Antibodies Against β2-Glycoprotein I in Anti-Cardiolipin Calibrators, Tie Quan Wei, Edward W. Bernes, Jr., and Mark A. Jandreski* (Dept. of Pathol., Rm. 0095, Loyola Univ. Med. Center, 2160 S. First Ave., Maywood, IL 60153; *author for correspondence: fax 708-216-4146, e-mail mjandre@luc.edu)

Measurement of anti-cardiolipin antibodies (aCLs) is part of the laboratory assessment for the anti-phospholipid antibody syndrome [1]. However, current aCLs assays have significant intermethod variation. Participant summary results from the College of American Pathologists Immunology Surveys have shown that CVs for quantitative aCL assays are large, ranging from 9.3% to 36.2% in the 1994 survey and from 18.7% to 31.4% in 1995 [2, 3]. In a separate study of five university-based and five commercial clinical laboratories in which the same 20 patients’ specimens were sent to each laboratory for qualitative aCL analysis, the number of specimens found to be positive for any isotype (IgG, IgA, or IgM) varied considerably among laboratories, with a range of 5 to 13 positive results. These 10 laboratories agreed (i.e., at least one isotype was positive or all were negative) for only 5 of the 20 specimens [4]. Considerable quantitative variation was also seen during an aCL wet workshop [5]. To elucidate the source of the variation, we looked for possible interference within commonly used aCL calibrators termed the “Harris Standards.”

The majority of aCL assays are based on ELISA methodology, involving the immobilization of cardiolipin molecules onto microtiter wells and the blocking of unoccupied binding sites with heat-inactivated fetal or newborn calf serum. When a serum sample is added to the wells, aCLs bind to the immobilized cardiolipin molecules. After the wells are washed, a second antibody (specific for human IgG, IgM, or IgA) conjugated with an enzyme is added, and detection is by spectrophotometry after development of a chromogenic substrate.

One endogenous factor in human and bovine serum important for this assay is β2-glycoprotein I (β2 GPI, or apolipoprotein H), a plasma protein with anticoagulant activity. Several laboratories have found that β2 GPI is required for aCLs to bind to cardiolipin [6–9]. β2 GPI may be important either by providing an epitope for aCLs when complexed with cardiolipin molecules [9, 10] or by enhancing the binding of cardiolipin molecules to the antibodies in some other fashion [11]. Thus, the real antigen may not be cardiolipin per se, but rather a cardiolipin–β2 GPI complex. Immobilization of β2 GPI molecules onto microtiter wells may also cause an assay to detect not only aCLs but also antibodies specific for β2 GPI. Fortunately, β2 GPI antibodies are not a common finding in aCL-positive sera [12]. However, serum samples with antibodies against β2 GPI could give false-positive results in an aCL assay. In experiments involving purified β2 GPI, we have found results that suggest that the current well-accepted aCL calibrators distributed by Louisville APL Diagnostics, Louisville, KY, the “Harris Standards,” may contain anti-β2 GPI antibodies or antibodies that specifically cross-react with β2 GPI.

Various amounts of human plasma β2 GPI (>99% pure, cat. no. 362225; Calbiochem–Novabiochem, San Diego, CA) were used to coat microtiter wells (Immulon-2 flat-bottom microelisa strips, cat. no. 011-010-6202; Dynatech, Chantilly, VA), which were subsequently blocked for 30 min with 110 μL of 100 mL/L newborn calf serum (GenBio, San Diego, CA) made up in 10 mmol/L phosphate-buffered saline, pH 7.2. These plates were used in the GenBio IgG and IgM aCL assays (cat. nos. B1029–984 and B1029-987, respectively) in place of the GenBio cardiolipin-coated plates included in the assay kits. aCL Standard 2.2 (Louisville APL Diagnostics), a positive calibrator (GPL = 60.3; MPL = 47.9)[1] was measured with the β2 GPI-coated plates in the GenBio aCL assay. When a fixed amount of aCL Standard 2.2 was used in wells coated with increasing amounts of β2 GPI, a β2 GPI dose–response curve for both anti-β2 GPI IgG and IgM was found, as shown in Fig. 1A. No response was found for plates coated with increasing amounts of bovine serum albumin (BSA, Fraction V, cat. no. A3912; Sigma Chemical Co., St. Louis, MO) and blocked with 100 mL/L newborn calf serum. The results indicate either that this calibrator contains anti-β2 GPI antibodies or that the aCLs in this calibrator can specifically cross-react with β2 GPI.

1 One GPL unit is defined as the cardiolipin binding activity of 1 μg/mL of an affinity-purified IgG aCL preparation from calibrator serum. One MPL unit is defined as the cardiolipin binding activity of 1 μg/mL of an affinity-purified IgM aCL preparation from calibrator serum.

Fig. 1. Detection of antibodies against β2 GPI.

Each data point is the average of experimental or control results from wells that were coated and tested in duplicate. (A) Wells in a blank microtiter plate were coated with different amounts of purified β2 GPI, blocked with 100 mL/L heat-inactivated newborn calf serum, and used to measure anti-β2 GPI IgM (●) and IgG (○) in aCL Standard 2.2. As controls, wells in a separate blank plate were coated with different amounts of BSA, blocked, and used to measure anti-BSA IgM (●) and IgG (○) in aCL Standard 2.2. (B) Wells in a GenBio aCL ELISA plate were coated with different amounts of purified β2 GPI and used to measure aCL IgM (●) and IgG (○) in aCL Standard 2.2. As controls, wells in a GenBio aCL ELISA plate were coated with different amounts of BSA and used to measure Standard 2.2 for aCL IgM (●) and IgG (○).
Figure 1B shows that different concentrations of β2GPI used to coat GenBio aCL microtiter plates changed the value of the Standard 2.2 calibrator for both IgG and IgM. The values increased with increasing amounts of β2GPI added to the wells. These data suggest that calibrator results could be affected by the number of functional β2GPI molecules used to supplement aCL assays.

In vitro β2GPI concentrations of 10 to 20 mg/L are needed to supplement aCL assays for effective aCL binding [7]. The concentration of β2GPI in human and bovine serum is similar [13] and normally ranges from 150 to 300 mg/L in humans [13–15]. Patients who are β2GPI deficient can have β2GPI concentrations as low as 9 mg/L [14]. Blocking plates with heat-inactivated calf serum is a common procedure to reduce the background of aCL assays and to supplement the assays with β2GPI for effective aCL binding. However, quality control can be very difficult with heat inactivation, which could result in variations in the number of β2GPI molecules in the calf serum capable of binding to anti-β2GPI antibodies. It is also not unreasonable to assume that β2GPI deficiencies similar to those seen in humans can occur in cattle. Because different manufacturers or clinical laboratories may use calf serum from different sources and may use different conditions to heat-inactivate calf serum, the number of functional β2GPI molecules bound onto aCL microtiter wells may vary among methods. This, coupled with calibrators that can contain antibodies to β2GPI or antibodies that can cross-react with β2GPI, may cause some of the intermethod variation seen in aCL assays.

On the basis of the above analysis, we suggest that (a) monoclonal calibrator materials free of anti-β2GPI antibodies and β2GPI-specific cross-reactivity may need to be developed for aCL assays and (b) aCL ELISAs should be supplemented with a standardized universally accepted amount of functional purified β2GPI to help minimize intermethod variation.

References


DNA Enzyme Immunoassay for Improved Molecular Detection of Deletions in Spinal Muscular Atrophies, Giuseppe Novelli,1–4,* Francesca Capon, 1 Chiara Novelli, 1 Anna Cavicchi, 2 and Bruno Dallapiccola1–5 (1) Cattedra di Genetica Umana, Edificio E Nord, Univ. Tor Vergata di Roma, Via di Tor Vergata 135, 00135 Roma, Italy; 2 Sorin Biomedica, Saluggia, Italy; and 3 Ospedale C.S.S., S. Giovanni Rotondo, Italy; *address correspondence to this author, at the Cattedra de Genetica Umana: fax +39-6-20427313, e-mail novelli@vtx1.ccd.utovrm.it

Childhood spinal muscular atrophies (SMAs I, II, III: MIM* 253300, 253400, and 253550, respectively) are a group of autosomal recessive disorders characterized by spinal cord anterior horn cell degeneration [1]. SMA is a relatively common disease, affecting ~1 in 6000 births with an estimated carrier frequency of 1 in 80 individuals [1]. The SMA mutation has recently been found to be a large genomic deletion involving the survival motor neuron (SMN) gene in 5q13 [2]. SMN lies in a 500-kb region that is duplicated and inverted in normal chromosomes, so that it encompasses a telomeric (SMNt) and a highly homologous centromeric gene copy (SMNc); the two copies differ only in five nucleotide positions. SMNt is lacking or truncated in 97% of SMN chromosomes, whereas SMNc deletions (observed in 5% of normal controls) do not seem to have any obvious phenotypic effect [2,3].

The strategies developed to screen SMA chromosomes for the SMNt gene deletion include single-strand conformation polymorphism (SSCP) [2], restriction enzyme analysis (REA) [4], and restriction-site-generating polymerase chain reaction (PCR) [4]. However, these methods are relatively expensive, time consuming, and difficult to use for routine screening. We have developed a microtiter plate-based method, which uses DNA enzyme immunoassay (DELA) to detect SMNt gene deletions.

After extracting genomic DNA from peripheral blood lymphocytes of 100 SMA patients diagnosed according to published consensus criteria [5], we tested the DNA for SMN gene deletion by SSCP and REA. Positive samples were further analyzed as follows. We amplified 200 ng of template by PCR, using primers R111 and S41770 according to Lefebvre et al. [2], then added ~15 μL of crude denatured PCR product to microtiter plates ("ETI-IEIA"; Sorin Biomedica) that had previously been coated with 20 ng/well of biotinylated SMN-specific oligonucleotide probes (CEN SMN: 5′-GTT TTT AGA CAA AAT CAA-3′ and TEL SMN: 5′-GTT TTC AGA CAA AAT CAA-3′) as described [6]. Plates were incubated at 46 °C for 1 h in 100 μL of hybridization buffer: 1× SSC (per liter, 8.765 g of NaCl and 4.41 g of sodium citrate), 2× Denhardt’s solution (per liter, 400 mg of Ficoll, 400 mg of polyvinylpyrrolidone, and 400 mg of bovine serum albumin), 10 mmol/L Tris-HCl (pH 7.5), and 1 mmol/L EDTA. Hybrids between SMN-specific probes and SMN gene sequences were