

Anticardiolipin Antibody Assay: a Methodological Analysis for a better Consensus in Routine Determinations

A Cooperative Project of the European Antiphospholipid Forum

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Keywords

Antiphospholipid syndrome, anticardiolipin antibodies, standardization

Summary

Despite the widely recognized practical importance of anticardiolipin (aCL) ELISA, the reliability of this test has been recently discussed. In order to investigate this area on European scale, we sent to 30 experienced centers a questionnaire focusing on the diagnostic procedures applied to patients with antiphospholipid syndrome (APS) and on the detailed protocols used to perform aCL. Anticardiolipin ELISA was found to be the most frequently performed test in patients with suspected APS, but significant difference was shown among the various protocols. The cross-laboratory multiple examination of ten serum samples evaluated independently by the 24 centers pointed out the difficulty in getting comparable results. Therefore a "consensus" protocol was derived from the aCL methods giving the best performance. The materials and reagents necessary to perform the "consensus" method, including, as putative standards, one IgG and one IgM monoclonal antibody (HCAL and EY2C9) were distributed to 19 Centers. The results of one IgG and one IgM aCL high positive sera measured in serial dilutions were compared. A progressive decrease in the variability of the values obtained for a given sample appeared evident when all the laboratories used the same standard, in their own in-house ELISA and even more in the "consensus" ELISA.

Our data show that aCL ELISA standardization is necessary in order to obtain comparable results in different laboratories.

Introduction

First described in 1983 (1), solid phase immunoassay for detection of anticardiolipin antibodies (aCL) has been widely applied and a number of studies underlined its relevance in the diagnostic definition of patients with the so-called antiphospholipid syndrome (APS).

This syndrome has been called "one antibody disease" that is to say that, in patients with suggestive clinical features, the positivity of aCL

or lupus anticoagulant (LAC) is enough to make a diagnosis and to apply a long-term treatment, not free from possible unwanted effects (2). In addition, the positive result of only one of these two tests is necessary as an inclusion criteria in clinical trials that obviously need to include and evaluate comparable patients. Lupus anticoagulant (LAC) has been standardized and should be performed according to well-defined procedures (3), but problems in obtaining comparable results still exist, due to the fact that many laboratories do not follow the proposed guidelines (4). On the other hand, no clear international consensus has been reached in the aCL ELISA methodology. In fact, attempts were originally made to compare the methods used by different workers in order to standardize procedures, reagents and materials (5-8). However, with the rapid widespread use of the test, due to its recognized value in the clinical practice, several commercial kits and home-made tests using different procedures were progressively introduced. In addition, in contrast to most other autoantibodies (9), no official standards recognized by the appropriate organizations (WHO, IUIS, CDC) have been defined for this assay.

Therefore, it is not surprising that a number of workers underlined the difficulty in getting consistent results on serum samples examined by different laboratories (10-13), raising some doubts on the real validity of the test itself. The difficulty in obtaining reproducible results in aCL ELISA might in fact explain, at least in part, its limited contribution to clinical studies occasionally reported (14).

Based on this historical background, it seemed to us of the greatest interest to perform a collaborative study, within the European Forum on Antiphospholipid Antibodies, in order to identify the different problems associated with the aCL ELISA and try to reach a consensus at least on the critical variables of the test performance.

The work proceeded through different steps: 1) analysis of the aCL ELISA used in different centers with their detailed methodology; 2) sera exchange in order to compare the results obtained by the different centers; 3) analysis of the methods giving comparable results and identification of a common protocol; 4) second sera exchange in order to verify the eventual improvement in the results and agreements between centers obtained by using a common protocol and common standards.

Material and Methods

Analysis of aCL ELISA Used in Different Centers

A survey was first performed in 1997 on a European scale after sending a questionnaire to 30 qualified laboratories. The addressed questions dealt with: a) the role of ELISAs in the diagnostic definition of patients with clinically sus-

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Table 1 Clinical and serological features of subjects from whom sera were used in the first serum exchange

Sample*	Sex	Age	Diagnosis	Clinical data	LAC
B1	F	36	Normal subject		neg
B2	F	31	PAPS §	Fetal loss, valve vegetations	pos
B3	F	33	SLE + APS ¶	Deep venous thromboses, low platelet count	pos
B4	F	42	SLE + APS	Deep venous thromboses, livedo reticularis	neg
B5	F	32	PAPS	Deep venous thromboses, fetal loss	neg
M1	M	54	PAPS	Deep venous thromboses, myocardial infarction	pos
M2	F	43	SLE + APS	Central nervous system thromboses	pos
M3	F	40	SLE + APS	Deep venous thromboses, valve vegetations, central nervous system thromboses	pos
M4	F	40	Normal subject		neg
M5	F	34	PAPS	Deep venous thromboses, low platelet count, livedo reticularis, renal failure	pos

* B = Sera selected in Brescia; M = Sera selected in Marseille. § PAPS = Primary Antiphospholipid Syndrome. ¶ SLE + APS = Systemic Lupus Erythematosus + Antiphospholipid Syndrome.

pected APS; b) the technical details of the tests currently used, including the type of microtitre plates, buffer compositions, sample dilutions, antiserum used, incubation time; c) result's expression, including: cut-off level definition, positivity ranges, calculation of units through the use of calibrators or standards.

Cross-laboratory Serum Samples Examination

In 1998, serum samples from 8 patients with well-characterized APS and 2 normal subjects were selected in Marseille and Brescia. The clinical and se-

rological features of the patients are summarized in Table 1. One aliquot of each serum was sent simultaneously to 24 centers, having accepted to enter this part of the study. Results from the different laboratories were compared for each sample, both in units (IgG antiphospholipid = GPL; IgM antiphospholipid = MPL) and in positivity range (negative; low, medium and high positive). A "consensus value" range was attributed to each serum derived from the majority of the results given by the various laboratories.

The protocols used by the centers giving the best performance in relation to "consensus values" of the 10 sera examined were carefully analyzed. A "consensus protocol" was subsequently derived from these protocols.

Test	First step	Second step
	Number of Centers	
aCL (anti-cardiolipin) - IgG - IgM	29	4
aCL - IgA	2	2
aPS (antiphosphatidylserine)	4	1
aPE (antiphosphatidylethanolamine)	3	4
aPC (antiphosphatidylcholine)	1	-
aPL - mixture (antiphospholipid mix)	1	16
aβ2GPI (anti-beta2-glycoprotein I)	7	3
aPT (anti-prothrombin)	1	1
aAN - V (anti-annexin V)	-	1
AMA - M5 (anti-mitochondria)	1	2
Anti-endothelial cells	1	-
LA (lupus anticoagulant)	25	2
BFP-ST5 (biologic false-positive test for syphilis)	-	1

Table 2 Tests routinely used by 30 European labs to study patients with APS-related clinical symptoms, in the first and second diagnostic step

Variable	Number of centers		
	not specified	specified	
Blocking buffers	5	11	PBS - 10% FCS
		6	PBS - 10% ABS
		1	TBS - 10% ABS
Blocking times	5	11 / 6 / 1	1h / 2h / 1h and 30'
Blocking temperature	4	16	room temperature
		1 / 1 / 1	4C° / 20°C / 37C°
Washing Buffers	5	14 / 1	PBS / TBS
		1	PBS - 10% FCS
		1	PBS - 10% ABS
		1	0.9% NaCl-0.02%Tween20
Sample type	1	15 / 5 / 2	serum / plasma / serum or plasma
Diluting buffer	5	11	PBS - 10% FCS
		5	PBS - 10% ABS
		1	TBS - 10% FCS
		1	TBS
Sample dilution	2	13 / 6 / 1 / 1	1:50 / 1:100 / 1:200 / 1:20
Sample distribution	1	16 / 4 / 2	duplicate / triplicate / single
Sample incubation time	6	7 / 4 / 2	60' / 30' / 90'
		6 / 3 / 1	3h / 2h / 2h 30'
Cut-off level determination	0	7 / 6 / 4	Mean \pm 3 SD / 2 SD / 5 SD
		3 / 2 / 1 / 1	99 / 97.5 / 98 / 95° percentile
		1	normal human serum mean OD x 2
		3	according to kit's instructions

PBS: phosphate buffer saline; TBS: tris buffer saline; ABS: adult bovine serum; FCS: fetal calf serum.

Second Exchange of Serum Samples

In spring 1999, 2 sera (1 aCL IgG positive, selected in Marseille, and 1 aCL IgM positive, selected in Brescia) were sent to the 24 participating centers. In addition each participating laboratory received 2 plates (Polysorp, Nunc, code 475094), cardiolipin (CL; Sigma, code C-1649), phosphatase-labelled affinity purified goat anti-human IgG (Sigma, code a-3188) and anti-human IgM (Sigma, code A-4337), heat-inactivated fetal calf serum (HyClone, code SH30073.03), together with a detailed protocol. All the reagents distributed were derived from the same batch. Two monoclonal antibodies chosen as standards were sent: a monoclonal chimeric antibody (HCAL) with human γ 1 constant region and variable region of WBCAL-1, a monoclonal antibody established from an antiphospholipid syndrome-prone mouse which has a specificity similar to that of anticardiolipin antibodies in sera from humans with antiphospholipid syndrome (15), and a human monoclonal anticardiolipin (anti- β 2GPI) IgM antibody established from a patient with antiphospholipid syndrome

(EY2C9,16), kindly provided by Dr. Koike. These materials and reagents were distributed as "consensus kit".

Participants were invited to test in serial dilution the two sera samples and the two monoclonal antibodies, using both their in-house aCL method and the "consensus kit and protocol". In both assays they were asked to include 10 normal sera derived from their own laboratory.

Results

Analysis of aCL ELISA Used in Different Centers

According to our survey, aCL ELISA was shown to be the immunoassay most frequently used in routine diagnosis of APS, although anti- β 2GPI ELISA was also widely applied. Table 2 summarizes the tests that the different centers declared to perform in a patient suspected to have APS as first and second diagnostic step.

Table 3 Main variables detected in blocking steps, incubation procedures and sample distributions

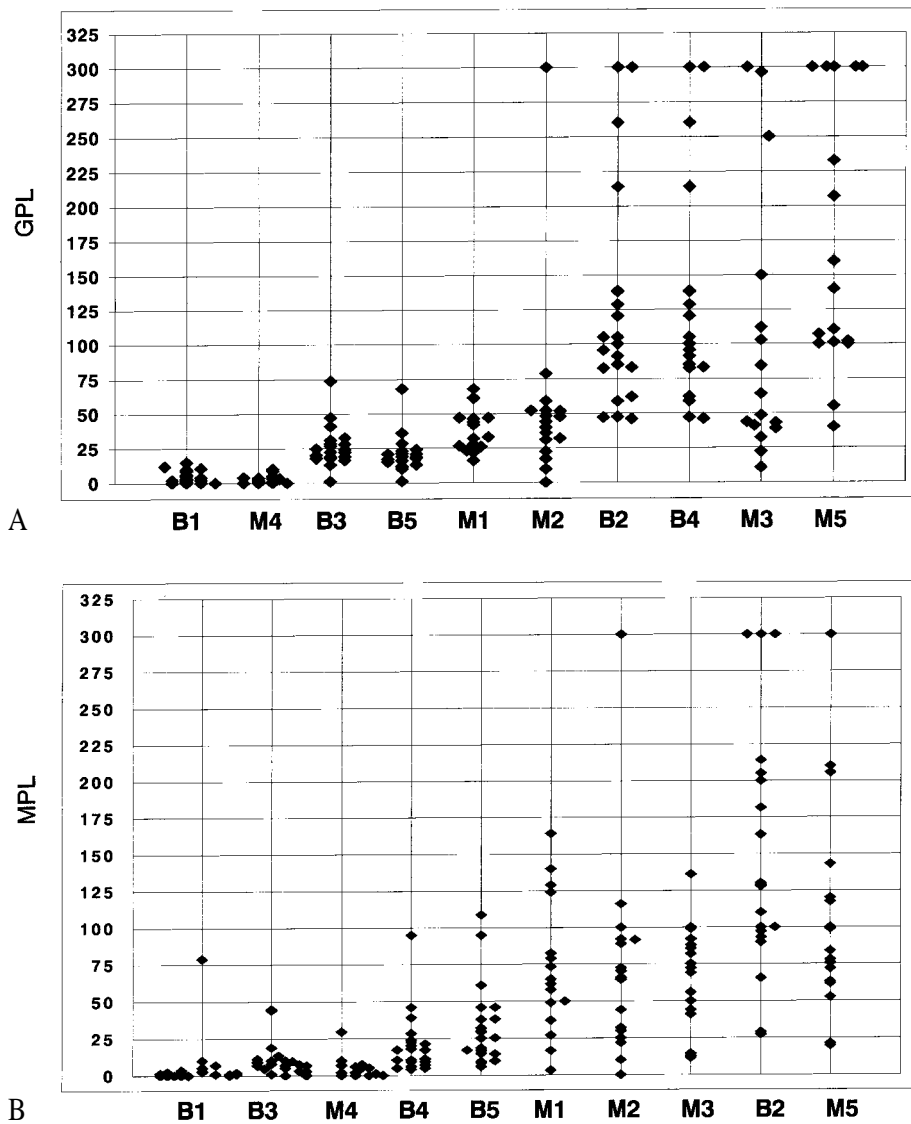


Fig. 1 Analysis of the same 10 sera performed in 24 different European centers. A, IgG aCL ELISA (results in GPL units). B, IgM aCL ELISA (results in MPL units). B1 to B5 = sera from Brescia; M1 to M5 sera from Marseille

Regarding aCL ELISA, most of the laboratories were using home-made assays (22/30, 73%) as reported in previous studies (7), while commercial kits were routinely used in 8 centers (Biomedical Diagnostics in 2; INOVA in 2; Novamed, Eurospital, Kallestad, Imtec each in 1).

Details obtained from 23 centers regarding the type of plate revealed that polystyrene plates were used in 14 laboratories (10 treated for high-antigen binding capacity), polyvinylchloride plates in 5 while 4 laboratories did not specify. The wells were flat in 15 laboratories, round in 1, combined in 1 and not specified in 6.

Coating was performed with CL from bovine heart in ethanol in 14 laboratories and in methanol in 3, while 1 laboratory was using recombinant CL from *E. coli* in methanol and 5 laboratories were not able to detail the features of the CL used.

Table 3 summarizes most of the variables detected in blocking steps, incubation procedures and samples' distribution.

Interestingly, 29 out of 30 centers expressed their results in GPL/MPL units and therefore were using calibrators or standards. Fourteen centers were using Harris' standards (Louisville APL Diagnostics, Inc.), 9 home-made standards calibrated on Harris' standards and 6 home-made standards per se.

Cut-off level determination received from 28 centers (Table 3) showed that actual values ranged from 0 to 25 GPL and from 0 to 25 MPL. Positivity ranges were defined by each Center according to its own experience: the highest level of low positivity varied from 10 to 40 GPL, and from 10 to 50 MPL; on the other hand, the highest values of medium positive ranges extended from 15 to 100 GPL and from 15 to 100 MPL respectively.

Cross-laboratory Serum Samples Examination

The 10 blind serum samples were distributed to 24 centers. Direct comparison of the results in GPL/MPL units is shown in Fig. 1, A and B. Because of the impressive scattering of values obtained in the same sample by different laboratories, we decided to compare the results, according to each laboratory definition, in term of negative, low, medium and high positivity. Finally a "consensus value" was identified for each serum, driven from the majority of results available. Table 4 summarizes the results obtained for IgG and IgM isotype respectively.

To evaluate the aCL ELISA performance of the different laboratories we looked at their results in term of agreement with the "consensus value" taken as a "gold standard" value (Table 5). According to this

Table 4 IgG aCL and IgM aCL ELISA: results of cross-laboratory sera examination. Results are classified in negative, low, medium and high positive

Sample	Answers		Results		"Consensus value"	
	N° labs #	Tot	in agreement	aberrant		
B1*	24					
M4§	20	44	41/44 (93%)	1 low, 1 medium, 1 high	Normal	
B3	22					
B5	22	44	22/44 (50%)	5 normal, 13 medium, 4 high	Low	
IgG	M1	17				
	M2	18	35	18/35 (51%)	5 normal, 5 low, 7 high	Medium
	B2	24				
	B4	24				
	M3	18	86	68/86 (79%)	2 low, 16 medium	High
M5	20					
B1	23					
B3	22	64	58/64 (91%)	3 low, 3 medium	Normal	
M4	19					
IgM	B4	22	22	7/22 (32%)	5 normal, 5 medium, 5 high	Low
	B5	23				
	M1	19	61	28/61 (46%)	8 normal, 6 low, 19 high	Medium
	M2	19				
	B2	23				
M3	18	60	42/60 (70%)	1 normal, 1 low, 16 medium	High	
M5	19					

* B = Sera selected in Brescia; § M = Sera selected in Marseille. # 4 Centers tested only 5 out of the 10 samples; some Centres didn't indicate their cut-off for low, medium and high positive.

analysis, 6 out of the 24 laboratories (25%) gave results in agreement with the "consensus value" for IgG isotype in all the 10 serum samples examined. On the other hand, for the IgM isotype, none of the laboratories gave 100% agreement with the "consensus value", possibly because of the high rate of overestimation (Table 5).

"Consensus Protocol" Draw

The different steps of the aCL ELISA performed in the 6 laboratories showing the best agreement with the samples consensus values were analyzed, in the attempt to derive a common protocol. The proto-

Table 5 Analysis of aCL ELISA performance of the 24 Centers participating to the inter-laboratory samples examination

Centres giving results for all 10 sera	IgG		IgM	
	n°	%	n°	%
In agreement with "consensus value"	6	25	0	0
Over-estimate	8	33.5	11	45.8
Under-estimate	7	29	7	29
Under-estimate and over-estimate	3	12.5	6	25

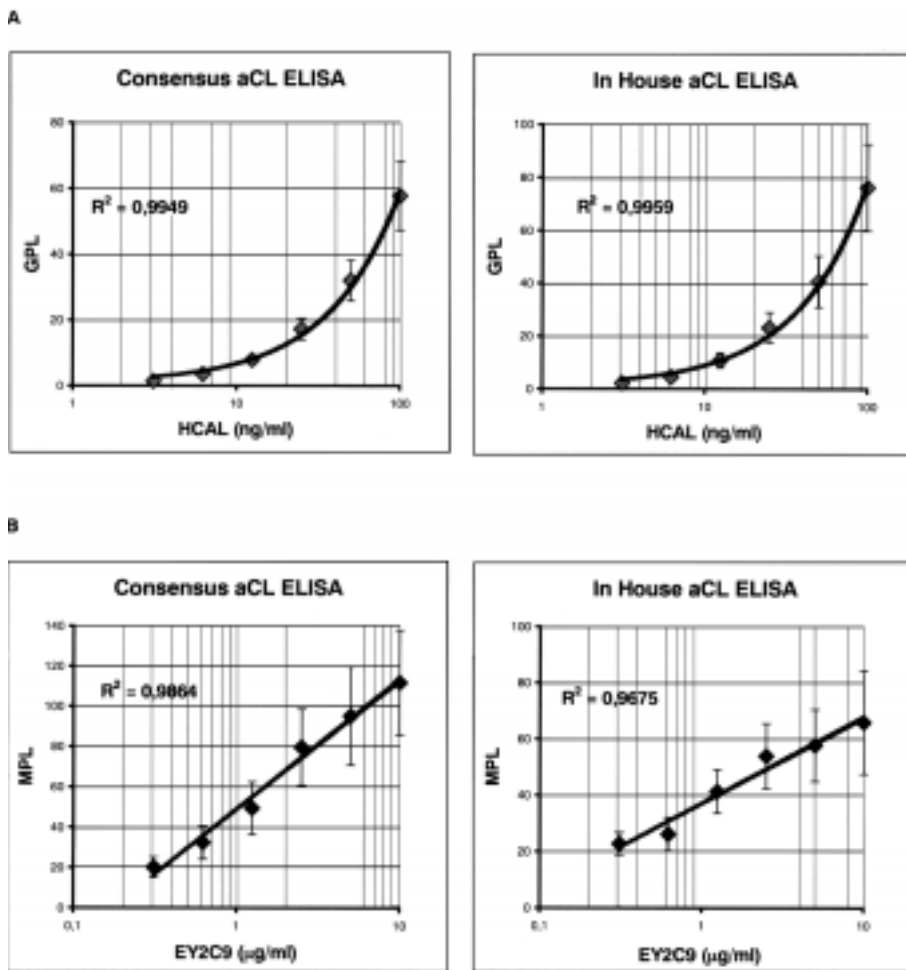


Fig. 2 Relationship between the concentration of monoclonal antibodies and phospholipid units. Serial dilutions of HCAL (A) and EY2C9 (B) were tested in 19 laboratories by both their in-house method and consensus method. The values in units are derived from the average values attributed by each center. For each point the standard error is reported

col was drawn choosing materials or procedures used by the majority of the laboratories or, alternatively, when a clear trend was not identified, we checked the different variables selecting the one giving the best performance in our hands (Table 6).

Second Exchange of Serum Samples

Nineteen laboratories sent their results of the last serum exchange. They were asked to test the 2 monoclonal and the 2 polyclonal aCL-positive samples in serial dilution and to give the results in GPL and MPL units, derived from their in-house standard curve as well as in optical density. The test had to be performed according to the in-house protocol as well as the consensus protocol.

Based on the average of the results obtained from the different laboratories, a value in GPL units was calculated for the different dilutions of HCAL and in MPL units for the different dilutions of EY2C9. The correlation between monoclonal protein concentrations and GPL/MPL units was analyzed by linear regression (Fig. 2). Each monoclonal was evaluated by both the in-house and the consensus method (Fig. 2, A and B). Therefore, for example, taking the average of the in-house method results, 100 ng/ml of HCAL were equivalent to 76 GPL, 50 ng/ml to 41 GPL and so forth and could well represent an additional standard curve. This new curve was in fact applied to the assay as an alternative measurement of the distributed serum samples.

The results obtained from the 19 centers testing the IgG and IgM aCL polyclonal serum samples were then analyzed. These results were pro-

vided in GPL/MPL units based on the in-house standard curve. In addition, since we calibrated the 2 monoclonal antibodies in GPL/MPL units, we could calculate the results also on the new standard curve based on the values attributed to the different dilutions of HCAL and EY2C9.

The standard error obtained from the evaluation of the same sample by the different laboratories is shown in Fig. 3. The figure compares the results obtained by the in-house method and in-house standard versus those obtained by the in-house method calculated on the monoclonal standards and versus those obtained by the consensus method including also the use of the monoclonal standards. The standard errors drawn on the top of each bar, show a progressive constant reduction although the overall difference does not reach a statistical significance.

Discussion

Among the tests used to detect antiphospholipid antibodies, aCL ELISA has probably had the greatest impact. In fact, from the 1950s, persistent false positive tests for syphilis and LAC have been reported in patients with recurrent abortions and thrombosis (17-21). Moreover, the aCL radioimmunoassay reported by Harris and coworkers (1) and later adapted to ELISA (22) immediately became a very popular tool for detecting aPL and for supporting the APS diagnosis. The widespread use of aCL in large cohorts of patients allowed the clinical observations leading to the generally accepted definition of a new syndrome (23-27).

As clearly shown by the present study and by other surveys (28, 29), physicians strongly rely upon aCL ELISA for diagnosis and decision regarding treatment.

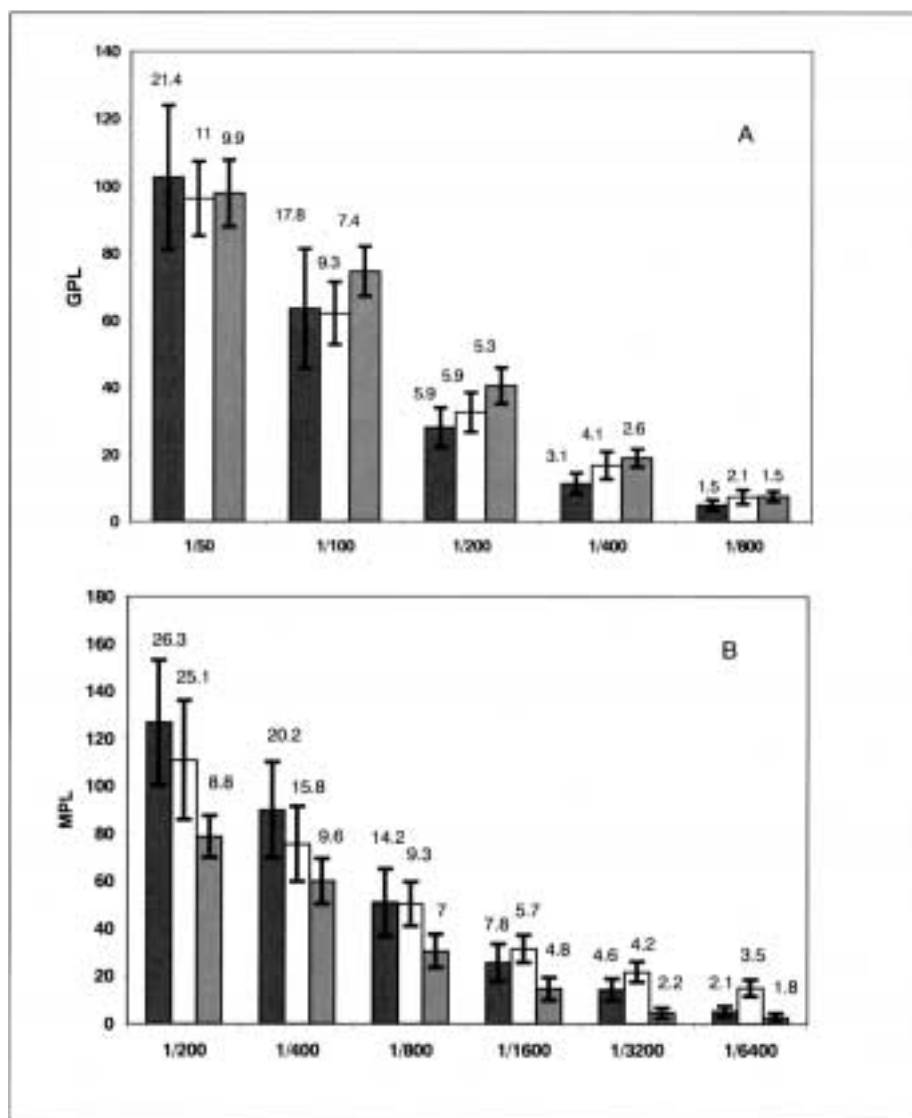


Fig. 3 Mean binding levels of serial dilution of IgG aCL high positive serum (A) and IgM aCL high positive serum (B), as determined by 19 independent laboratories using their own method and their own standards (dark grey bar), their own method and monoclonal standards (white bar), and finally, consensus method and monoclonal standards (light grey bar). Values of standard error are given on the top of each bar

However, when comparing the inter-laboratory variability in the measurement of the same serum samples we revealed how difficult it is to get a good agreement, not only in terms of GPL/MPL units, but also in terms of positivity range. In the present study, the “consensus value” given to the examined sera, and taken as gold standard, was derived from the majority of the values attributed by the participating laboratories. Nevertheless, for the IgG isotype only 6 out of 24 participating laboratories attributed values in total agreement to the consensus value, while for the IgM isotype none of the participating centers was in total agreement with it. This poor performance might be due to a possible aspecific binding, since the results show a high rate of overestimation (Table 5). This finding, according to a previous report (12), underlines the difficulty in performing aCL ELISA when compared to other better standardized tests (14, 29). In this view, the historically well-known weaker correlation of IgM aCL compared to IgG aCL with thrombosis episodes and recurrent miscarriages (30) may also be explained by the particular difficulty to measure the IgM aCL isotype, as clearly demonstrated by this paper.

In any case, it is worthwhile to remember that problems of accuracy, specificity and sensitivity are not rare in ELISA determinations: in fact such problems have been recently pointed out also among ELISAs routinely applied to the detection of other autoantibodies (31).

According to our analysis, the main sources of inter-laboratory variations seemed to be the variety of calibrations used in the test and the different way used to calculate the cut off point. In fact, although all the tests had to be theoretically performed using Harris’ standards, a careful analysis showed that this was the situation in only 14 out of 30 centers. Interestingly, 9 laboratories declared to calibrate their own standards on Harris’ standards, and this operation, especially if repeated more than once, could well lead to the adoption of more or less different calibrators, that do not even share the same dilution curve slope. On the other hand, different performances of different batches of Harris’ standards have been reported (12). This observation is not surprising because of the objective difficulties in finding absolutely identical positive serum samples. In fact, in different sera, even if chosen with the same positivity value, antibodies with different avidity can be present in different ratio among themselves, leading to different slopes when used in twofold dilution, like in a calibration curve. For all these reasons we decided to introduce in the consensus protocol a common calibration system. In the attempt to have a stable standard solution, IgG and IgM aCL human monoclonal antibodies were considered. The recent demonstration that HCL was proven to be useful as a standard for human IgG aCL and anti- β 2GPI antibody assays (15) prompted us to use this monoclonal. On the other hand, EY2C9 (16), here used for the

Table 6 Analysis of the 6 aCL ELISA protocols giving the best performance in the cross-laboratory serum samples examination and the “consensus protocol”

Variable	Centres' protocol number of laboratories	Experimental evaluation	"Consensus protocol"
Cardiolipin: C 1649 SIGMA	4 out of 6	nd §	C 1649 SIGMA
Cardiolipin diluent: ethanol	5 out of 6	nd	Ethanol
Cardiolipin incubation: overnight	4 out of 6	nd	Overnight
Wells without antigen: not included	5 out of 6	nd	Not included
Blocking buffer: PBS-10% FCS	4 out of 6	nd	PBS-10%FCS
Microtiter plates:	5 different brand*	yes	NUNC 475094
Washing buffers:	3 labs with and 3 without proteins	yes	PBS-10%FCS
Washing after blocking:	twice (2 labs), three times (1 lab), not specified (3 labs)	yes	1 wash after blocking
3 washes after samples distribution:	5 out of 6	nd	3 times
Washing after antiserum:	from 3 to 6 times	yes	3 times
Samples dilution: 1\50	5 out of 6	nd	1\50
Samples incubation:	from 60' to 180'***	yes	60'
Phosphatase-labelled anti-human IgG-IgM	various***	nd	SIGMA
Antiserum incubation:	from 45' to 180'****	yes	120'

§ nd: not done. *NUNC 475094; Flow 717205; Greiner 655001; Costar 2595; Corning 25801. ** 60' (3 labs), 120' (2 labs), 180' (1 lab). *** DAKO, Jackson IR, SIGMA (2 labs), Tago, not specified (1 lab). **** 45' (1 lab), 90' (3 lab), 180' (1 lab), 15 hrs (1 lab).

first time as a standard for the IgM aCL assay, was handled with similar methodology and appeared suitable although, probably because of its IgM isotype, more scattered determinations were observed (Fig. 2). In this paper the GPL/MPL units attributed to HCAL and EY2C9 derived from the average of the value attributed by the 19 laboratories measuring them. Therefore 100 ng/ml of HCAL resulted equivalent to 76 GPL using the in-house aCL and 58 GPL using the consensus protocol. This reaction pattern may be slightly different from that previously reported (15), but this difference can well be attributed to the over or under estimation given by some of the participants. In any case, even if the GPL and MPL units are totally arbitrary, Fig. 3 shows that the sharing of a common standard clearly decreases the standard error, when the same serum samples are measured by different centers, even if they use their own in-house assay, that we proved to be performed in rather different ways.

Therefore the introduction of monoclonal standards was able “per se” to improve the performance of aCL assay.

As expected, the agreement among the results obtained in different laboratories is further increased when, beside standards, they shared a common protocol (Fig. 3).

In the attempt to minimize the difference among different assays, we tried to define a common method derived from a careful analysis of the methods used by the centers obtaining the best performance in terms of consensus results. Our consensus protocol includes the materials and procedures adopted by the majority of those centers or, in the absence of a clear major trend, giving the best performance in our hands. Nineteen laboratories in the last part of our work have used this protocol. Therefore a number of workers could try its different variables in comparison with their own in-house method.

At the present time we try to gather comments and criticisms derived by this common experience in order to write a consensus document of aCL ELISA guidelines, that can provide a guidance for the performance of the assay.

Addendum on the Role of Authors

A. Tincani: main writer; data collection; M. Sanmarco and G. Balestrieri: statistical analysis and providing patients and sera; F. Allegri, M. Cinquini and M. Taglietti: performance of experiments necessary to obtain “consensus protocol”, distribution “consensus kit”, and correspondence with different Laboratories; K. Ichikawa and T. Koike: production of monoclonal antibodies; P. Meroni: data analysis; M. C. Boffa: general coordinator and participant in manuscript preparation.

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Appendix

Members of aPL European Forum Participating to the Work

In addition to the authors, the following workers actively co-operated in this project: Abbate R, and Farsi A (Università di Firenze, Italy), Arvieux J, Youinou P (Laboratoire d'Immunologie, Brest, France), Bengoufa D and Piette JC (Pitié-Salpêtrière University Hospital, Paris, France), Biron C (Hopital Saint-Eloi, Montpellier, France), Bombardieri S and Gianola D (Università di Pisa,

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