

The Evolution of aPL Assay Development (continued from page 3)

A patient is considered to have APS if at least one clinical and one laboratory criterion is met. There has been disagreement over whether the criteria are intended for use in diagnosing patients for treatment, or to identify patients in order to study the syndrome further. The Sapporo Criteria have been discussed at many scientific meetings, and debate continues on the addition of other clinical and laboratory criteria, in particular, the addition of testing specifically for anti-B2GPI and anti-prothrombin antibodies, the importance of IgA isotypes and the significance of low levels of anti-B2GPI antibodies. This will be a topic for discussion during the SSC committee meeting at the International Society on Thrombosis and Haemostasis Congress in Sydney, Australia (August 2005).

Part II of "Anti-Phospholipid Testing 101" will continue in a subsequent issue of THE READER with a review of standardization and performance issues associated with different available methods.

References;

1. Moore JE. Biologically False Positive Serologic Tests for Syphilis. JAMA. 1952;150:467-473.
2. Harris EN. Anticardiolipin Antibodies: Detection by Radioimmunoassay and Association with Thrombosis in Systemic Lupus Erythematosus. Lancet. 1983; 26: 1211-1214.
3. Harris EN. Evaluation of the Anti-cardiolipin Antibody Test: Report of an International Workshop Held 4 April 1986. Clin Exp Immunol. 1987; 68:215-222.
4. Lopez LR. Anti-B2-Glycoprotein I and Antiphosphatidylserine Antibodies are Predictors of Arterial Thrombosis in Patients With Antiphospholipid Syndrome. Am J Clin Pathol. 2004;121:142-149.
5. Wilson WA. International Consensus Statement on Preliminary Classification Criteria for Definite Antiphospholipid Syndrome. Arth Rheum. 1999; 42:1309-1311.
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UPCOMING CONFERENCES

• The Mayo Clinic and the North American Specialized Coagulation Laboratory Association (NASCOLA) are jointly sponsoring a new conference, "Coagulation Testing Quality: Lessons and Issues from Quality Assessment, Standardization and Improvement Programs & Studies," June 15-17, 2005 in Rochester, MN. **Corgenix** invites you to stop by our display at the meeting to learn about our Coagulation products. For more information, call 800-533-1710 or visit the Mayo Clinic Website: (www.mayoreferenceservices.org/mml).

READER PRODUCT FEATURE

REAAADS Anti-Beta 2 Glycoprotein I Semi-Quantitative Test Kits For *In Vitro* Diagnostic Use

Assay format -	96-well microtiter plate (8 x 12 strips) with breakaway wells
Sample matrix -	Human serum or plasma collected in 3.2% sodium citrate
Sample dilution -	1:50
Antigen -	Purified human Beta 2 Glycoprotein I
Conjugate -	Horseradish peroxidase (HRP) / goat anti-human IgG, IgM, or IgA
Chromogenic substrate -	TMB (single component)
Stopping solution -	0.36N sulfuric acid
Assay incubations	
Sample -	15 min @ room temperature
Conjugate -	15 min @ room temperature
Substrate -	10 min @ room temperature
Wavelength -	450 nm
Assay calibration -	single point or multi-point curve prepared from 3 serum calibrators included in kit
Clinical specificity-	IgG 100%; IgM 93%; IgA 95%
Clinical sensitivity-	Autoimmune population: IgG 28%; IgM 23%; IgA 27%
Product numbers:	037-001: 96 well IgG aB2GPI test kit 038-001: 96 well IgM aB2GPI test kit 039-001: 96 well IgA aB2GPI test kit

UPCOMING CONFERENCES (cont.)

• The joint meeting of the XIX International Congress of Clinical Chemistry (ICCC) and the 2005 Annual Meeting of the American Association for Clinical Chemistry (AACC) will be held July 24-28, 2005, at the Orange Co. Convention Center, in Orlando, Florida, USA. Visit booth #1352 during the exhibit, where **Corgenix** representatives will be available to discuss your ELISA testing needs.

• The Association of Medical Laboratory Immunologists (AMLI) will hold their Annual Meeting at the Hyatt Regency Hotel in Denver, CO, August 21-24, 2005. The meeting brings together basic and clinical scientists who have developed expertise in clinical laboratory immunology.

Corgenix is sponsoring a Luncheon Symposium on Sunday, Aug. 21, at 12:00-1:30pm where Luis R. Lopez, MD, will discuss "Oxidized Low Density Lipoprotein and Beta 2 Glycoprotein I - Novel Risk Factor for Autoimmune Mediated Atherosclerosis."

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THE READER

Volume 15, Number 3

June 2005

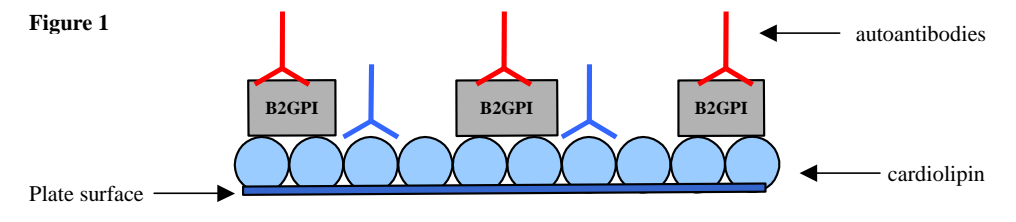
Anti-Phospholipid Testing 101—Past, Present & Future Part I: The Evolution of aPL Assay Development

In a 1952 JAMA article (J Moore et al.), the topic of false positive syphilis screening was discussed¹. The article identified one of the "chemically definable substances" used in the VDRL assay as the phospholipid cardiolipin (diphosphatidylglycerol). The article claimed that up to half of positive patients in a population of US Army personnel did not have syphilis but were false positives, caused by antibodies unrelated to syphilis. Many of these false positive results were due to other infectious diseases, and typically disappeared within six weeks. There was, however, a subgroup of patients that demonstrated persisting false positive results; these patients were identified as having systemic lupus erythematosus (SLE). Further investigation revealed that the SLE patients had autoantibodies that were binding to cardiolipin. It wasn't until a 1983 Lancet article (EN Harris et al.) that a radioimmunoassay for the detection of anti-cardiolipin (aCL, also abbreviated as ACA) antibodies was described². Elevated levels of aCL antibodies were demonstrated in 61% of SLE patients, many of whom had a history of thrombotic events (venous and/or arterial). This suggested that elevated levels of aCL antibodies were a risk factor for thrombosis. Subsequently, enzyme linked immunosorbent assays (ELISA) were developed for aCL detection. In the summary of a 1986 international workshop³, GPL (IgG anti-PhosphoLipid) and MPL units were first introduced for reporting IgG and IgM aCL levels. A GPL or MPL unit was defined as the equivalent of 1 ug/mL of affinity-purified antibody. The first attempt to standardize aCL methods developed from this workshop. Thirty laboratories tested aCL IgG and IgM levels in a set of seven serum samples prepared by mixing various proportions of normal serum and sera from two patients with lupus anticoagulant and a history of multiple thrombotic episodes. The results were consistent with expected values in the laboratories whose methods included fetal calf serum or adult bovine serum and PBS in the diluent formulation, but not with methods using PBS, PBS-Tween or 0.3% gelatin as diluents. These serum samples were later made commercially available as the "Harris" or Louisville APL standards. Most aCL methods (both commercial and in house) were standardized against the Louisville standards, but not all against the same preparation. Standards are now also available for IgA aCL (values are reported in APL units).

Today, the importance of beta 2 glycoprotein I (B2GPI, cofactor) in the detection of clinically relevant anti-phospholipid antibodies is accepted and better understood. In early assay development, it was recognized that the inclusion of fetal calf or bovine serum in the test system was important, but the role it played was not fully understood. Most methods incorporated bovine serum into the aCL microtiter plate coating process and/or in the sample diluent. It was later revealed that B2GPI from bovine serum binds to cardiolipin immobilized in the wells and B2GPI dependent aCL antibodies in patient samples bind to the B2GPI (figure 1). B2GPI dependent antibodies have been shown to correlate with a history of thrombosis. However, using bovine serum on the microplate as a source of B2GPI also introduces other non-relevant bovine antigens to the test system that may cause positive patient results unrelated to thrombosis.

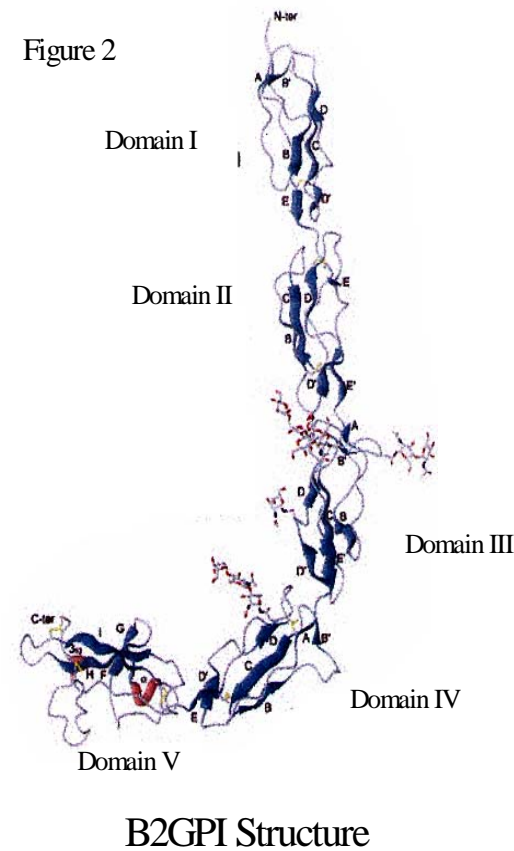
(continued on page 2)

Figure 1



ACL assays also detect antibodies that bind directly to cardiolipin (non-B2GPI dependent). These antibodies are typically not associated with thrombosis, and may be due to infections or other conditions. With the identification of B2GPI and its association with phospholipid binding, researchers have developed ELISA methods to detect anti-B2GPI antibodies directly, in the absence of cardiolipin. Anti-B2GPI assays have been shown to be more specific for autoantibodies related to thrombosis. However, since more patients test positive for aCL antibodies than for anti-B2GPI antibodies (including some with a history of thrombosis), some investigators consider the aCL assay to be more sensitive than anti-B2GPI testing.

B2GPI is a serum protein found in circulation and associated with several lipids such as chylomicrons, LDL and HDL-cholesterol. For this reason, it is also known as apolipoprotein H. B2GPI is a single chain polypeptide of 326 amino acids with a molecular weight of approximately 50KD, which binds negatively-charged phospholipids and various macro-molecular structures including DNA, heparin and platelet membranes. The molecule is made up of five distinctive domains (Figure 2). Domain V binds to the phospholipid surface. When bound to a phospholipid, a tertiary structural change may occur, creating a neoepitope(s) that is recognized by autoantibodies. Many researchers believe there is a very well defined amino acid sequence in domain I where most, if not all, anti-B2GPI antibodies bind. Other studies (see December 2004 READER) demonstrated that though most patients react similarly with different methods, some patients do not. This supports the notion of a heterogeneous group of antibodies that recognize different binding epitopes, possibly in different domains of B2GPI. Whether antibodies to different domain locations have different clinical importance remains to be demonstrated. In vivo, B2GPI acts both as a procoagulant when activated by phospholipid binding (domain V cleavage), down-regulating intrinsic fibrinolysis, and as an anticoagulant, binding factor XI and inhibiting its activation (XI → XIa). Anti-B2GPI antibodies enhance the binding of B2GPI to negatively charged phospholipids on cell membranes, promoting the cleavage of B2GPI, amplifying the procoagulant effect.



There are many other phospholipids and phospholipid binding proteins besides cardiolipin and B2GPI (Table 1, page 3). Though cardiolipin was the first phospholipid identified in association with autoantibodies, it is known that cardiolipin is not contained in the platelet membrane, nor is it involved in the coagulation cascade. The platelet membrane is actually a combination of phosphatidylcholine (50-60%), phosphatidylethanolamine (20-30%), phosphatidylserine (10-15%) and phosphatidylinositol (<5%). It has been demonstrated that phosphatidylserine is the phospholipid that binds B2GPI in vivo. Phosphatidylserine is unique not only because of its direct involvement in the coagulation cascade, but also because it is the only phospholipid located exclusively in the interior of the bilayer membrane in a resting platelet. Phosphatidylserine is externalized by way of a flip-flop mechanism during cell activation (injury). Cardiolipin is found in the membrane of the mitochondria. The molecular structure of cardiolipin is very similar to phosphatidylserine and both have demonstrated the ability to bind B2GPI in solid-phase assays. Because of these similarities, comparisons of antibody detection of ELISA methods that utilize cardiolipin versus phosphatidylserine result in approximately 90% agreement. However, the anti-phosphatidylserine method actually correlates better than aCL testing for the presence of anti-B2GPI antibodies, resulting in a higher positive predictive value⁴.

(continued on page 3)

Phospholipids	Binding Proteins
Cardiolipin	B2GPI
Phosphatidylserine	Prothrombin
Phosphatidylethanolamine	Annexin V
Phosphatidylglycerol	Kininogen
Phosphatidylcholine	Protein C
Phosphatidylinositol	Protein S
Phosphatidic Acid	

The issue of using human source B2GPI in aCL test systems rather than bovine has been the topic of much debate since B2GPI was identified as a phospholipid binding protein and a specific target for autoantibodies. Bovine B2GPI has about 85% identical amino acid sequence with human B2GPI and binds similarly to aCL antibodies in ELISA methods. Human B2GPI is always present in aCL test methods from the patient sample itself. Endogenous B2GPI can bind to the cardiolipin surface during the reaction step and therefore bind autoantibodies. The advantage of using purified human B2GPI on the coated cardiolipin surface may result in better consistency and control of its quantity (density), without introducing other bovine antigens to the test system. Accordingly, many aCL methods now use human B2GPI in the plate coating process.

The presence of aCL antibodies was initially associated with SLE and lupus like disorders, but a number of patients with aCL antibodies without SLE were also identified. Many different specific disease names were proposed: Hughes' syndrome, anti-cardiolipin syndrome, and others. Later, the antiphospholipid syndrome (APS) was adopted to describe this disease, and a set of diagnostic criteria was defined. During the 8th International Symposium on Antiphospholipid Antibodies held in Sapporo, Japan in October of 1998, the Congress members and associated researchers determined the minimal requirements for APS (Table 2). This definition for APS has come to be known as the Sapporo Criteria.

(continued on page 4)

Preliminary Classification Criteria for Definite APS International Workshop; October, 1998 - Sapporo, Japan	
<u>Clinical Criteria</u>	
Vascular thrombosis: One or more clinical episodes of: arterial, venous or small vessel thrombosis in any tissue or organ; confirmation by imaging, doppler or histopathology (except for superficial VT); histopathology - thrombosis without inflammation of vessel wall.	
Pregnancy morbidity:	
<ul style="list-style-type: none"> • One or more unexplained deaths of normal fetus at or beyond 10 weeks - documented by ultrasound or direct examination, or • One or more premature births of normal neonate at or before 34 weeks due to severe eclampsia or placental insufficiency, or • Three or more unexplained consecutive spontaneous abortions before 10 weeks with anatomic, genetic or hormonal causes excluded. 	
<u>Laboratory criteria:</u>	
<ul style="list-style-type: none"> • Anticardiolipin antibody of IgG and/or IgM isotype in medium or high titer on 2 or more occasions at least 6 weeks apart, measured by standardized ELISA for B2GPI-dependent antibodies. • Lupus anticoagulant in plasma on 2 or more occasions at least 6 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Hemostasis (Scientific Subcommittee on Lupus Anticoagulants/Phospholipid-Dependent Antibodies)⁶: <ul style="list-style-type: none"> - prolonged coagulation time on phospholipid-dependent screening test i.e. aPTT, KCT, dRVVT, dPT - failure to correct prolonged coagulation time by mixing with normal PPP - shortening or correction of prolonged coagulation time by addition of phospholipid - exclusion of other coagulopathies i.e. factor VIII inhibitor, heparin, as appropriate 	