



Minimal requirements for antiphospholipid antibodies ELISAs proposed by the European Forum on antiphospholipid antibodies

Angela Tincani^{a,*}, Flavio Allegri^a, Genesio Balestrieri^a, Guido Reber^b, Marielle Sanmarco^c, Pierluigi Meroni^d, Marie-Claire Boffa^e

^aRheumatology and Clinical Immunology, Ospedale Civile di Brescia, Piazza Spedali Civili 1, 25125 Brescia, Italy

^bGeneva University Hospital, Switzerland

^cHopital de la Conception, Marseille, France

^dIstituto Auxologico, Università di Milano, Italy

^eDepartment Int. Med., Hopital de la Pitié, Paris, France

Received 2 June 2004; received in revised form 20 June 2004; accepted 23 June 2004
Available online 26 August 2004

KEYWORDS

Antiphospholipid antibodies immunoassays; Anticardiolipin; Anti-beta2 glycoprotein I; Standardization; Cut-off; Antiphospholipid Antibodies Syndrome

Abstract Antiphospholipid ELISAs are part of the Antiphospholipid Antibodies Syndrome classification criteria, having the same diagnostic value as lupus anticoagulant. However, sometimes their results appear scarcely meaningful especially when wide metanalyses studies are performed, probably because of their well-known inter-laboratory variability. The application of a common protocol was shown to improve the test reproducibility, but this observation did not have any influence on the routine performances. After discussion among experts at the European level, we identified four conditions named “minimal requirements” considered useful to decrease the inter-laboratory variability:

- (1) to run the samples in duplicate;
- (2) to determine the cut off level in each laboratory analysing at least 50 samples from normal subjects, possibly age- and sex-matched with the patient population usually attending the Centre;
- (3) to calculate the cut-off level in percentiles;
- (4) to use stable external controls in the test.

Abbreviations: aPL, antiphospholipid; aCL, anticardiolipin; Anti- β 2GPI, anti-beta2 glycoprotein I; APS, antiphospholipid antibody syndrome; SD, standard deviation; OD, optical density; IU, international units; MoC, monoclonal concentration.

* Corresponding author. Tel.: +39 340 5551971; fax: +39 030 3995085.

E-mail address: tincani@bresciaumatologia.it (A. Tincani).

A collaborative study involving 36 European centres proved that the use of monoclonal anti-beta2 glycoprotein I antibodies, HCAL (IgG) and EY2C9 (IgM) as standards, can help to reduce the inter-laboratory coefficient of variation both in anticardiolipin (aCL) and anti- β 2GPI (anti-beta2 glycoprotein I) ELISA. Therefore, we propose HCAL and EY2C9 as external controls, but other monoclonal or polyclonal preparations may be considered. During an interactive workshop held last May in Italy, 16 companies producing these tests agreed to consider the introduction of the "requirements" in their products. We suggest to adopt these "requirements" particularly in clinical studies, in order to compare more easily the literature data.

© 2004 Elsevier Ltd. All rights reserved.

Introduction

During the last 20 years, the wide application of antiphospholipid (aPL) ELISAs and in particular of anticardiolipin (aCL) antibody assays was determinant in the definition of the antiphospholipid antibody syndrome (APS) and of its pathogenic mechanisms, mainly consisting of antibody-mediated thrombosis. Consequently, aCL ELISA was introduced as one of the laboratory classification criteria of the syndrome [1].

Despite its recognized practical importance, and several standardization workshops [2,3], aCL immunoassay, 20 years after the publication of the first paper, still shows a high inter-laboratory variability [4–6]. The reasons for this poor performance can be linked to the difference among the protocols applied by either different companies producing commercial kits or workers performing homemade assays [4,5].

A limited number of studies focused on anti-beta2 glycoprotein I (anti- β 2GPI) ELISA, but a significant variability was shown also in the results of this test [7], that still lacks accepted rules in the reporting of results.

Consequently, it is not surprising that large meta-analyses recently performed were not able to give a clear clinical significance to aCL and anti- β 2GPI immunoassays, underlying the need for standardization or harmonization of the methods [8,9].

After 3 years of common work within the European APL Forum, we showed that the use of a common protocol and of common standard preparations can improve the reliability of aCL assay; however, this effort did not have any practical consequences, because no improvement was achieved in the routine performance of these tests.

Identification of European APL forum minimal requirements

In preparation of the IV European Forum Meeting (London, 16–17 January), the Standardization

Committee members actively collaborated, by mail exchanges, to identify methodological improvements that could be applied to aPL ELISAs on a large scale. The conclusion of this discussion was presented at the Forum Meeting and approved by the delegates, underlining their concern particularly for the publication of clinical studies.

Taking into account the studies performed during the previous years, the committee was aware that radical changes such as buffers, samples dilutions, kind of antigen, etc., although probably advisable in some cases, could not be pursued. In fact, now a number of laboratories performed their in-house test and also a number of companies produce their own kits with their own methods, already approved by FDA and EEC authorities, that cannot be changed without entering another process review. The committee concentrated the discussion on changes that were thought important to improve the standardization of the tests but that could easily be accepted by all workers in the field without major problems.

The committee agreed to suggest the following minimal requirements.

(1) To run the samples in duplicate. To cover the cost of the kits, which is in some occasions rather high compared to the cost of the materials enclosed in the package, some companies advise their customers to run the samples singly. This advice is also included in a recent report of consensus guidelines on aCL antibody testing and reporting [10]. After careful consideration of these positions, the committee still decided to ask to run the samples in duplicate because of the unpredictable high inter-assay coefficient of variation sometimes observed.

(2) To determine the cut off level in each laboratory. Since APS was first described in connective tissue diseases and women suffering recurrent miscarriages, the current data (specificities of the test) refer to the study of a young adult normal population. In fact, several companies base their cut-off level on the analysis of a

wide blood donors population. It is now clear that, in some age groups, aPL immunoassays can have a different profile. An example is that of children with low levels of aCL and, in particular, of anti- β 2GPI antibodies that can be linked to transient infections [11]. Also in the healthy elderly population, the detection of low positive is not rare and may not necessarily underline an associated APS [12]. The committee therefore proposed that each centre should be advised to study their own normal population examining 50 (minimum) or 100 (optimum) subjects age- and sex-matched with the majority of the patients attending.

(3) To calculate the cut off in percentiles. Despite the number of reports stating that the results of aPL ELISA do not have a normal distribution [13], still some laboratories as well as some Companies calculate the results according to different methods (mean +2 or 3 or 5 S.D., double of the mean optical density (OD) of the normal samples, etc.) [5]. Therefore, it was considered important to underline that the use of percentiles is mandatory both for aCL and for anti- β 2GPI immunoassays. The centres should specify if they use 95°, 98° or 99° percentiles.

(4) To use stable external controls for the test. Our previous work confirmed that the results of aCL immunoassays are always given in IU (GPL/MPL). Consequently, a common set of International Calibrator or Standards (or Secondary Standards derived from them) were always included to allow calculation in the Units. However, a high variability of the results was observed. Therefore, we distributed two anti- β 2GPI monoclonal preparations (HCAL, IgG and EY2C9, IgM, kind gifts of Takao Koike [14,15] to the Forum) to be used as calibrators and we observed a decrease of the inter-laboratory standard errors in the aCL assay [5]. Because of this experience and because of the lack of recognized international calibrators in the anti- β 2GPI assay, we suggest at least to include reproducible external controls in the runs at different concentrations: one under the cut-off and one in the range of medium positive. Several polyclonal and monoclonal aPL preparations may be helpful in this respect: for example, the College of American Pathologists produced and distributed some "quality control survey samples" more or less with this intent. However, since we had the opportunity to use HCAL and EY2C9 [5] and to verify their performance both in aCL and anti- β 2GPI assay (see next paragraph) we suggest to use them as external control at two dilutions to be fixed.

HCAL and EY2C9 use as calibrators

To validate the efficacy of HCAL and EY2C9, suggested by our previous study [5], we undertook a collaborative work on European scale involving 36 laboratories. All the participants received five serum samples and were asked to study them by their routine aCL and anti- β 2GPI assay. The samples were previously selected and analysed in three laboratories (Marseille, Geneva and Brescia) involved in the European Forum Standardization Committee. One sample was from a normal subject and four from APS patients with aCL and anti- β 2GPI in the range of medium positive (IgG and/or IgM). In addition HCAL and EY2C9 were also distributed and participants were invited to test them in both tests by performing a dilution curve starting at a concentration of 100 ng/ml for HCAL (IgG) and at 1200 ng/ml for EY2C9 (IgM). The results of the two curves and of the serum samples were requested not only in international units (IU) or arbitrary units but also in OD. This allowed us to read the sample values also in monoclonal concentration (MoC, ng/ml), on the curve obtained with HCAL and EY2C9. The coefficient of variation among the aCL assay results was calculated using data expressed in OD, IU and MoC (Table 1a and b). Anti- β 2GPI coefficients of variation were calculated in OD and MoC only for IgG isotype, because in 1/3 of the laboratories the EY2C9 curve starting at 1200 ng/ml resulted in too high OD (Table 2). These data, showing a significant decrease in the coefficients of variation in the titre of some sample, suggest that the introduction of HCAL and EY2C9 may be of some help to reduce the inter laboratory variations. Therefore we propose the introduction of HCAL and EY2C9 (or other accepted polyclonal or monoclonal reference preparations) as external controls that can be made two to three times a year in routine determinations of aCL and anti- β 2GPI assays.

Interactive Forum-companies workshop

During the IVAPL European Forum Meeting (London, 16–17 January 2004), the minimal requirements were presented to the few companies producing kits for the two assays that were present there. Acting on a proposition from Marie-Claire Boffa, we decided to share our ideas with all Companies producing aCL and anti- β 2GPI immunoassays, that we could reach. With this intent, the Standardization Committee invited 17 companies to an interactive workshop, that was held on the 3rd of May 2004 and organized by Pierluigi Meroni in Cusano

Table 1 Coefficients of variation of results of IgG (a) and IgM (b) aCL reported in optical density (OD), international units (IU) and monoclonal concentration (MoC)

Samples	IgG aCL coefficients of variation %			Squared rank test
	Results in OD	Results in IU	Results in MoC	
<i>(a)</i>				
1	27	24	25	ns
2	32	34	36	ns
3	23	25	28	ns
4	54	65	15	<i>P</i> <0.001
5	35	48	14	<i>P</i> <0.001
<i>(b)</i>				
1	83	118	17	<i>P</i> <0.001
2	35	36	40	ns
3	62	64	34	<i>P</i> <0.007
4	64	68	22	<i>P</i> <0.001
5	46	41	56	ns

Milanino (Milan, Italy) at the Biomedical Research Centre, Istituto Auxologico Italiano.

Sixteen companies attended the workshop (Table 3) and were represented, as requested, by collaborators directly involved in the production rather than in the kit distribution. Most of these companies participated in the previous standardization.

For this workshop, the Standardization Committee presented “proposals” derived from the “minimal requirements”.

To calculate the cut-off in percentiles was the first proposal. Some companies printed in their instructions a differently derived cut-off and this method has been already approved by authorities responsible for the license. These problems were discussed coming to the conclusion that the percentile derived cut-off value could be added to the previous one. Customers will have both data available and in any case will be advised to calculate their in house cut-off, running their own normal samples.

The possibility of using a semiquantitative (high, medium, low, negative) result expression was also discussed. Several companies asked to

have samples measured by the Standardization Committee and classified in the different ranges. It was decided to collect samples, to analyse them at least in three laboratories in order to reach a consensus in their classification. Hopefully by the end of July, the Standardization Committee will be able to provide a set of 5–20 classified samples to the companies.

Finally, an external reference was proposed. According to our experience, we suggested the use of HCAL and EY2C9. Some companies already had experience with them and reported a satisfactory performance. However, the discussion underlined the possibility to use other monoclonal antibodies, apparently available to some companies.

The delegates agreed that the reference preparations could be validated by a common work of companies and experts in the field.

Lastly, the companies raised the problem of HCAL and EY2C9 source. According to our recent

Table 2 Coefficients of variation of results of IgG (a) and IgM (b) anti-β2GPI reported in optical density (OD) and monoclonal concentration (MoC)

Samples	IgG anti-β2GPI coefficients of variation		Squared rank test
	Results in OD	Results in MoC	
1	25	30	ns
2	34	28	ns
3	28	33	ns
4	47	6	<i>P</i> <0.001
5	61	24	<i>P</i> <0.001

Table 3 List of the companies participating in the first Forum-Companies Interactive Workshop

- AESKU.LAB DIAGNOSTICA
- BIO-RAD
- BMD
- CORGENIX
- DIASORIN
- EURODIAGNOSTICA
- EUROHOSPITAL
- HYPHEN BIOMED
- IMTEC IMMUNODIAGNOSTICA
- INOVA DIAGNOSTICS
- MENARINI
- ORGENTEC DIAGNOSTICA
- PHARMACIA DIAGNOSTICS
- STAGO
- THE BINDING SITE
- THERA TEST

contacts, these monoclonals have several possible distribution modalities: from Prof. Koike himself, at least in selected circumstances; from CDC (Atlanta), that will soon be ready to distribute them as reference preparations; from INOVA (San Diego), that is already selling them.

Conclusions

In the area of aPL ELISAs standardization, the Standardization Committee of the APL European Forum is focusing on a realistic possible improvement in the assays performance. Our efforts are concentrating on two distinct aspects, both critical in the general improvement of the knowledge in the area and in the patients' classification. The first one underlines the "minimal requirements" of aCL and anti- β 2GPI immunoassays necessary to get comparable results in clinical studies. It appears important for homemade assays to adhere to these proposed rules because of the larger inter-laboratory variations between home-made than between kits, sometimes reported [6].

The second aspect is devoted to the companies producing aCL and anti- β 2GPI kits. In fact, the committee is aware that many laboratories involved in the APS diagnosis are now using commercial kits. By interactive discussion, the committee and the companies identified together some possible changes in the commercial methods that could really improve their diagnostic relevance. Hopefully, such innovative discussion between experts and companies will be further enlarged during the International Symposium, to reach a general consensus and provide concrete answers to the need of reliable and comparable results of aCL and anti- β 2GPI immunoassays.

Acknowledgments

The authors want to thank Takao Koike for generously providing HCAL and EY2C9 to the Forum; all the companies attending the Interactive Workshop, for their prompt answers and their interest; Franco Franceschini, Robert Roubey and Yehuda Shoenfeld for their helpful discussion.

In addition, we want to acknowledge the Chairmen of the International Institutions devoted to the Autoantibody Tests Standardization: Marvin Fritzler (AF/IUIS/CDC/WHO Committee for Standardization of the Autoantibody Diagnostic Tests

for the Rheumatic Diseases); Flip G de Groot (ISTH Scientific Standardisation Subcommittee Lupus Anticoagulants/Phospholipid dependent antibodies); Allan Wiik and Ruud Smeenk (Standardization Committee of the European Workshop of Rheumatology Research). All these institutions supported our work with their interest and their advice.

References

- [1] Wilson WA, Gharavi AE, Koike T, Lockshin D, Branch DW, Piette J-C, et al. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome. *Arthritis Rheum* 1999;42:1309–11.
- [2] Harris EN, Gharavi AE, Patel SP, Hughes GRV. Evaluation of the anti-cardiolipin antibody test: report of an international workshop held April 4 1986. *Clin Exp Immunol* 1987;68:215–22.
- [3] Pierangeli SS, Stewart M, Silva LK, Harris EN. Report of an anticardiolipin wet workshop during the VIIth International Symposium on antiphospholipid antibodies. *J Rheumatol* 1998;25:156–62.
- [4] Reber G, Arvieux J, Comby E, Degenne D, de Moerloose P, Sanmarco M, et al. Multicenter evaluation of nine commercial kits for the quantitation of anticardiolipin antibodies. *Thromb Haemost* 1995;73:444–52.
- [5] Tincani A, Allegri F, Sanmarco M, Cinquini M, Taglietti G, Balestrieri G, et al. Anticardiolipin antibody assay: a methodological analysis for a better consensus in routine determinations. A cooperative project of the European Antiphospholipid Forum. *Thromb Haemost* 2001;86:575–83.
- [6] Harris EN, Pierangeli SS. Revisiting the anticardiolipin test and its standardization. *Lupus* 2002;11:269–75.
- [7] Reber G, Schousboe I, Tincani A, Sanmarco M, Kveder T, de Moerloose P, et al. Inter-laboratory variability of anti- β 2-glycoprotein I measurement. A collaborative study in the frame of the European Forum on antiphospholipid antibodies standardization group. *Thromb Haemost* 2002;88:66–73.
- [8] Galli M, Luciani D, Bertolini G, Barbui T. Lupus anticoagulants are stronger risk factor of thrombosis than anticardiolipin antibodies in the antiphospholipid syndrome: a systematic review of the literature. *Blood* 2003;101:1827–32.
- [9] Galli M, Luciani D, Bertolini G, Barbui T. Anti- β 2-glycoprotein I, antiprothrombin antibodies, and the risk of thrombosis in the antiphospholipid syndrome. *Blood* 2003;102:2717–23.
- [10] Wong RCW, Gillis D, Adelstein S, Baumgart K, Favaloro MJ, Hendle MJ, et al. Consensus guidelines on anticardiolipin antibody testing and reporting. *Pathology* 2004;36:63–8.
- [11] Avcin T, Cimaz R, Meroni PL. Recent advances in antiphospholipid antibodies and antiphospholipid syndrome in pediatric populations. *Lupus* 2002;11:4–10.
- [12] Piette JC, Cacoub MD. Antiphospholipid syndrome in the elderly: caution. *Circulation* 1998;97:2195–6.
- [13] Shi W, Krilis SA, Chong BH, Gordon S, Chesterman CN. Prevalence of lupus anticoagulant and anticardiolipin antibodies in a healthy population. *Austr NZ Med* 1990;20:231–6.

- [14] Ichikawa K, Tsutsumi A, Atsumi T, Matsuura E, Kobayashi S, Hughes GRV, et al. A chimeric antibody with the human γ 1 constant region as a putative standard for assays to detect IgG β -2-glycoprotein I-dependent anticardiolipin and anti- β 2-glycoprotein I antibodies. *Arthritis Rheum* 1999;42: 2461–70.
- [15] Ichikawa K, Khamashta M, Koike T, Matsuura E, Huges GRV. β -2-Glycoprotein I reactivity of monoclonal anticardiolipin antibodies from patients with antiphospholipid syndrome. *Arthritis Rheum* 1994;37:1453–61.