

Development of Automated Assays for Anticardiolipin Antibodies Determination: Addressing Antigen and Standardization Issues

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ABSTRACT: The lack of reliable standardization tools as well as the poorly defined nature of the “Cardiolipin antigen” makes the development of the anticardiolipin antibody (ACA) assays (for anti-IgG and IgM detection) highly challenging. This article describes how several issues have been solved during the development of an automated ACA immunoassays, based on a technology that includes paramagnetic microbeads as solid-phase reagents and chemiluminescence as a signal. The technology is adapted to an automatic immunoanalyzer, called LIAISON, which performs, in an automatic manner, the whole assay, starting from the primary tube of the bleeding to the display of the assay result. Briefly, the magnetic microbeads were coated with an ethanolic solution of cardiolipin (CL) followed by an affinity-purified, cross-linked human β 2-glycoprotein I. CL-coated paramagnetic microbeads, after incubation with an ACA-positive sera plus addition of immunogold-protein A, were visualized by SEM, showing the presence of well-defined protein clusters on the microbeads surface as an indication of the successful occurrence of the “antigen” coating. The assay standardization was achieved on the basis of human samples containing various amount of ACA, which were previously classified according to consensus doses. The evaluation of the optimized LIAISON Cardiolipin assays (IgG and IgM) was conducted by using clinically characterized APS sera. The results of the evaluation showed that the LIAISON assays perform at least similar to certain well-established ACA enzyme-linked immunosorbent assay (ELISA) products.

KEYWORDS: anticardiolipin antibodies; immunoassay; LIAISON β 2-glycoprotein I purification; antiphospholipid syndrome; SEM

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INTRODUCTION

The aim of this work was to study the development of reliable and reproducible immunoassay for both IgG and IgM anticardiolipin antibodies (ACA) detection. The reasons that prompted us to start the project were mainly a diffused disappointment of current ACA ELISA users caused by the poor agreement among different manufacturers' assays, an increasing demand of fully automated ACA test kits, and the lack of universally acknowledged approaches and/or materials for standardization. We judged that our available immunoassay technology, called LIAISON, was able to overcome such issues. This technology, which has been described for infectious diseases applications, as well as for other applications, is based on paramagnetic microbeads as solid-phase reagents and a chemiluminescent signal, the amino-butyl-ethyl-isoluminol (ABEI).¹⁻⁴ It is adapted to an automatic immunoanalyzer, which is capable of performing all the assay steps starting from the primary bleeding tube till the display of the final result. All the assay components (solid-phase, signal solution, sample diluent, assay calibrators) are contained in a cartridge, which can be inserted on-board of the instrument. This feature requires that the whole set of components is ready to use and is stable at 6–8°C along the shelf-life of the product. Another feature of the instrument is the capability to retrieve a resident dose–response slope, by dosing the two calibrators present in the cartridge. This implies that it is not necessary to calibrate every assay run by measuring a calibration curve along with unknown samples; instead two calibrators are tested to retrieve actual calibration. The design goal for the project was the development of 30 min, fully automated ACA assay for the detection of IgG and IgM ACA, offering semiquantitative results allowing the best possible match between sample categorization and antiphospholipid syndrome (APS) clinical classification, with a fully described correlation with the proposed standards and minimal cross-reactions with cardiolipin (CL)-related non-APS antibodies. To this end, the main directions of our project were the control over the solid-phase preparation, over the standardization (GPL/MPL “in-house” standards maximizing agreement with peers and correlation with Sapporo proposed standard) and the conduction of prospective and expert clinical studies.

MATERIALS AND METHODS

Preparation of the “Antigen”

The “antigen” consisted of a CL mixture (purchased from Sigma-Aldrich [Milan, Italy], code C1649) and of a preparation of purified β 2-glycoprotein I. The protein was purified from citrated human plasma. To ensure the recovery of the intact protein, we started from plasma pool units, which underwent

no more than one freeze-thaw cycle. The plasma pool was treated with 1.4% (v/v) HClO_4 followed by affinity chromatography on heparin-Sepharose, as reported previously.⁵

Preparation of the Solid Phase

Paramagnetic carboxylated-microparticles (purchased from Merck Chimie SAS, Estapor Microspheres [Fontenay Sous Bois, France], code EM1–100/40) are coated with the CL mixture, subsequently coupled with a β 2-glycoprotein I preparation, which has been cross-linked through N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide, purchased from Sigma, code E7750. After an overcoating process, based on PBS/ BSA buffer, the microbeads are stored at 4°C in MES buffer until use.

Scanning Electron Microscopy

Uncoated magnetic beads or CL-coated beads were left unblocked. We prepared a sample, in which the CL beads were incubated with a high-titer ACA-positive serum and further incubated with 12 nm colloidal gold-affiniPure Goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA). Beads were coated with a thin gold layer by sputter coater unit (Edwards, Irvine, CA) and the surface topography was analyzed with a Quanta 3D DualBeam™ Scanning Electron Microscope (FEI Company, Hillsboro, OR).

Assay Calibration and Standardization

Inhouse calibrators, that we labeled as LIAISON PL/mL (Diasorin, Salugia, Italy), were constructed by pooling and serially diluting three overranged positive plasma units. Doses to each dilution were assigned upon consensus of various commercial kits. Furthermore, following the recommendation of the Eleventh International Congress on Antiphospholipid Antibodies, we carried out studies to compare LIAISON GPL U/mL as well MPL U/mL concentrations with IgG and IgM Sapporo standard expressed as $\mu\text{g/mL}$ concentrations.⁶

Standards were treated as a specimen and serially diluted from 1:2 to 1:256. A linear relationship was found between the two slopes. This system was used, rather to have a conversion table, to convey about the performances of the assay components and to ensure reliability and reproducibility of the assay.

Sera

To calculate the diagnostic specificity, 120 blood donor serum samples were collected from voluntary blood donors in Tel-Hashomer Hospital, Tel Aviv, Israel. The diagnostic sensitivity was initially assessed through a retrospective

study: a serum panel has been prepared at a rheumatology center (Spedali Civili–Brescia, Servizio di Reumatologia ed Immunologia Clinica) made of 35 serum samples from patients affected by primary APS and of 35 serum samples from patients affected by secondary APS, in conjunction with systemic lupus erythematosus (SLE).

Furthermore, an additional prospective study was conducted by testing, in parallel with commercially available ACA ELISA method (QUANTA Lite INOVA Diagnostics, San Diego, CA), a population of 200 serum samples collected at the above rheumatology center.

RESULTS AND DISCUSSION

To date, there is not an absolute consensus about the nature of the “Cardiolipin antigen.” However, there is an increasing evidence that $\beta 2$ -glycoprotein I is the principal antigenic target for antiphospholipids in patients with the APS, while autoantibodies directed uniquely to CL are mainly associated with infection.^{7–9} It has been postulated that the interaction between $\beta 2$ -glycoprotein I and CL determines the appearance of a cryptic $\beta 2$ -glycoprotein I immunodominant epitope.¹⁰ Taken all this information together, we derived that one of the key factors for the development of a well-performing ACA assay, is the inclusion of the $\beta 2$ -glycoprotein I in the assay. Furthermore, the purified protein must maintain the phospholipid-binding property. As it came up from crystallographic studies, the protein structure revealed four complement control modules and a distinctly folded fifth C-terminal domain, which contains a large patch of 14 positively charged amino acidic residues on the half of domain and by a flexible loop of Serine 311 and Lysine 317.¹¹ This domain provides potential electrostatic interactions with the anionic phospholipids.

In order to dispose of a protein preparation possessing the defined structure, we selected among the various possible purification methods, the one used for the crystal preparation, based upon human plasma citrated treatment with HClO_4 followed by heparin affinity chromatography.⁵

Another concern was about the control of factors that might affect the proper structure of human-derived heparin affinity-purified $\beta 2$ -glycoprotein I. It was known there are two single nucleotide polymorphisms (SNPs) present in the $\beta 2$ -glycoprotein I gene, which disrupt the binding of the apolipoprotein H ($\beta 2$ -glycoprotein I) to the phospholipids: these naturally occurring mutations are located in the fifth domain and are respectively Cys306Gly and Trp316Ser.^{12,13}

Finally, a further factor that might hamper the correct functionality of the $\beta 2$ -glycoprotein I is the occurrence of proteolysis during the phases of the protein purification. Matsuura *et al.*¹⁴ demonstrated loss of antigenicity for the autoantibodies detected in ELISA caused by plasmin or factor X treatment. The cleavage provoked a $\beta 2$ -glycoprotein I nicked in the fifth at the site between K³¹⁷ and T³¹⁸, thereby determining a conformational change predicted by an

X-ray analysis, molecular modeling, and epitope mapping conducted with a monoclonal antibody.¹⁴

To circumvent these potential risks, we developed a procedure by which the protein purification starts from fresh human citrated plasma pools or which underwent no more than one freeze-thaw cycle.

Once a proper β 2-glycoprotein I purification process was established, we focused on the coating process. Two, not incompatible, hypotheses were taken as a guide for solid-phase preparation:

1. Initial microbeads coating with CL, to allow the formation of the cryptic epitopes of β 2-glycoprotein I upon its addition (Hyp.1-cryptic epitopes).¹⁵
2. Addition of cross-linked β 2-glycoprotein I, to allow its dimerization that has been shown to better bind the ACA (usually of low affinity) than the monomeric form (Hyp.2-clustering).¹⁶

To characterize the outcome of this coating procedure, we carried out scanning electron microscopy (SEM) analysis. We analyzed respectively plain beads, CL-coated magnetic beads, beads incubated with a high-titer ACA-positive serum and the same beads further incubated with protein A conjugated with colloidal gold.

FIGURE 1 shows the result of the SEM analysis carried out on immunogold-labeled CL-beads: densely packed protein clusters, disseminated throughout the whole bead surface can be easily seen. In our interpretation, this pattern should demonstrate the occurrence of a successful coating carried out with the first phospholipid layer, followed by the addition of the cross-linked β 2-glycoprotein I. Furthermore, we have found that for an optimal “antigen” coating, the use of cross-linked β 2-glycoprotein I is essential; moreover, we noted that the coating functionality of a native β 2-glycoprotein I is dramatically reduced (data not shown). This finding is in line with a previous report of Sheng *et al.*, which showed that a dimer construct of β 2-glycoprotein I is bound more avidly than native β 2-glycoprotein I by polyclonal human anti- β 2-glycoprotein I antibodies.¹⁷

Once the method to prepare an optimal solid-phase had been established, we focused on the standardization aspects. To date, two international standard calibrators have been introduced to generate calibration curves from which levels of unknown samples can be derived: (1) the Harris standards (GPL/mL, MPL/mL, APL/mL), prepared by mixing varying quantities of high positive with normal sera; and (2) the Sapporo HCAL/EY2C9 Standards, derived from chimeric monoclonal antibodies.^{18,19}

However, both types of calibrators present disadvantages that render the determination of a precise and reproducible anti-CL level difficult. For example, taking the Harris calibrator (named as LAPL-G-200) and dosing it with various commercial assays we found a low agreement.

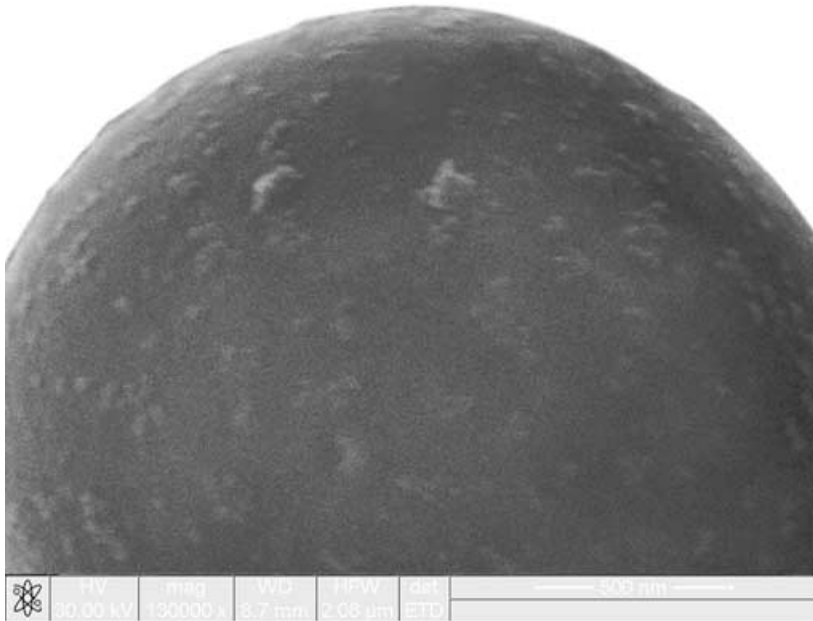


FIGURE 1. SEM analysis of immunogold-labeled CL-beads incubated with high titer ACA sera. The beads were coated with a thin gold layer by sputter coater unit (Edwards) and the surface topography was analyzed with a Quanta 3D DualBeam Scanning Electron Microscope (FEI company). Densely packed protein clusters, disseminated throughout the whole bead surface can be visualized.

This finding prompted us to develop our “in-house” standard that was made through the selection of an high-titer specimen (according to various manufacturers), preparation of seven specimen, diluted with ACA negative samples, dose assignments with consensus doses (GPL/mL), and construction of a dose–response curve.

Following the recommendation of Harris and Pierangeli, the utilization of the dose–response curve was intended for a semiquantitative measure (low, medium, high) rather than a quantitative measure.²⁰ This may suffice in most clinical settings and would be less subject to error. Furthermore, we have standardized the LIAISON GPL/mL units versus the HCAL: a good parallelism between the two slopes has been found; the threshold at 20 GPL/mL showed to be equivalent to about 50 ng/mL of HCAL. However, we intend to use the latter information for internal quality monitoring procedures rather than proposing it as a basis for a proposed correlation factor.

We then conducted the clinical studies to calculate the specificity and the sensitivity of the assays. Specificity was calculated on 120 blood donors collected at the Tel-Hashomer Hospital, Tel Aviv, Israel. The LIAISON ACA IgG

TABLE 1. Retrospective study

	LIAISON Cardiolipin				ACA ELISA			
	IgG	IgM	IgG + IgM		IgG	IgM	IgG + IgM	
	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	95%C.I.	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	95%C.I.
Negative	4/70 (5.7%)	47/70 (67.4%)	2/70 (2.9%)	0.3–10.0	8/70 (11.4%)	33/70 (47.1%)	4/70 (5.7%)	1.6–14.0
Equivocal	–	–2/70 (2.9%)	–	–	3/70 (4.3%)	12/70 (17.1%)	1/70 (1.4%) (1.4%)	0.0–7.7
Positive	66/70 (94.3%)	21/70 (30.0%)	68/70 (97.1%)	90.0–99.6	55/70 (84.3%)	25/70 (35.7%)	65/70 (92.9)	84.1–97.6

assay showed a 99th percentile at 10.4 GPL U/mL with the upper result at 16.6 GPL U/mL. The LIAISON ACA IgM assay showed a 99th percentile at 12.8 MPL U/mL. One specimen, positive with other three CE-marked commercial immunoassays, had a concentration of 42.4 MPL U/mL.

To assess the diagnostic sensitivity we conducted two studies, retrospective the former, prospective the latter. In the retrospective one (shown in TABLE 1), we tested 35 specimens obtained from patients affected by primary APS as well as 35 specimens obtained from patients affected by secondary APS in conjunction with SLE. The specimens were tested with LIAISON Cardiolipin IgG and IgM in parallel with a commercially available ACA IgG and IgM ELISA methods.

Four negative and 66 positive results were observed in the population affected by APS by using LIAISON Cardiolipin IgG assay. In contrast, 8 negative results, 3 equivocal results, and 59 positive results were observed in the same population using ACA IgG ELISA method. Diagnostic sensitivity was 94.3% (95% confidence interval: 86.0–98.4%).

Forty-seven negative results, 2 equivocal results, and 21 positive results were observed by using LIAISON Cardiolipin IgM assay. In contrast, 33 negative results, 12 equivocal results, and 25 positive results were observed by using ACA IgM ELISA method. Diagnostic sensitivity was 30.0% (95% confidence interval: 19.6–42.1%).

In the prospective study we tested a population made of 200 specimens collected randomly in a rheumatology reference center. Testing was made with the LIAISON ACA IgG assay in parallel with a commercially available ACA IgG ELISA method as well as with the LIAISON ACA IgM together with an ACA IgM ELISA. The discrepant samples were tested with two additional commercially available assays.

After retesting, the results of the study, fully illustrated in TABLE 2, showed that 4 samples out of 200 were unresolved and that the LIAISON ACA IgG assay detected 176 versus 177 negative samples by ACA IgG ELISA

TABLE 2. Prospective study*

ACA IgG ELISA after resolution of discrepancies						ACA IgM ELISA after resolution of discrepancies					
		–	±	+	Tot			–	±	+	Tot
LSN CL IgG	–	176	3	4	183	LSN CL IgM	–	174	8	7	189
	+	1	1	15	17		±	0	0	0	0
	Tot	177	4	19	200		+	0	1	10	11
							Tot	174	9	17	200

*IgG study: 4 samples remained unresolved after resolution of discrepant results, and therefore were not included in the data analysis.

IgM study: 9 samples remained unresolved after resolution of discrepant results, and therefore were not included in the data analysis.

LSN CL = LIAISON Cardiolipin.

(99.4% agreement), 15 LIAISON ACA IgG positive samples versus 19 ACA IgG ELISA positive (78.9% agreement), and the overall agreement between LIAISON and ELISA IgG was 97.4%.

Further investigation of the four samples that resulted LIAISON ACA IgG negative compared to ACA IgG ELISA is ongoing; however, it must be pointed out that these four samples were resulting low positive with all the other assays.

As for the IgM testing, 9 out of 200 samples were unresolved; the same number of negative was found between the 2 assays (174/174, 100% agreement), and the LIAISON ACA IgM detected 10 positive samples out of 17 scored positive by ACA IgM ELISA (58.9% agreement); the overall agreement was 96.1%.

Also in this case, a further investigation on the seven samples that were resulting negative by LIAISON IgM is ongoing. Furthermore, it should be emphasized that 6 out of 7 negative samples were scoring low positive with the ELISA assays, while the remaining sample was obtained from a SLE patients, with no clinical sign of APS; this observation suggested that the LIAISON ACA assays are highly specific, without compromising the assay sensitivity. Obviously, this statement will require additional clinical data to be confirmed.

In conclusion, we showed that the immunochemical performance of the LIAISON Cardiolipin assays is at least comparable to the well-established ELISA products. To our knowledge, these are the first ACA immunoassays that can be performed on a fully automatized immunoassay analyzer.

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