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A chromogenic substrate method for detecting and titrating anti-factor VIII antibodies in the presence of lupus anticoagulant

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Background and Objectives. The development of neutralizing anti-factor VIII antibodies (a-fVIII) is a major clinical complication. Lupus anticoagulant (LA) might affect detection of a-fVIII, since both inhibitors may act on the same coagulation pathway. Our aim was to accomplish unequivocal detection and titration of a-fVIII even in the presence of LA.

Design and Methods. We evaluated a-fVIII activity by a chromogenic substrate (CS) method in samples with a-fVIII (n=6), LA (n=12) and presumably both LA+a-fVIII (n=5). The inhibition index before (Ii) and after incubation at 37°C (Ii_{37}) was estimated. We also performed factor VIII assays (one-stage and CS) and titration methods (Bethesda and CS) in parallel.

Results. Inhibition in the a-fVIII group (Ii=5-3200) was potentiated by incubation (Ii_{37}=27-5200) as it was in LA+a-fVIII (Ii=9-21; Ii_{37}=50-903). LA samples showed no or meaningless inhibitory effect (Ii=0-7; Ii_{37}=0-4) or a-fVIII activity (0.00-0.06 CSU/ml) by the CS method; on the contrary, very low to moderate (0.52-7.00 BU/ml) a-fVIII activity was recorded by the Bethesda method. The two titration methods did not correlate (p>0.100) in the presence of LA, or LA+a-fVIII. Differences between factor VIII:C and factor VIIIcs were significant only in LA samples (p=0.005); however, patients with residual factor VIII activity from the LA+a-fVIII group also showed higher factor VIIIcs values than factor VIII:C ones.

Interpretation and Conclusions. Results indicate the possibility of detecting and titrating a-fVIII without interference of LA by the CS method. This marks a difference with respect to the Bethesda method, in which a measurable effect can be expected in the presence of a strong LA.

Key words: anti-factor VIII antibodies, lupus anticoagulant, factor VIII, chromogenic substrate, Bethesda method

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Neutralizing anti-factor VIII antibodies (a-fVIII) are immunoglobulins directed against different factor VIII epitopes, mainly located at A2, A3 and C2 factor VIII domains, that specifically block factor VIII activity in a time-dependent manner.[1,2] They may arise as allo- or autoantibodies[3-5] and are mostly of the IgG isotype with a predominance of the IgG_4 subclass.[1,6] Antibodies appear in the course of replacement therapy in hemophilia A patients; the incidence (approximately 20% in severe hemophilia A) varies depending on the population analyzed, the methodology applied and the inhibitor threshold level.[1,7,8] In non-hemophilic patients a-fVIII although rare (0.2-1 cases/million population/year) may develop in a variety of settings. Almost half of the cases are idiopathic arising in elderly patients; a-fVIII are also associated with the postpartum period, immunologic diseases and malignancies, among other situations.[3] The development of neutralizing a-fVIII is a major clinical complication, interfering with hemophilic replacement therapy[1] and prompting life-threatening bleeding complications in non-hemophilic patients.[3]

Lupus anticoagulants (LA) are immunoglobulins (IgG, IgM or IgA) directed against phospholipid-bound protein epitopes that inhibit one or more phospholipid-dependent coagulation reactions.[9-11] They may interfere with dosage of factors, such as factor VIII measured by the one-stage method.[10, 12] The clinical relevance of LA is that these immunoglobulins are associated with thrombosis, obstetric complications, neurologic and/or cutaneous symptoms.[13,14] They are not related to hemorrhage unless they are combined with thrombocytopenia or prothrombin deficiency.[15]

Recently, there have been descriptions of several cases of possible simultaneous occurrence of a-fVIII and LA in hemophilic[16,17] and in non-hemophilic patients.[18-21] There is concern about the current difficulties in discriminating between them,

which is important because of their different clinical and therapeutic implications.[16,22,23] Even though both inhibitors have different target epitopes, they can inhibit the same coagulation pathway interfering with the factor VIII one-stage method. Hence, LA could affect detection of a-fVIII and even titration by the Bethesda method.

Time-dependent inhibition appears to be restricted to a-fVIII. Although a progressive effect has been described in some LA series.[10,24,25] Exner recently showed that time dependency in LA is most probably an artifact due to a shift in the pH of the plasma during incubation.[26]

In order to detect and titrate a-fVIII specifically, without interference from LA, we tried a system in which the inhibitory effect on factor VIII activity was evaluated by a chromogenic substrate (CS) method, based on the previous observation[12] that this method could avoid interference from LA. Samples with a-fVIII, with LA and samples with presumably both LA+a-fVIII were evaluated.

Design and Methods

Subjects

We studied samples from subjects with the following antibodies, according to laboratory results:

a-fVIII: we analyzed 6 plasma samples with a-fVIII, 3 of them were alloantibodies and the other 3 autoantibodies. Samples showed a neutralizing effect exclusively against factor VIII and were negative for the criteria for LA.

LA: twelve samples were evaluated, all of them fulfilling the LA criteria proposed by the *Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody* of the SSC-ISTH (prolongation of the screening tests, not corrected by addition of normal plasma and positive neutralization). Both the APTT and dRVVT systems were affected in eight samples, only the dRVVT in two, and solely the APTT system in the other two. Most samples displayed a strong effect on the APTT system (P/N median: 2.62; range: 1.33-5.07).

LA+a-fVIII: we also studied 5 patients positive for LA and presumably positive for a-fVIII; such samples displayed a strong time-dependent inhibitory effect. Three hemophilia A patients had a previous history of a-fVIII and the other two non-hemophilic patients showed bleeding symptoms (hematomas, ecchymoses). The samples included in the study were taken for routine controls or diagnostic studies requested from our laboratory.

Samples

Blood was collected by venipuncture in 0.11M sodium citrate (9:1, v/v). Platelet-poor plasma (residual platelet counts $<3\times10^9$/L) was obtained by double centrifugation at 1,500 × g for 15 min. Aliquots were analyzed either immediately or frozen at -70ºC for future analysis.

Laboratory methods

Coagulation studies. Global tests. Thrombin time, prothrombin time, activated partial thromboplastin time (APTT) and diluted Russell viper venom time (dRVVT) were measured in every sample, according to established methods previously described.[27] The APTT was carried out using a reagent highly sensitive to LA (PTT-LA Diagnostica Stago, France). A lyophilized fraction of Russell viper venom®, in cephalin, containing factor X activating enzyme (SIGMA Chemical Co., USA) was used for the dRVVT test.

Mixing studies. The inhibitory effect on both APTT and dRVVT was assessed performing mixing studies on 1:1 mixtures with normal plasma. Rosner's $APTT_{index}$ and $dRVVT_{index}$ were calculated in order to assign the inhibitory status of the samples.[16,28]

Time-dependent effect on the APTT. Patient's plasma, normal plasma and a mixture of both plasmas were incubated for 1 h at 37ºC (Rosner's $APTT_{index37}$); the immediate mixture of the patient's plasma and normal plasmas after they had been separately incubated were used as a control.[29]

Factor VIII (*one-stage method*). The assay was performed on progressive dilutions of samples and controls (IL Test™ factor VIII deficient plasma, Instrumentation Laboratory, Italy).

LA confirmatory tests. Platelet neutralization procedures on both APTT (ΔAPTT-PNP) and dRVVT (ΔdRVVT-PNP) were carried out in all samples, and also on 1:1 mixtures with normal plasma. Diagnosis of LA in patients with a-fVIII or presumed a-fVIII was based mainly on dRVVT, since the APTT system is more likely to be affected by a-fVIII.

The Bethesda method was applied to titrate neutralizing a-fVIII (BU/mL).[30]

Chromogenic substrate

Factor VIII activity (factor VIIIcs) was measured by the manual end-point assay (COAMATIC® Factor VIII, Chromogenix, Sweden). The reaction takes place in the presence of factor IXa, thrombin, Ca^{++}, phospholipids and an excess of factor X. The generation of factor Xa is evaluated by its amidolytic activity on a chromogenic substrate (S-2765); a chromophoric group is released, and the absorbance at 405 nm is proportional to the amount of factor

VIII present in the sample.[31]

Inhibition index before and after incubation. Factor VIIIcs was assayed on at least four progressive dilutions of patients' and normal plasmas and their mixture before (P+N) and after (P+N)i a 2 h incubation at 37ºC, in order to evaluate the time-dependent effect. The mixture of patients' and normal plasmas after they had been separately incubated (P_i+N_i) was tested as well. The amount of factor VIIIcs present in the mixtures, was expressed as a percentage with respect to the normal curve. The proportion of patient's plasma that inactivates approximately 50% of the activity present in the mixture was chosen to calculate the inhibition index (Ii):

$$Ii = [1-(fVIII_{(P+N)}/fVIII_{(P+N)expected})] \times 1/R \times 100$$

where $fVIII_{(P+N)}$ is the amount of factor VIIIcs measured in the mixture; $fVIII_{(P+N)expected}$ is the theoretical amount of factor VIIIcs (patient plus normal) and R is the proportion of patient to normal plasma present in the mixture.

The inhibition index after incubation for 2 h at 37°C (Ii_{37}) was estimated using a control equivalent to the one employed to evaluate the APTT time-dependent effect:

$$Ii_{37}= [1- (fVIII_{(P+N)i}/ fVIII_{(Pi+Ni)})] \times 1/R \times 100$$

where $fVIII_{(P+N)i}$ is the factor VIIIcs present in the mixture after incubation and $fVIII_{(Pi+Ni)}$ the factor VIIIcs present in the immediate mixture of patient's and normal plasmas separately incubated.

Chromogenic substrate titration. The mixture of one part of patient's plasma (diluted or not), plus an equal part of normal plasma was incubated for 2 h at 37°C, factor VIIIcs was then measured. The control was an incubation mixture (1:1) of normal plasma and Owren buffer, pH 7.35. A patient's plasma leaving a residual factor VIIIcs activity of 50% in the mixture was considered to contain 1 chromogenic substrate unit of inhibitor per mL (CSU/mL).

Frequency of probable LA + a-fVIII

The frequency of patients with probable LA+a-fVIII, defined according to the criteria stated above, was estimated considering a total of 5,796 patients evaluated during a period of 4 years (1997-2000).

Statistical analysis

Data were expressed as the median obtained for the different tests in each group, indicating minimum and maximum values. The following non-parametric tests were applied: the Kruskal-Wallis one-way analysis for comparison between groups, the Mann-Whitney rank sum test to compare values between 2 groups and the Wilcoxon signed rank test to analyze differences within groups. Correlations between methods were determined with Spearman's rank correlation test. Calculations were performed with the Statistical Package for Social Sciences (SPSS Inc., Chicago IL, USA, version 9.0 for Windows) software; statistical significance was taken at the 5% level.

Results

Coagulation studies

Results are displayed in Table 1. APTT values did not differ (p=0.110) among groups. Abnormal APTT values showed no correction in mixtures with normal plasma, either immediately or after incubation at 37ºC. Differences (p=0.017) between groups in the $APTT_{index}$ became non-significant (p=0.635) after incubation. A time-dependent inhibitory effect (higher $APTT_{index37}$ than $APTT_{index}$) was

Table 1. Results of coagulation studies.

Test (normal values)	*a-fVIII n=6*	*LA n=12*	*LA+a-fVIII n=5*
APTT (35-50 sec)	133 *75;142*	94 *41;213*	119 *96;135*
$APTT_{index}$ (≤0.10)	0.25 *0.11;0.59*	0.41* *0.11;0.78*	0.08 *0.06-0.14*
$APTT_{index}37$ (≤0.10)	0.46 *0.15;0.57*	0.42* *0.10;0.70*	0.25 *0.19;0.52*
ΔAPTT-PNP (≤7 sec)	1 *–5;7*	21* *11;35*	15 *1;20*
DRVVT (ratio≤1.13)	1.09 *0.93;1.20*	2.15† *1.30;2.57*	1.34 *1.24;1.48*
$dRVVT_{index}$(≤0.08)	0.04 *–0.08;0.08*	0.40† *0.09;0.49*	0.14 *0.10;0.22*
ΔdRVVT-PNP (≤0.09)	0.03 *–0.03;0.08*	0.27† *0.15;0.78*	0.25 *0.16;0.28*

*Coagulation results are expressed as median values, with minimum and maximum values indicated in italics, for each group: plasmas with a-fVIII, with LA and with presumably both LA+a-fVIII. Normal values for each test are displayed in parentheses in the left column. *Tests were performed only in those samples with prolonged APTT (n=10). †Only samples affecting dRVVT were considered (n=10).*

observed in a-fVIII (p=0.042) and LA+a-fVIII (p=0.042), but not in LA (p=1.000). Negative results for LA tests were found in a-fVIII samples. No significant differences were observed between LA and LA+a-fVIII, except for $APTT_{index}$ (p=0.008) and dRVVT (p=0.019): higher values were obtained in LA samples.

Chromogenic substrate inhibition assays

Suitable curves (parallel to the normal) were obtained for patients' samples and mixtures, allowing estimation of the percentage of factor VIIIcs with respect to the normal, and the calculation of the inhibition index before (Ii) and after incubation (Ii_{37}). Figure 1 displays the inhibition index results obtained for the different groups. The inhibitory effect detected in a-fVIII samples was potentiated by incubation at 37ºC; allo (Ii:5-3200; Ii_{37}:27-5000) and autoantibodies (Ii:17-513; Ii_{37}:40-5200) followed a similar pattern. None of the LA samples (even those strongly affecting the APTT) displayed an inhibitory effect (Ii:0-7), not even after incubation (Ii_{37}:0-4). Samples from patients with presumably both LA+a-fVIII showed a time-dependent inhibition (Ii:9-21; Ii_{37}:50-903).

Factor VIII detection and a-fVIII titration

Factor VIII:C levels were significantly lower in a-fVIII (p<0.001) and LA+a-fVIII (p<0.001) samples than in LA samples; no significant difference (p=0.931) was observed between the a-fVIII and LA+a-fVIII groups. A similar behavior was found for factor VIIIcs: lower values were obtained in a-fVIII (p<0.001) and LA+a-fVIII (p<0.001) samples than in LA ones, displaying no significant difference (p=0.792) between a-fVIII and LA+a-fVIII groups. Figure 2 shows individual factor VIII levels (one-stage and chromogenic substrate assays) for every group analyzed. In samples with LA, most of the factor VIIIcs values were higher than factor VIII:C; similar results were seen in patients with residual factor VIII activity from the LA+a-fVIII group (2/5 non-hemophilic patients). Unlike in a-fVIII and LA+a-fVIII, differences between factor VIII:C and factor VIIIcs levels were significant in LA, without correlation between them (Table 2).

Titration results, performed in parallel by the Bethesda and chromogenic substrate methods, are shown in Figure 3. LA samples displayed negligible a-fVIII chromogenic substrate activity (0.00-0.06 CSU/mL) but they could interfere with the Bethesda method (0.52-7.00 BU/mL) yielding significant differences between Bethesda and chromogenic substrate results (Table 3). Titration methods did

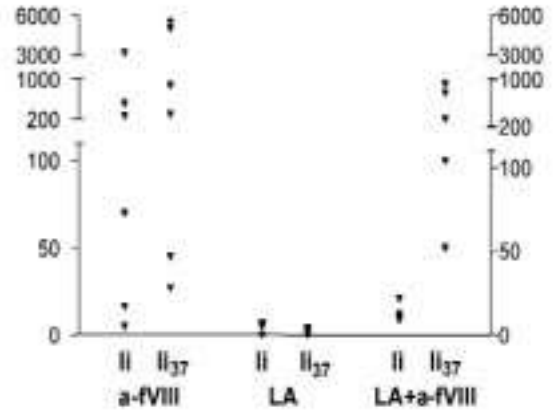

Figure 1. Chromogenic substrate assay. The inhibitory effect, expressed as an inhibition index before (Ii) and after incubation (Ii_{37}), was evaluated measuring the percentage of residual factor VIIIcs activity present in mixtures of samples with normal plasma. Unlike samples with a-fVIII (alone or in concurrence with LA), in which the time-dependent inhibitory effect was evident, LA samples did not show a progressive effect.

Table 2. factor VIII levels in the 3 groups of samples.

	a-fVIII n=6	*LA n=12*	*LA+a-fVIII n=5*
fVIII:C‡ (U/dL)	0.01	45.0	1.5
	0.01;15.0	*12.0;100.0*	*0.01;5.7*
fVIIIcs‡ (U/dL)	0.01	129.0	0.01
	0.01;17.0	*64.0;200.0*	*0.01;13.0*
fVIII:C vs fVIIIcs			
$Wilcoxon_{signed\ rank}$ (p)	0.180	0.003	0.109
Spearman's rho	1.000	–0.004	1.000
p	<0.001	0.991	<0.001

Factor VIII values are expressed as median values with minimum and maximum values in italics for each group: plasmas with a-fVIII, with LA and with presumably both LA+a-fVIII. ‡For statistical purposes, factor VIII activity measured by one-stage (fVIII:C) or chromogenic substrate (fVIIIcs) methods with values lower than 1 U/dL were considered as 0.01 U/dL. Statistical significances of the differences between both methods in each group are indicated (Wilcoxon signed rank). In addition, correlations (Spearman's rho) between both methods and the corresponding statistical significance (p) are also shown.

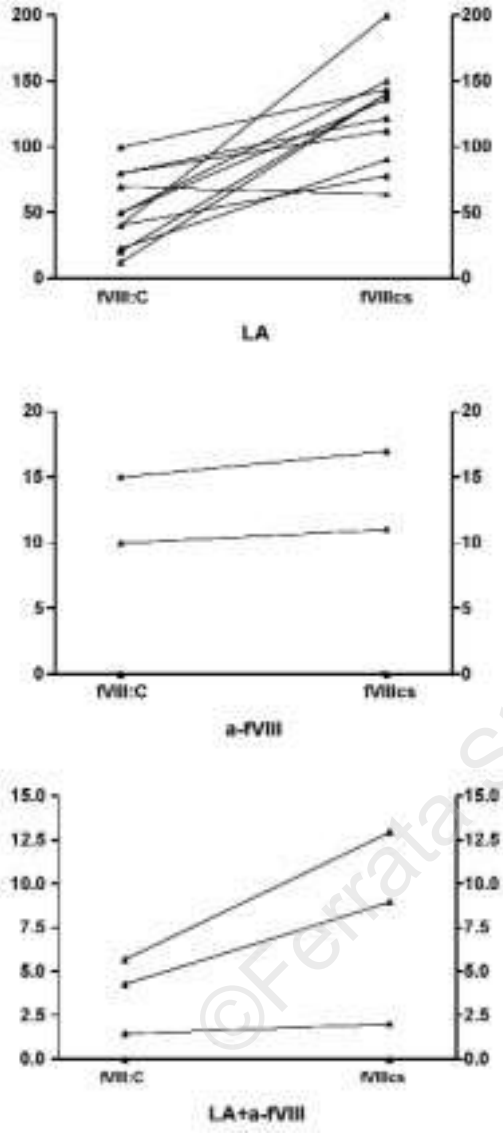

Figure 2. Factor VIII measured by the one-stage assay (fVIII:C) and by the chromogenic substrate assay (fVIIIcs). Results of samples with LA (n=12), samples with a-fVIII (n=6) and with presumably both LA+a-fVIII (n=5).

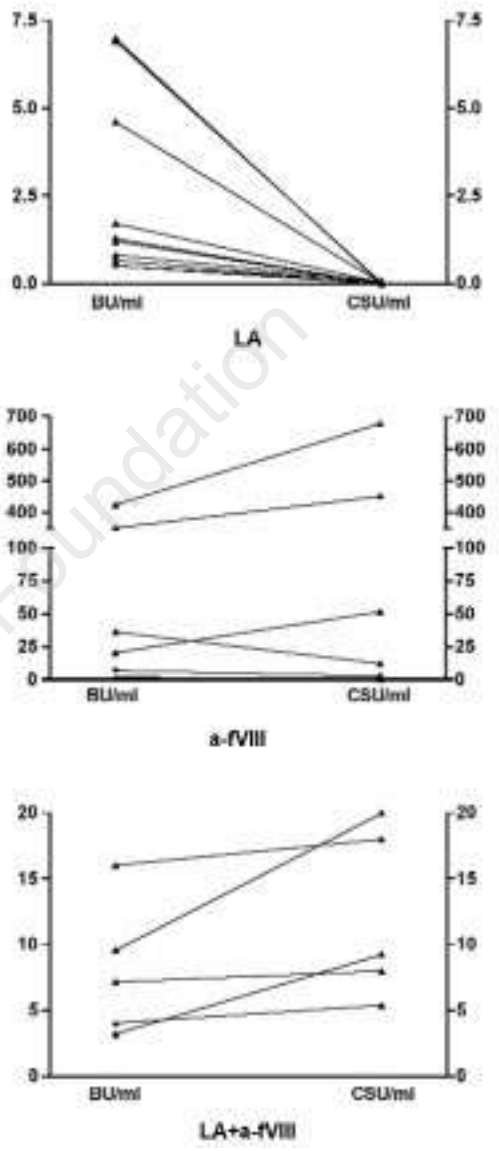

Figure 3. Bethesda (BU/mL) and chromogenic substrate titration methods (CSU/mL). Results of samples with LA (n=10), samples with a-fVIII (n=6) and with presumably both LA+a-fVIII (n=5). Only 10 out of 12 LA samples were available for performance of both titration methods in parallel.

Table 3. Comparison between Bethesda and chromogenic substrate results.

	a-fVIII n=6	*LA n=10**	*LA+a-fVIII n=5*
a-fVIII (BU/mL)	28.00 *1.00;423.00*	1.20 *0.52;7.00*	7.20 *3.20;16.00*
a-fVIII (CSU/mL)	32.00 *0.80;680.00*	0.04 *0.00;0.06*	9.30 *5.40;20.00*
BU/mL vs CSU/mL			
$Wilcoxon_{signed\ rank}$ (*p*)	0.345	0.005	0.043
Spearman's rho	0.943	–0.241	0.600
p	0.005	0.503	0.285

*Titration of a-fVIII by Bethesda (BU/mL) and chromogenic substrate (CSU/mL) methods is expressed as median values with minimum and maximum values in italics for each group: plasmas with a-fVIII, with LA and with presumably both LA+a-fVIII. Statistical significances of the differences between both methods in each group are indicated ($Wilcoxon_{signed\ rank}$). In addition, correlations (Spearman's rho) between both methods and the corresponding statistical significance (p) are also shown. *Only 10 out of 12 samples were available for performance of both titration methods in parallel.*

not correlate when LA was present either alone or combined with a-fVIII (Table 3). Despite the correlation observed in a-fVIII and even though differences were not significant (Table 3), most of the a-fVIII CSU values were higher than the BU values (Figure 3). In LA+a-fVIII, slight differences were found (Table 3); but like in the a-fVIII group, CSU appeared to be higher than BU (Figure 3).

Frequency of probable LA+a-fVIII

A frequency of 1.6‰ (9/5796) was estimated, considering a period of 4 years. This represents a total of 24% (9/34) of the patients in which a-fVIII inhibitors were detected during the same period.

Discussion

Given the implications of the neutralizing a-fVIII inhibitors in the clinical and therapeutic management of patients, it is important to detect such inhibitors unequivocally and titrate them correctly, even in the presence of LA. In order to avoid false positive results caused by plasma factor deficiencies, we considered confirmatory tests positive for LA only if a neutralizing effect was seen both on the patient's plasma and on its mixture with normal plasma.[10,32] The possibility of false positive LA confirmatory tests in the presence of specific factor inhibitors was also considered.[10,32,33] The group defined as a-fVIII had no positive LA results.

No distinguishing coagulation results between LA and LA+a-fVIII were observed, except for the time-dependent effect and lower factor VIII, $APTT_{index}$ and dRVVT values seen in the latter group. An apparent severe factor VIII deficiency without evidence of any dilutional effect could be found in the presence of a high titer LA.[10] However, the progressive inhibitory effect observed suggests the concomitant presence of a-fVIII, further sustained by the previous history of a-fVIII in hemophilia A patients (3/5) or by severe bleeding symptoms in non-hemophiliacs. From the laboratory point of view, since LA could affect factor VIII:C detection, and consequently the Bethesda method, a simultaneous a-fVIII cannot be unequivocally detected and correctly titrated unless a specific a-fVIII assay can be carried out. Moreover, it must be considered that although rare, in some situations a-fVIII may not display a clear time-dependent effect.

Brandt *et al.* suggested measuring factor VIII by employing tests based on other principles, such as amidolytic assays, to differentiate LA interference from LA from a true deficiency[10] and de Maistre *et al.* reported that factor VIII activity evaluated by a chromogenic substrate method is not affected by LA.[12] Considering this, we tested whether an a-fVIII system based on the chromogenic substrate principle could be specific, excluding LA interference in the detection and titration of a-fVIII.

Chromogenic substrate results revealed inhibition of factor levels (VIIIcs) by a-fVIII or LA+a-fVIII samples. An immediate effect might not be unequivocal when very low Ii values are obtained; nevertheless, the effect becomes clear after incubation. It is worthy of remark that the Ii_{37} allows detection of an otherwise missed a-fVIII activity (false negative results).[10] None of the LA plasma samples displayed substantial inhibition on factor VIIIcs.

As expected, factor VIIIcs values were higher than factor VIII:C in LA samples; a similar behavior was observed in plasmas with LA+a-fVIII and residual factor VIII activity (2/5). This discrepancy between factor VIII:C and factor VIIIcs could represent an additional sign of the presence of LA, to be considered mainly in LA+a-fVIII samples.

The results indicate the possibility of detecting and titrating a-fVIII by their inhibitory effect on factor VIIIcs without interference by LA. Differences between Bethesda and chromogenic substrate titration methods were noticed in the presence of LA and no significant correlation was observed either in the LA group or in the LA+a-fVIII one. Results compatible with low to moderate a-fVIII titer by the Bethesda method (0.52-7.00 BU/mL) were found, in contrast to no or meaningless a-

fVIII activity detected by the chromogenic substrate method (<0.06 CSU/mL) in LA samples.

A measurable effect by the Bethesda method mediated by a strong LA can be expected when a sensitive LA reagent is used; this could be particularly worrying in patients with simultaneous presence of both inhibitors. No clear effect was found on the Bethesda titer in LA+a-fVIII samples; slight differences were detected, in contrast to the differences observed between both titration methods in LA patients. A weak LA effect could be suspected in our LA+a-fVIII group, since APTT or dRVVT were less prolonged in LA+a-fVIII samples compared to in LA ones. As Nuss *et al.* reported, in a study on hemophilia A children,[17] weak LA interference in the Bethesda method could be diluted out.

Difficulties in determining the coexistence of a-fVIII and LA beyond question with the currently established methods are evident in most papers that address the subject.[16-23] We point out that both inhibitors should be suspected in patients fulfilling rigorous LA criteria and a strong progressive inhibitory effect.[16] Following these criteria, we found a frequency of probable LA+a-fVIII of 1.6‰, which represents a total of 24% of the patients with a-fVIII. In this subset of patients it would be more reliable to detect and titrate a-fVIII activity by the chromogenic substrate assay.

Contributions and Acknowledgments

ANB planned and supervised the study; she was responsible for evaluation of the data and writing the manuscript. AAP carried out the chromogenic substrate tests and contributed to data collection, analysis and writing of the manuscript. SHG and LCG carried out the coagulation studies. RPB was the clinician responsible for the patients' clinical management. MAL designed, supervised and critically revised the final version of the paper.

The authors are listed according to a criterion of decreasing individual contribution to the work with the exception of the last author who had a major role as senior author in designing the study, interpreting the data and preparing the article.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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Peer Review Outcomes

Manuscript processing

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What is already known on this topic

The presence of lupus anticoagulant may affect titration of anti-FVIII antibodies.

What this study adds

The authors develop a chromogenic substrate method for an accurate detection and titration of anti-FVIII antibodies in patients with lupus anticoagulant.

Potential implications for clinical practice

The new method simplify the titration of anti-FVIII antibodies in presence of lupus anticoagulant.

Vicente Vicente, Deputy Editor