responses (see Fig. 1) compared to type IIA vWD patients (Fig. 2). Most striking in the DDAVP response in type I vWD patients was the incremental increases observed in CBA values, which markedly overshadowed the vWF response detected using any other assay type (Fig. 1). This is further strikingly depicted in Figure 3, where changes have been shown as -fold increases over baseline (i.e., pre-DDAVP) levels. Even in type IIA patients, where the absolute quantitative increase in CBA was not as marked (Fig. 2), changes, when expressed as -fold increase over baseline levels, tended to be most striking (Fig. 4). As a result of this differential pattern, the ratio of CBA-derived values to vWF:Ag-derived values for each patient is observed to increase following DDAVP infusion (and particularly for type I vWD patients).

Changes in FVIII Parameters Following DDAVP

All patients evaluated in the current study showed increases in the levels of FVIII in patient plasma following DDAVP, as detected by the two FVIII assays employed (see Figs. 1, 2). Differences detected between patients in their response to DDAVP in terms of factor VIII levels were not as marked as those observed for vWF above. Thus, all patients gave a good response in terms of both FVIII coagulant activity and antigen, with similar upward changes between patients (even between type I and type IIA patients). Responses were generally more striking with respect to functional (FVIII coagulant) activity (see Figs. 1, 2). Changes expressed as -fold increase over baseline levels also tended to be fairly consistent between patients (see Figs. 3, 4).

Time Course of Changes to vWF and FVIII

Peak responsiveness to DDAVP was observed for most patients, and using most assays, at between 30 min and 2 hr post-DDAVP (see Figs. 1–4). Levels remained elevated even at 6 hr post-DDAVP infusion (Figs. 3, 4). While there were minor differences between patients and between assays, there did not appear to be any consistent major differences in the pattern of changing levels with time as assessed by using the different assays. Thus, peak levels of vWF, or of FVIII, as assessed by all assays, and for each patient, occurred at a similar time point (usually between 30 and 60 min post-DDAVP), and fell subsequently at rates that we could not determine to be different. One patient's responsiveness was assessed for a longer time frame (type I vWD). In this patient, values for vWF and FVIII fell to baseline levels by 24 hr; at this time the patient was rechallenged with DDAVP and showed a smaller, but significant, response as detected by all assays (data not shown).

SBT Changes Following DDAVP

All three type IIa patients evaluated had initial prolonged SBTs (each >15.0 min), and two of these (patients shown in Fig. 2A,B, respectively) showed some correction (to 8.5 min and 12.0 min) following DDAVP. Of the four type I patients evaluated in this study, only one (patient shown in Fig. 1C) was determined to have an

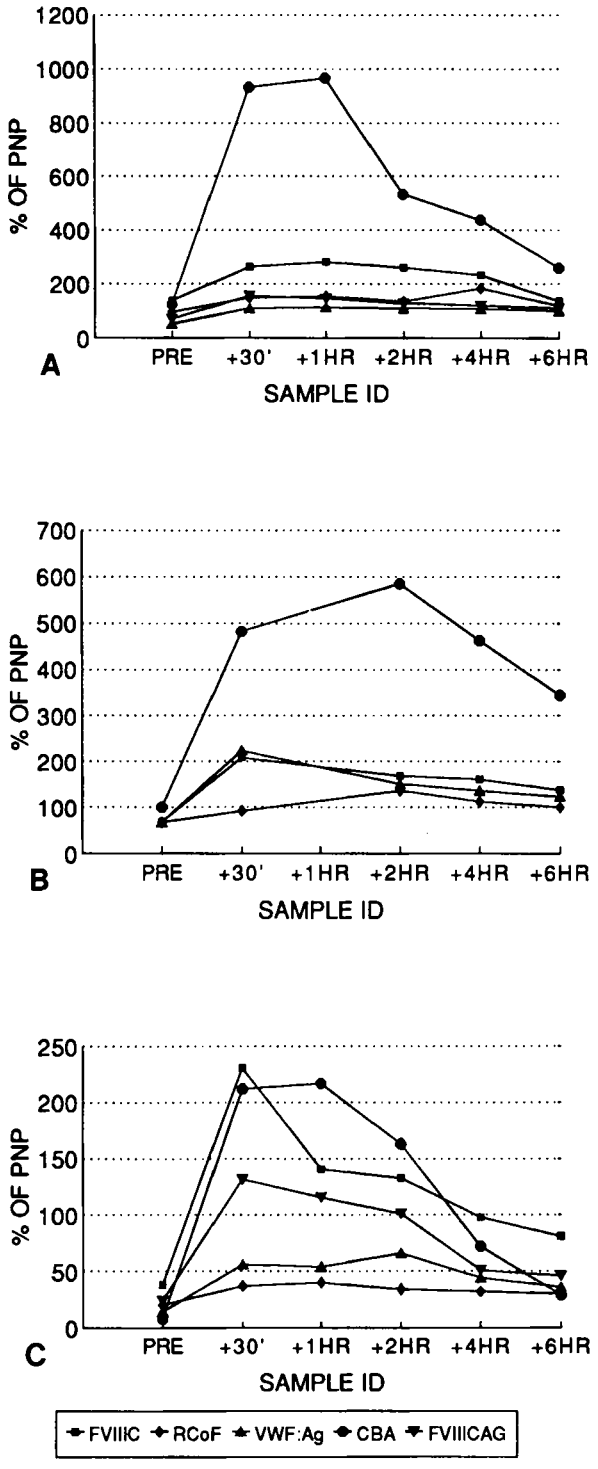


Fig. 1. Changes in vWF and FVIII levels following DDAVP infusion, as detected using five separate assay procedures, for three separate patients (A–C) with type I vWD. Results are expressed as a percentage of PNP value (assigned to equal 100%). Samples were collected preinfusion (PRE) and at indicated times following infusion (+30 min [= +30]; + 1 hr; + 2 hr; + 4 hr; + 6 hr). All patients showed initial upward increases using all tests but consistently showed the greatest upward change in CBA assay values. A fourth type I vWD patient evaluated yielded data essentially similar to those for the patient shown in C.

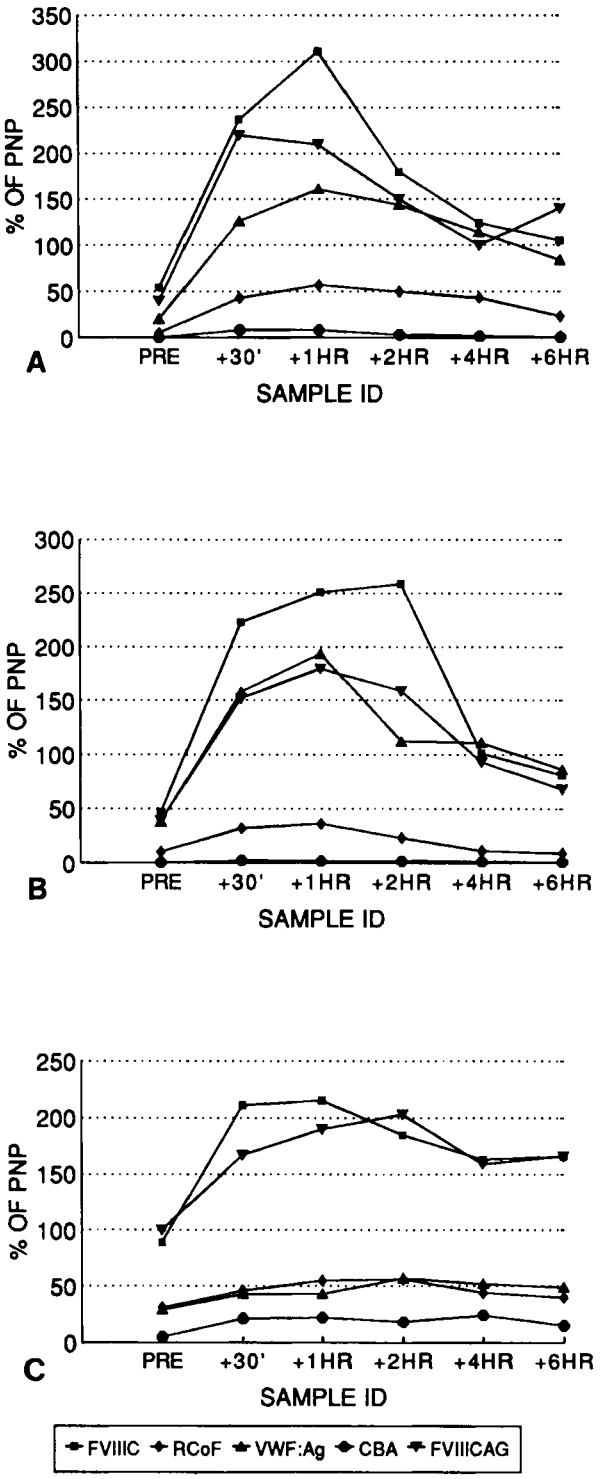
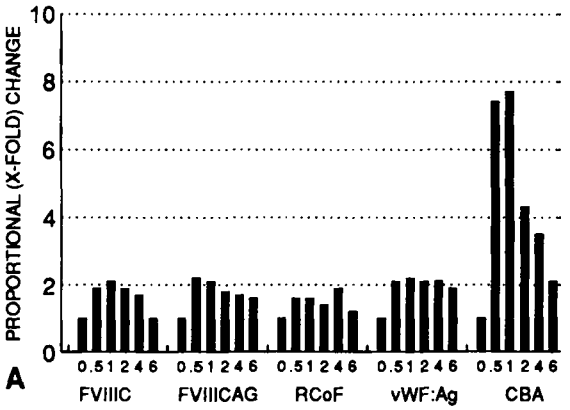
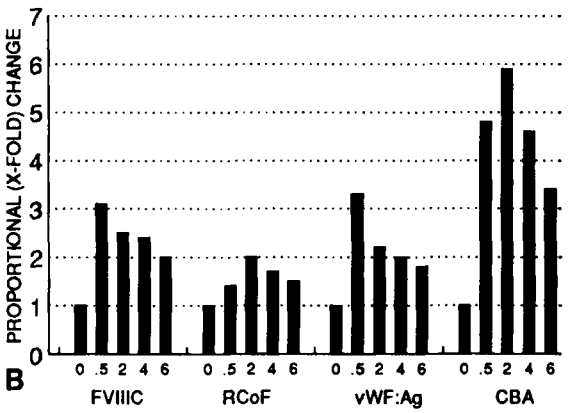


Fig. 2. Changes in vWF and FVIII levels following DDAVP infusion, as detected using five separate assay procedures, for three separate patients (A–C) with type IIA vWD. Results are expressed as a percentage of PNP value (assigned to equal 100%). Sampling times are as in Figure 1. All patients showed initial upward increases using all tests.





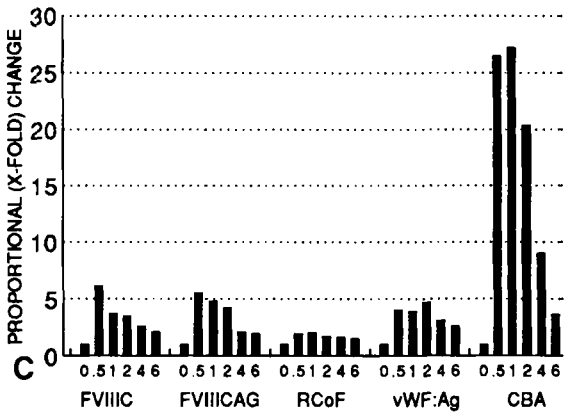
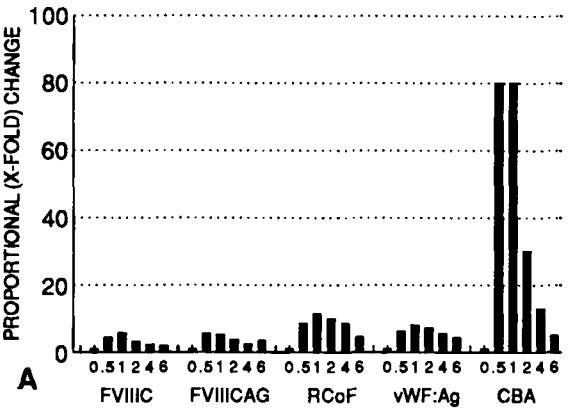
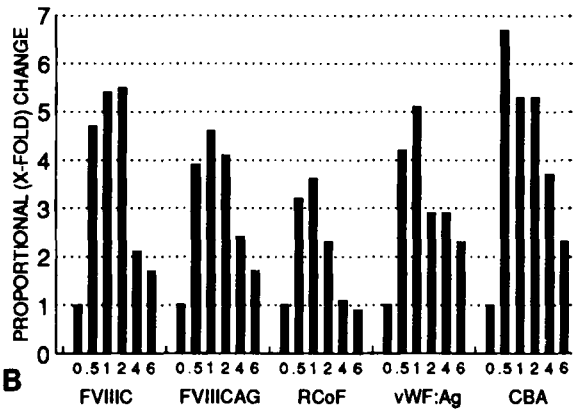


Fig. 3. Changes in vWF and FVIII levels following DDAVP infusion for vWD type I patients (A–C) shown in Figure 1, with results expressed as proportional changes compared to baseline values (i.e., -fold change over baseline = 1.0). All patients showed initial upward increases using all tests, but note the consistently greater upward change using the CBA. X axis abbreviations: 0 = pre DDAVP; 0.5 = + 30 min post-DDAVP; 1 = + 1 hr post-DDAVP; 2 = + 2 hr post-DDAVP; etc. Fourth type I vWD patient evaluated yielded data essentially similar to those for the patient shown in C.





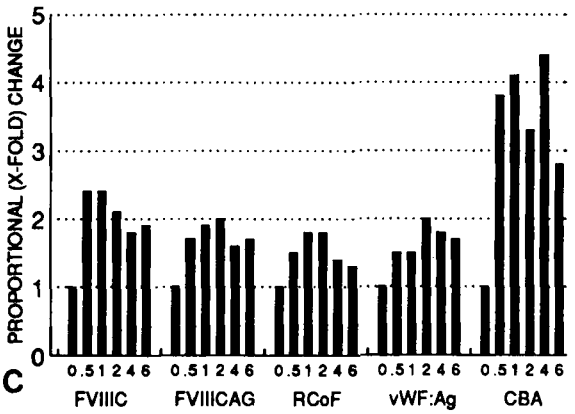


Fig. 4. Changes in vWF and FVIII levels following DDAVP infusion for vWD type IIA patients (A–C) shown in Figure 2, with results expressed as proportional changes compared to baseline values (i.e., -fold change over baseline = 1.0). All patients showed initial upward increases using all tests and, despite the low absolute CBA levels depicted in Figure 2, showed consistently high upward change (as -fold increase) using the CBA compared to baseline values. Abbreviations are as in Figure 3.

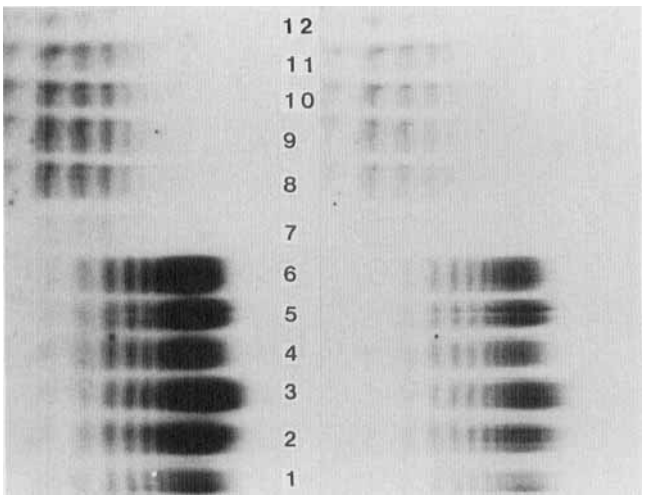


Fig. 5. Sample multimer patterns, obtained using plasma from two vWD patients (type I vWD patient) [depicted in Figs. 1A, 3A] and lanes 1–6 this figure; type IIA vWD patient [depicted in Figs. 2A, 4A] and lanes 7–12 this figure). Left and right segments show the same gel results with high and low autoradiography exposure to improve resolution of both qualitative and quantitive changes observed following DDAVP infusion. Lanes 1–6: Type I vWD patient: lanes 1 = pre-DDAVP sample; 2 = +30 min post-DDAVP; 3 = +1 hr; 4 = +2 hr; 5 = +4 hr; 6 = +6 hr. Lanes 7–12: = Type IIA vWD patient: lanes 7 = pre-DDAVP sample; 8 = +30 min post-DDAVP; 9 = 1 hr; 10 = +2 hr; 11 = +4 hr; 12 = +6 hr.

abnormal SBT prior to DDAVP (>15.0 min), and this showed correction (to 7.0 min) following DDAVP.

Multimer Analysis

Sample results are depicted in Figure 5. DDAVP infusion resulted in the expression within plasma of both increased levels of vWF (quantitative change; represented in Fig. 5 by the darker intensity of resultant multimer bands in post-DDAVP sample lanes) and in the expression or appearance of higher molecular weight multimer forms of vWF (qualitative change; represented in Fig. 5 by the appearance of multimer bands in post-DDAVP sample lanes, which are not visible in pre-DDAVP sample lanes).

DISCUSSION

This report is the first to describe the use of the newly developed [9] collagen binding assay, a novel functional assay for von Willebrand factor, in the assessment of the responsiveness of patients with von Willebrand's disease to DDAVP infusion. This assay has previously proved its worth [9,10] by its unique ability to allow effectively separation of type I and type II vWD patients (the two most common subtypes of vWD).

In type I vWD patients (four evaluated in the current study), responsiveness to DDAVP in terms of vWF release, as detected using the CBA, consistently overshadowed levels of vWF assessed by other standard laboratory procedures (Figs. 1, 3). Interestingly, in this group of patients, responsiveness as assessed using the standard functional vWF assay, the ristocetin cofactor assay, showed least effect among the group of tests employed. Responsiveness to DDAVP, as assessed using factor VIII coagulant assays, were generally concordant with that assessed using the vWF:Ag assay. That the CBA showed the most striking initial proportional changes in these patients following DDAVP infusion is significant for a number of reasons. First, and simply, the CBA appears to be more sensitive to such changes (i.e., detection of vWF levels following DDAVP) than existing standard and utilised assay systems (vWF:Ag, RCof). Second, the CBA is a functional assay, which measures an "adhesive" ability of vWF and selectively enables the measurement of (collagen) binding to higher molecular weight (i.e., more functional) forms of vWF. It follows, then, that levels of vWF detected using the CBA more likely reflect a true functional responsiveness of the patient to DDAVP infusion. In simple terms, the CBA is detecting vWF more functionally relevant than that detected using other vWF assays.

In type IIA vWD patients (three evaluated), absolute quantitative changes in CBA values following DDAVP (Fig. 2) were less marked than those observed in type I vWD patients (Fig. 1). Despite this, however, initial proportional (i.e., -fold) increases in CBA tended to be more striking than those observed using other assays (Fig. 4). In this group of patients, proportional changes in vWF or factor VIII assessed by other assays tended to be similar to each other.

While our CBA has not been previously used to evaluate vWD patients' responsiveness to DDAVP, there are many reports of studies utilising the other assay systems described here [see, e.g., 5–8]. In two recent reviews [6,7], the use of DDAVP is advocated for all suitable (and trialed) vWD patients (e.g., most type I, and some type IIA patients benefit haemostatically following DDAVP infusion, but DDAVP is not effective in type III vWD and is contra indicated in type IIB or pseudo-vWD). It is also generally concluded that, if DDAVP is able to correct factor VIII coagulant levels, this might be sufficient to ensure satisfactory haemostasis in most cases of haemorrhagic complications. The obvious exceptions are cases of mucosal bleeding, which are more difficult to predict, and it is in these cases when the use of the CBA to evaluate DDAVP responsiveness may be of further relevance, particularly as a proposed marker of vWF/platelet/subendothelial activity. It is a functional vWF assay, and it is capable of far more sensitive discrimination of DDAVP-induced changes than the standard vWF

functional assay (the RCof assay). Since it has never been previously evaluated, one cannot be certain what correction in the CBA would be required to enable prediction of satisfactory haemostasis in such cases.

With respect to the current study, some measure of clinical responsiveness (i.e., correction of potential mucosal bleeding) following DDAVP was evaluable in three patients. While it may be premature to attempt to draw any firm conclusion regarding the clinical relevance of these findings, we can confirm that (at least for these patients) effective haemostasis was evident post-DDAVP. In a similar manner, correction of SBT was also evident in some patients. Only further work in this area will allow clarification of the relationship between CBA levels and effective haemostasis for both minor and major surgery/trauma.

In conclusion, the CBA is a novel functional assay for vWF, whose use should now be extended from that of a test to distinguish type I and type II vWD patients [9,10] to that of monitoring the responsiveness of such patients to DDAVP therapy. We propose that it may reflect more accurately the true functional vWF responsiveness of such patients, and we have determined it to be a sensitive procedure for detection of such changes.

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