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An ELISA system to detect anti-factor VIII antibodies without interference by lupus anticoagulants. Preliminary data in hemophilia A patients

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ABSTRACT

Background and Objectives. **Difficulties in identifying the coexistence of neutralizing anti-factor VIII antibodies (anti-fVIII) and lupus anticoagulant (LA) are mainly due to the interference of LA on anti-fVIII assays. Our aim was to reveal the presence of anti-fVIII using a system that is not affected by LA.**

Design and Methods. **We developed an enzyme-linked immunosorbent assay (ELISA) method that uses phospholipid-free recombinant factor VIII as the antigen. A monoclonal anti-fVIII was tested as a positive control, excluding non-specific binding by using two unrelated monoclonal antibodies. The ELISA was performed on hemophilic plasmas with anti-fVIII and negative LA (n=12) or without inhibitors (n=12). Two hemophilic plasmas with LA and presumably anti-fVIII were also assayed. Positive LA (n=12) and normal (n=10) plasmas were tested as negative controls.**

Results. **All (12/12) plasmas with anti-fVIII and 5/12 hemophilic plasmas without inhibitors were positive; LA and normal plasma controls were negative.**

Interpretation and Conclusions. **Results presented here show that LA does not interfere with the anti-fVIII ELISA. However, the assay detects both neutralizing and non-neutralizing anti-fVIII antibodies, therefore a neutralizing effect must be confirmed through functional tests.**

Key words: factor VIII, inhibitors, lupus anticoagulant, hemophilia, ELISA

Hemophilic patients can develop both neutralizing anti-factor VIII antibodies (anti-fVIII) and anti-phospholipid-protein antibodies, such as lupus anticoagulants (LA).[1] Neutralizing anti-fVIII are immunoglobulins (Ig), mostly of the IgG4 subclass, that specifically block factor VIII activity in a time-dependent manner and are associated with hemorrhagic complications.[2] LA are antibodies directed against phospholipid-protein epitopes that inhibit one or more phospholipid-dependent coagulation tests; they are not linked to hemorrhagic events, unless they are associated with thrombocytopenia or factor II deficiency.[3] Given the implications of neutralizing anti-fVIII inhibitors in the clinical and therapeutic management of hemophilic patients,[4] it is important to discriminate between LA and anti-fVIII. Despite the fact that the antibodies have different target epitopes,[2,3] such diagnosis is difficult, because both are capable of inhibiting the same coagulation pathway.[3,5,6]

In a previous study[7] we reported that 21% of hemophilia A patients had LA, half of them showing a time-dependent inhibitory effect. This is a high prevalence of time-dependency in LA, in comparison with previous reports,[8,9] and suggests a possible coexistence of LA and anti-fVIII,[5-7,10] mainly in those patients in whom such an effect is very strong.

Since LA may interfere in factor VIII one-stage assays, the presence of neutralizing anti-fVIII antibodies cannot be specifically proven with the current tests based on such assays (e.g. the Bethesda method).[9]

The time-dependent effect does not seem to be restricted to anti-fVIII antibodies, because Triplett *et al.* reported time-dependency in LA.[11] Since then, different series showed that time-dependency in LA varies between 10 and 40%.[8,9]

In addition, a dilutional effect on one-stage factor VIII assays may be of help in the identification of the inhibitory effect. Usually, in the presence of specific factor inhibitors, the very low factor levels do not change with dilution. However, in patients with severe hemophilia A, the dilutional effect cannot be evaluated because of the lack of factor VIII activity. Moreover, other situations could lead to misdiagnosis. An apparent severe factor deficiency without evidence of any dilutional effect could be found in the presence of a high titer of LA and a sensitive APTT reagent.[9] Also, a potent neutralizing anti-fVIII may produce a dilutional effect on the apparent level of other factors,[9] such as factor IX, similar to that observed in the pres-

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ence of LA.

Brandt *et al.* stated that specific factor inhibitors (e.g. factor VIII inhibitors) may still cause false positive results with confirmatory studies for LA.[9] In a previous study we found hemophilic patients (n=7) with anti-fVIII antibodies (8-107 BU/mL) and negative criteria for LA; dilute Russell viper venom time and Staclot® LA tests were negative, suggesting the absence of false positive results at least with these tests.[7]

Not only are rigorous criteria needed to establish the presence of LA, but also a specific anti-fVIII assay is needed to detect an underlying anti-fVIII antibody which could be masked by LA. Misdiagnosis could delay appropriate therapy and even result in a severe bleeding episode.[5]

Since there is no specific method for detecting the neutralizing anti-fVIII antibodies, our aim was to reveal the presence of such antibodies without interference from LA by an ELISA system. We used phospholipid-free recombinant factor VIII as the antigen in order to exclude the possibility of detecting false positive or false negative results. Anti-phospholipid antibodies attached to phospholipids bound to factor VIII could yield false positive results; on the other hand, false negative results could be due to anti-fVIII antibodies not capable of recognizing factor VIII bound to phospholipids.[12] To our knowledge, interference by LA on the previously described anti-fVIII ELISA methods, has not been evaluated.

In order to evaluate the usefulness of the ELISA we analyzed plasmas from hemophilic patients with anti-fVIII or without inhibitors, plasmas that were positive for LA and plasmas from normal subjects. In addition, we tested two plasma samples that were positive for LA and presumably positive for anti-fVIII.

Design and Methods

Samples

Blood was collected in plastic tubes containing 1/10 volume of 0.11M sodium citrate and centrifuged for 15 min. at 1,500 x g; the supernatant was then recentrifuged. The platelet-poor plasma was divided into aliquots that were either tested immediately or frozen at –70°C for future analysis.

Subjects

The performance of the anti-fVIII ELISA was evaluated on positive and negative anti-fVIII plasma samples. Twelve plasmas from hemophilic patients positive for anti-fVIII (8-310 Bethesda Units (BU)/mL) and negative for LA were included as positive controls. The ELISA was also carried out on 2 samples positive for LA and presumably positive for anti-fVIII,[7] displaying a strong time-dependent inhibitory effect. In addition, plasmas from 12 hemophilic patients displaying neither anti-fVIII nor LA were analyzed. Negative controls comprised plasmas from 12 non-hemophilic patients positive for LA and 10 plasmas from normal subjects. All the patients included in the study were taken from routine control or diagnostic studies requested from our laboratory.

Coagulation studies

The following series of tests were performed in order to assign the inhibitor status of the samples: thrombin time, activated partial thromboplastin time (APTT), diluted Russell viper venom time (dRVVT), mixing studies and platelet neutralization of both APTT and dRVVT. Established methods previously described were applied.[3,9,13-16] In order to evaluate the correction of the defect by the addition of normal plasma, we calculated the $APTT_{index}$ = [mixture (1:1)-normal]/patient. We evaluated the time-dependent effect by measuring APTT on the mixture of patient and normal plasmas incubated for 1 hour at 37°C ($APTT_{index37}$), as well as a control consisting of a mixture of patient and normal plasmas after they had been separately incubated.[17] The platelet neutralization procedure on the APTT (ΔAPTT-PNP) was performed as previously described.[14] dRVVT was assayed on plasma samples and on a 1:1 mixture with normal plasma ($dRVVT_{index}$); the neutralizing effect of lysed platelets was evaluated both on samples and mixtures (ΔdRVVT-PNP).[9,15,16] One-stage assays for factor VIII and factor IX were performed on progressive dilutions of patient samples and control samples.[9]

LA samples fulfilled the criteria proposed by the *Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardization Committee-International Society on Thrombosis and Haemostasis*.[9] Abnormal or positive results were defined according to criteria previously stated using non-LA plasmas.[15] Samples diagnosed as containing anti-fVIII showed a time-dependent neutralizing effect exclusively against factor VIII and negative criteria for LA.[7] The Bethesda method was applied to titrate neutralizing anti-fVIII antibodies.[18,19]

Anti-fVIII ELISA system

Recombinant factor VIII (Antihemophilic Factor Recombinant, Kogenate®, Miles, USA) was used as the antigen. Phospholipid was quantified by a colorimetric method on the mineralized ethanol:ethyl ether (3:1, v/v) extracts, and confirmed the concentrate's phospholipid-free status (<0.15 μg/UfVIII).

Binding of recombinant factor VIII. Recombinant

factor VIII (2U/mL/tube; 100 µL/tube) diluted in 0.1 M bicarbonate pH 9.6, was immobilized (20 h at 4ºC) in polystyrene tubes. The remaining free surface was blocked with 2M imidazole, 0.12M NaCl, bovine serum albumin (BSA) (5%; 150 µL/tube; 2 h at 37ºC). The tubes were subsequently incubated with purified von Willebrand factor (vWF) diluted in 0.01M imidazole, 0.01M $CaCl_2$ (1U/mL; 100 µL/tube; 2 h at 37ºC), followed by incubation with anti-vWF_{rabbit} (von Willebrand, rabbit, anti-human Dako A/S, Denmark) (0.5%; 100 µL/tube; 2 h at 37ºC) and finally with anti-IgG_{rabbit}-phosphatase conjugate (anti-rabbit IgG, γ-chain specific, alkaline phosphatase conjugate, Sigma Immuno Chemicals, USA) (0.2%; 100 µL/tube; overnight at 4ºC). A specific substrate (104 phosphatase substrate, Sigma Immuno Chemicals, USA) was added (1 mg/mL in 0.2 M carbonate, 1 mM $MgCl_2$, pH 9.8 buffer; 100 µL/tube) and after 30-60 min. at room temperature, the reaction was stopped by the addition of 3N NaOH (50 µL/tube); optical density (OD) at 405 nm was then measured. To eliminate unbound fractions, tubes were washed 5 times with 2M imidazole, 0.12M NaCl, BSA 0.1% buffer (500 µL/tube each washing step) after the immobilization of recombinant factor VIII and after every incubation with each antibody. The following blanks were included in all assays: A-tubes without recombinant factor VIII, B-tubes without purified vWF, C-tubes without anti-vWF_{rabbit}, D-tubes without anti-IgG_{rabbit}-phosphatase.

Factor VIII and anti-fVIII interaction. Immobilized recombinant factor VIII was incubated (2 h at 37ºC) with a monoclonal anti-human factor VIII antibody (Monoclonal antibody FVIIIC Ag, Immunotech, France) diluted in 2M imidazole, 0.12 NaCl, BSA 5% buffer (1/500, 1/1,000 and 1/2,000; 100 µL/tube) and was revealed by overnight incubation at 4ºC with an anti-IgG_{mouse}-phosphatase conjugate (anti-mouse IgG, alkaline phosphatase conjugate, Sigma Immuno Chemicals, USA) diluted 1/100 in 2M imidazole, 0.12M NaCl, BSA 0.1% (100 µL/tube). The specific substrate was added; after 30-60 min. at room temperature, the reaction was stopped by 3N NaOH, and the OD was measured. Unbound fractions were removed by washing 5 times, after the immobilization of recombinant factor VIII and after each incubation with all the different antibodies. Blanks comprised: A-tubes without recombinant factor VIII, B-tubes without monoclonal antibody, C-tubes without anti-IgG_{mouse}-phosphatase conjugate.

Non-specific binding. This was excluded by replacing the monoclonal anti-human factor VIII antibody with two unrelated monoclonal antibodies (negative controls), anti-vWF_{human} (von Willebrand, monoclonal, anti-human, Dako A/S, Denmark) and anti-$GPIIIa_{human}$ (glycoprotein IIIa, monoclonal, anti-human, Dako A/S, Denmark), both diluted 1/100 in 2M imidazole, 0.12M NaCl, BSA 5% buffer and tested under the same experimental conditions as described above for the monoclonal anti-human factor VIII antibody. Blank tubes (as described under *factor VIII and anti-fVIII interaction*) were analyzed in each assay.

Anti-fVIII assay. Polystyrene tubes were coated with phospholipid-free recombinant factor VIII and the remaining free surface blocked as previously described. Following 2 h incubation at 37ºC of plasma samples (diluted 1/25-1/800 in imidazole, BSA 5%; 100 µL/tube), an anti-IgG_{human}-phosphatase conjugate (anti-human IgG, γ-chain specific, alkaline phosphatase conjugate, Sigma Immuno Chemicals, USA) diluted 1/100 in imidazole, BSA 1% was added (100 µL/tube) and incubated overnight at 4ºC. After 30-60 min incubation at room temperature with its specific substrate, the reaction was stopped by addition of 3N NaOH and the OD was measured at 405 nm. After coating and after incubation with either the plasma samples or the anti-IgG_{human}-phosphatase conjugate, tubes were washed 5 times. Each assay included the analysis of progressive dilutions (1/50-1/200 in imidazole, BSA 5%) of a plasma sample from an untreated hemophilic patient with a high titer of anti-fVIII (20 BU/mL). Blank tubes were included in each run. Sample dilutions were analyzed in duplicate and results were expressed as the difference between the OD of the sample and the OD_{mean} of the blanks (ΔOD). Any value greater than the mean of the normal controls plus 3 standard deviations was considered as positive.

Different concentrations of recombinant factor VIII (0.1-20 U/mL) and blocking reagents (phosphate buffer saline/Tween 0.1-1% or 2M imidazole, 0.12M NaCl, BSA 1-10%) were tested before the assay conditions were chosen. The polystyrene surface was saturated at 0.2 U/mL; no further binding of recombinant factor VIII was observed after blocking the surface with 2 M imidazole, 0.12 M NaCl, and BSA 5%.

Assays to verify the binding of recombinant factor VIII to the surface, solid-phase recombinant factor VIII-anti-fVIII monoclonal antibody interaction and non-specific binding were performed at least 3 times and each sample dilution was analyzed in duplicate.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Multiple comparisons were performed with analysis of variance, followed by

paired t tests with Bonferroni correction (*post-hoc* comparison of means). Within-run and between-run coefficients of variation (CV) were calculated to evaluate the precision of the assays. Linear regression analysis was applied to analyze the dose-dependent response of both monoclonal anti-fVIII and patients' neutralizing antibodies.

Results

Anti-fVIII ELISA system

Binding of recombinant factor VIII. Tubes containing immobilized recombinant factor VIII showed a significantly higher (p<0.0001) ΔOD_{mean} than the blanks, supporting the fact that recombinant factor VIII had bound to the polystyrene tubes, interacted with the purified vWF and had subsequently been recognized by the anti-vWF. Three sets of assays were performed and each tube was run in duplicate. Within-run CV ranged from 1.02 to 1.85%, between-run CV from 1.84 to 3.78%.

Factor VIII and anti-fVIII interaction. After determining the binding of recombinant factor VIII onto the polystyrene surface, we verified that the system was able to recognize anti-human factor VIII antibodies. Tubes containing progressive dilutions of monoclonal anti-factor VIII antibody were assayed. The ΔOD values from the tubes containing the monoclonal anti-factor VIII were higher (p<0.001) than those from the blanks. Moreover, results were proportional to the amount of monoclonal anti-human factor VIII antibody present in the reaction tube (Figure 1). Experiments were performed 3 times, in which samples were analyzed in duplicate. Within-run CV for this assay ranged from 2.05 to 5.85% and between-run CV from 2.73 to 5.85%.

Non-specific binding. Unrelated monoclonal antibodies were tested under the same experimental conditions. Neither the anti-vWF nor the anti-GPIIIa showed significant ΔOD values allowing us to exclude non-specific binding. Assays were carried out 3 times and each tube analyzed in duplicate. Within-run CV ranged from 1.85 to 2.99%, between-run CV from 2.73 to 3.88%.

Anti-fVIII assay. We analyzed the behavior of the anti-fVIII ELISA on progressive plasma dilutions from a hemophilic patient with anti-fVIII (20 BU/mL). A dose-dependent response was observed upon plotting ΔOD_{mean} against the anti-fVIII titer (BU/mL) (Figure 1). Dilutions (1/50, 1/100 and 1/200 in imidazole, BSA 5%) of this sample were included in every assay as a *standard* curve. Within-run CV ranged from 1.85 to 5.85% and between-run CV from 2.73 to 5.85%.

In addition, we analyzed 12 samples from hemophilic patients with anti-fVIII (8-310 BU/mL), 12 samples from hemophilic patients without inhibitors, 12 LA samples and 10 normal samples. A correlation (r=0.876; p=0.0002) between the ΔODmean and the anti-fVIII titer (BU/mL) was observed in hemophilic patients with anti-fVIII inhibitors; the anti-fVIII ΔOD_{mean} was significantly (p<0.0001) higher than that of the blanks. Plasmas from hemophilic patients without inhibitors also showed a higher (p<0.01) ΔOD_{mean} than the blanks. Neither LA nor normal samples yielded readings that dif-

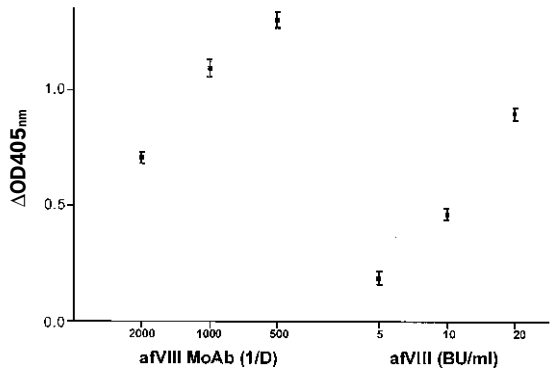

Figure 1. Recombinant factor VIII-anti-fVIII antibody interaction. The figure displays the ELISA results on progressive dilutions of either monoclonal anti-factor VIII antibody (afVIII MoAb) (dilutions 1/2000, 1/1000 and 1/500) or a positive neutralizing anti-fVIII hemophilic plasma (afVIII) (5-20 BU/mL). Experiments were performed 3 times, in which samples were analyzed in duplicate. The figure exhibits the ΔOD_{mean} at 405 nm ± SEM. Results were proportional to the amount of either monoclonal or human antibody present in the reaction tube.

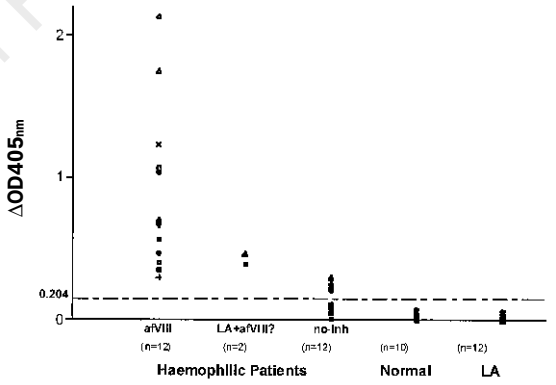

Figure 2. Anti-fVIII ELISA assays. This figure presents the ELISA results expressed as values at 405 nm (afVIII: hemophilic patients with anti-fVIII; LA+afVIII?: samples positive for LA and presumably positive for anti-fVIII; no-Inh: hemophilic patients without inhibitors; Normal: normal samples; LA: positive LA samples). The dot-dashed line indicates the cut-off value (0.204), calculated as the ΔOD_{mean} of normal samples plus 3 standard deviations.

Table 1. Coagulation studies.

	afVIII *n=12*	*no-Inh* *n=12*	*LA* *n=12*	*Normal* *n=10*	*LA +afVIII?* *Case #1*	*Case #2*
APTT (>50 sec)	118.8±3.3 (100.0-134.0)	116.8±3.5 (100.0-134.0)	66.3±4.8 (53.0-115.0)	41.8±1.0 (36.0-47.0)	116	150
$APTT_{index}$ (>0.10)	0.177±0.017 (0.11-0.30)	0.038±0.005 (0.02-0.07)	0.225±0.032 (0.12-0.54)	0.039±0.007 (0.00-0.07)	0.14	0.11
Δ APTT-PNP (>7 sec)	3.6±0.5 (1.0-6.0)	4.0±0.5 (1.0-6.0)	13.4±2.0 (8.0-32.0)	1.4±0.4 (-1.0-3.0)	12	16
$APTT_{index37}$	0.428±0.049 (0.20-0.74)	0.039±0.004 (0.02-0.07)	0.222±0.029 (0.13-0.50)	0.041±0.007 (0.01-0.07)	0.43	0.41
δRVVT (ratio>1.13)	1.022±0.007 (0.98-1.07)	1.020±0.011 (0.94-1.08)	1.230±0.031 (1.14-1.51)	1.027±0.010 (0.99-1.08)	1.42	1.15
$dRVVT_{index}$ (>0.08)	0.018±0.007 (-0.02-0.06)	0.013±0.008 (-0.03-0.06)	0.142±0.018 (0.10-0.33)	0.016±0.008 (-0.03-0.06)	0.22	0.09
ΔdRVVT-PNP (>0.09)	0.022±0.005 (-0.01-0.05)	0.026±0.006 (-0.01-0.06)	0.196±0.024 (0.10-0.38)	0.019±0.008 (-0.02-0.06)	0.27	0.10
a-fVIII (BU/mL)	62.85±25.19 (8.0–310.0)	0.02±0.01 (0.00-0.10)	Not done	Not done	9.6	39

Results are expressed as the mean±SEM from each group: hemophilic plasmas positive for anti-fVIII (afVIII); hemophilic plasmas negative for anti-fVIII and LA (no-Inh); non-hemophilic plasmas positive for LA (LA) and normal plasmas (Normal). Minimum and maximum values are given in parentheses. Results from 2 patients positive for LA and probably positive for anti-fVIII (LA+afVIII?) are also detailed (cases #1 and 2).

fered significantly from the blanks (p>0.1). Figure 2 shows the results obtained in each group. A cut-off value equivalent to the ΔOD_{mean} of the normal samples plus 3 standard deviations was adopted. Results higher than the cut-off value (>0.204) were considered positive. All (12/12) hemophilic plasmas with neutralizing anti-fVIII (8-310 BU/mL) and the 2 samples with LA and presumably anti-fVIII were positive. However, 5 out of 12 hemophilic patients without inhibitors were also positive. None of the LA plasmas (0/12) or the normal samples (0/10) showed positive results.

Coagulation studies

Table 1 displays the laboratory results from the different groups studied and 2 patients positive for LA and probably positive for anti-fVIII.

Discussion

The factor VIII binding assays in which the solid-phase recombinant factor VIII was incubated with purified vWF, and the tests performed using a monoclonal anti-human factor VIII, showed that recombinant factor VIII is able to bind to the polystyrene tube as well as interact with its carrier, the vWF or with a specific antibody such as monoclonal anti-human factor VIII (Figure 1). On the contrary, it does not react with monoclonal antibodies directed against other proteins, related (vWF) or not (GPIIIa) to factor VIII, which excludes the possibility of a non-specific interaction between the monoclonal antibodies and the tube surface. Furthermore, the anti-fVIII ELISA results (ΔOD) are proportional to the anti-fVIII concentration, whether the monoclonal anti-human factor VIII or plasmas positive for the neutralizing anti-fVIII inhibitor are used (Figure 1). These results agree with previous reports about the possibility of detecting anti-fVIII by ELISA methods.[20,21]

The anti-fVIII ELISA enabled us to detect all anti-fVIII antibodies (Figure 2). Plasmas from hemophilic patients with the neutralizing anti-fVIII inhibitor were positive (100% sensitivity) and a correlation between the ΔOD and the anti-fVIII titer was found. Unlike results reported by Vianello *et al.*,[20] no patients with neutralizing anti-fVIII (≥8BU/mL) had negative results by immunoassay. Neither the normal nor the LA plasmas gave positive anti-fVIII ELISA results (100% specificity) confirming that there is no interference by LA.

The assay was not specific for neutralizing antibodies. Discrepancies between Bethesda and ELISA results were observed and they were not due to LA interference. Plasmas from hemophilic patients without inhibitors (5/12) yielded values (ΔOD) above the cut-off, denoting the presence of antibodies capable of specifically reacting with factor VIII but unable to neutralize its coagulant activity (Figure 2); similar results have been previously described.[21]

The anti-fVIII ELISA assay seems to be specific for anti-fVIII, since we did not observe any interference from LA. However, it appears to have the limitation of recognizing not only neutralizing anti-fVIII antibodies, but also non-neutralizing ones. Therefore, a positive anti-fVIII ELISA test indicates the presence of antibodies against factor VIII but does not define their neu-

tralizing status. In LA positive samples in which the presence of neutralizing anti-fVIII is suspected due to clinical and/or laboratory evidence,[5-7] a negative anti-fVIII ELISA result would exclude the presence of anti-fVIII. On the other hand, a positive anti-fVIII ELISA test would confirm the presence of the anti-fVIII antibody; further experiments should then be conducted to verify the neutralizing effect of the isolated anti-fVIII IgG-fraction on factor VIII coagulant activity.

Contributions and Acknowledgments

ANB was responsible for the development of the ELISA test, the analysis of the data and writing the manuscript, AAP carried out the ELISA test and contributed to collecting data and writing the manuscript, SHG and LCG carried out the coagulation studies, RPB was the clinician responsible for the patients' clinical management, and MAL supervised and critically revised the final version of the paper. The authors wish to thank Dr. Ana Catalina Kempfer for her expert advice concerning the ELISA tests.

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Disclosures

Conflict of interest: none.

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Manuscript processing

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Potential implications for clinical practice

- The herein described ELISA would be useful to exclude the presence on anti-fVIII antibodies in patients in whom there is a suspicion of the simultaneous presence of anti-fVIII and LA.
- It would influence clinical and therapeutic management of patients, allowing a major clinical complication in replacement therapy and life-threatening hemorrhagic events in non-hemophilic patients to be ruled out.

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