Antiphospholipid Syndrome Antibodies — Snippets from the Laboratory

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ABSTRACT

Antiphospholipid (aPL) antibodies refer to antibodies associated with the clinical syndrome of vascular thrombosis and/or pregnancy morbidity, termed as aPL syndrome (APS). aPL antibodies include lupus anticoagulants (LA), anticardiolipin (aCL) antibodies and anti-β2-glycoprotein 1 (anti-β2GP1). aCL antibody is more sensitive than LA and anti-β2GP1 even though the latter are more specific for APS. Interpretation of aPL results should be done collectively together with the clinical presentation of each individual. The latest recommendations and consensus on aPL antibody testings should be closely adhered to by laboratories in order to achieve an accepted standard and to ensure accuracy of test results.

Keywords: Antiphospholipid syndrome, Antiphospholipid antibodies, Anticardiolipin antibodies, Anti-β2-gycoprotein 1, Lupus anticoagulant, Thrombosis

INTRODUCTION

aPL Syndrome (APS) is a thrombophilic disorder characterised by arterial and/or venous thrombosis and/or pregnancy morbidity, associated with the presence of a heterogenous group of antibodies called aPL.

aPL antibodies are heterogenous groups of antibodies directed against phospholipid (PL)-binding proteins. aPL antibodies include LA, aCL antibodies, and anti-β2GP1. The current classification criteria for APS mandate that the presence of at least one of these antibodies is necessary in making the diagnosis of APS.

CLASSIFICATION CRITERIA FOR APS[1]

APS is present if at least one clinical criteria and one laboratory criteria are met.

Clinical Criteria

1. Vascular thrombosis: one or more clinical episodes of arterial, venous, or small vessel thrombosis in any tissue or organ, confirmed by an objective validated criteria such as imaging studies and histopathology.

2. Pregnancy morbidity: one or more unexplained deaths of a morphologically normal foetus at or beyond 10 weeks of gestation, one or more premature births of a morphologically normal neonate before 34th week of gestation because of eclampsia/severe pre-eclampsia or placental insufficiency, or three or more unexplained consecutive spontaneous abortions before 10 weeks of gestation.

Laboratory Criteria

1. LA present in plasma on two or more occasions at least 12 weeks apart.

2. aCL antibody of immunoglobulin (Ig) G and/or IgM isotype in serum or plasma, present in medium or high titre (i.e. >40 IgG PL [GPL] or IgM PL [MPL], or >99th percentile), on two or more occasions, at least 12 weeks apart, measured by a standardised enzyme-linked immunosorbent assay (ELISA).

3. Anti-β2GP1 antibody of IgG and/or IgM isotype in serum or plasma (in titre >99th percentile), present on two or more occasions, at least 12 weeks apart, measured by a standardised ELISA.

LUPUS ANTICOAGULANT

To date, there are no specific tests for LA. LA testing remains complex. LA is detected indirectly by using PL-dependent tests that must fulfill a set of diagnostic criteria set by the International Society of Thrombosis and Haemostasis (ISTH). Lack of uniformity of LA testing and variable application of the ISTH criteria, leading to false negative and false positive results, has been a foremost concern over the past decade. This issue is addressed in the latest recommended guidelines for LA testing published in 2009[2].

WHY DO WE TEST FOR LA?

LA is the most specific assay for APS though not as sensitive as aCL ELISA. The presence of LA has the strongest association with thromboembolism risk[3] and foetal loss[4]. Swadzba J et al[5] suggested that the association with thrombosis is stronger if a 'double LA positivity' is detected by a combination of any tests (diluted Russell viper venom time [dRVVT], activated partial thromboplastin time [aPTT], diluted prothrombin time [dPT], or anti-β2GP1). Isolated LA positivity is significantly more frequent in individuals without clinical sequelae.

WHO SHOULD UNDERGO LA TESTING?[2]

1. Patient with a Significant Probability of Having APS

In order to minimise false positive results, it has been recommended that the appropriateness of LA testing can be decided using the grading of clinical characteristics as follows:

a. Low: venous thromboembolism (VTE) or arterial thromboembolism (ATE) in elderly patients.

b. Moderate: incidental finding of prolonged aPTT in asymptomatic individuals, recurrent spontaneous early pregnancy loss, provoked VTE in young patients.

c. High: unprovoked VTE and unexplained arterial thrombosis in young patients (<50-years-old), late pregnancy loss, thrombosis at unusual sites, or any thrombosis or pregnancy morbidity in patients with autoimmune disease (eg. systemic lupus erythematosus, autoimmune haemolytic anaemia).

2. Patient with Unexplained Prolonged aPTT

For those with positive LA, it is compulsory to repeat the test more than 12 weeks after the first test.

SAMPLE AND TEST PREPARATION

Blood collection is preferably done before the initiation of any anticoagulant drugs or a sufficient period after their discontinuation. Fresh venous blood is collected in a sodium citrate tube. The sample subsequently undergoes double centrifugation to ensure the sample is platelet poor, with a residual count $<10 \times 10^9$/L. This involves the regular centrifugation of blood (2,000g, 15 minutes, room temperature), followed by centrifugation of the supernatant plasma at higher speed (>2,500g, 10 minutes). If the test is postponed, the plasma needs to be rapidly frozen at -70°C or below. Frozen plasma needs to be thawed at 37°C.

LA DIAGNOSTIC CRITERIA

The rationale of using PL-dependent tests in LA testing arises from the fact that these anticoagulants bind to negatively charged PLs combined with specific proteins and this prolongs the coagulation test time.

LA testing consists of three steps in order to maximise its diagnostic yield:

a. Screening test

The prolongation of the clotting time of one (or more) PL-dependent test(s) beyond the upper limit of the reference interval is the first criteria. There are many PL-dependent tests that can be used for this step, for which dRVVT is the first recommended test and a sensitive aPTT is the second recommended test. These two tests are chosen as they are done based on different principles[2]. This helps in augmenting the diagnostic yield. dRVVT is recommended due to its higher specificity for detecting LA in patients with high risk of thrombosis[6]. dRVVT is also the most robust test in detecting LA[7]. aPTT is used due to its higher sensitivity for LA[8]. Positivity for at least one of the two tests is adequate to proceed with the following mixing test. Other tests eg. kaolin clotting time (KCT) and dPT are not recommended.

b. Mixing test

The above prolongation is not corrected after mixing equal portions of patient and normal plasmas, suggesting presence of inhibitors in the patient's plasma rather than deficiency of coagulation factors. This test cannot be concluded if the thrombin time of the test is significantly prolonged.

c. Confirmatory test

Prolongation is reduced upon repetition of the test with increased concentration of PL. Bilayer and hexagonal phase PL should be used to increase the concentration of PL.

CUT-OFF VALUES

Different laboratories use different assays and different instruments, resulting in different cut-off limits. Standardisation in LA testing remains a challenge. Based on the latest guidelines, the cut-off level is defined as the value above the 99th percentile from the distribution of LA levels in healthy controls[2]. By using the 99th percentile as cut-off, the specificity of the test will improve. Results are suggestive of LA when their clotting times are longer than the cut-off level. This is applicable to screening and mixing tests.

The use of normalisation ratios (patient sample:pooled normal plasma sample) is the best way to compensate for inter- and intra-assay variation[1]. For example, clotting time ratios (test/control) of >1.1 for dRVTT and >1.2 for KCT are used in many laboratories[9,10].

FALSE POSITIVE AND FALSE NEGATIVE RESULTS

False positive results happen when plasma contains specific antibodies to coagulation factors such as factor VIII or when the plasma has factor deficiencies[11,12]. The presence of unfractionated heparin may mimic the presence of LA as well, resulting in false positive results[13]. This can be ruled out with prolonged thrombin time, which is normal in the presence of LA. False positive results can be found in elderly patients or if it is the first positive result[14].

The presence of residual platelets in test plasma, as little as 16 x 10^9/L, may shorten the coagulation time. This may result in false negative results, especially in those with weak LA[15]. This can be overcome by double centrifugation[16].

The reagents used to test for syphilis may contain PLs. These can result in a false positive result in those with aPL antibodies. Therefore, aPL antibodies testing may be ordered to help determine the cause of a positive venereal disease research laboratory/ rapid plasma reagin test for syphilis.

SPECIAL CIRCUMSTANCES

Patients with Acute Thromboembolic (TE) Events

During an acute TE episode, LA results do not alter the initial management of a patient with acute TE. Nevertheless, most of the coagulation tests used for LA testing can still be done, as they are not affected if the patient is on low molecular weight heparin. If the patient is on unfractionated heparin, LA status is important because aPTT, used to monitor treatment when the patient is on unfractionated heparin, will be affected by LA. Thus, such patients will require alternative laboratory tests for monitoring.

Patients on Vitamin K Antagonist Treatment

LA testing is challenging when a patient is on vitamin K antagonist treatment for thrombosis as vitamin K antagonists mimic the presence of LA. LA testing is important for a patient who has thrombosis but is on vitamin K antagonist treatment, as the persistence of LA is an indication for long-term anticoagulation. Recommended strategies include discontinuation of treatment for one to two weeks before testing, addition of normal pooled plasma into patient's plasma with ratio 1:1 (provided the international normalised ratio is <3) to reverse coagulation defect induced by vitamin K antagonists, and the use of coagulation assays not affected by vitamin K antagonist such as textrarin/ ecarin clotting time ratio. For those who require the discontinuation of vitamin K antagonist for one to two weeks, low molecular weight heparin can be used, as it does not interfere with most of the coagulation assays used in LA testing.

aCL AND anti-β2GP1 ANTIBODIES

Presence of aCL and anti-β2GP1 antibodies are indispensable in the diagnosis of APS. The 13th International Congress on Antiphospholipid Antibodies published the internal consensus guidelines on aCL and anti-β2GP1 testing in 2012[17].

IgG aCL and anti-β2GP1 have stronger association with clinical features of APS as compared to IgM isotypes[18–20]. In the latest APS classification criteria, IgA aCL and anti-β2GP1 are not included. However, there are several reports of isolated IgA anti-β2GP1 positivity with APS[21,22]. Thus, it is now recommended that the IgA isotypes, for both aCL and anti-β2GP1, to be done in cases where IgG and IgM isotypes are negative but APS is still suspected[17]. It is known that most patients positive for IgA anti-β2GP1 are also positive for IgG and/or IgM anti-β2GP1.

WHY DO WE TEST FOR aCL AND ANTI-β2GP1 ANTIBODIES?

The aCL test is more sensitive and the anti-β2GP1 test more specific for APS diagnosis[23,24]. aCL antibody is positive in more than 80–90% of patients with APS[25]. However, in 3–10% of APS patients, anti-β2GP1 may be the only positive test[26]. Hence, testing for anti-β2GP1 is imperative in patients suspected of APS in whom aCL IgG and IgM and LA testing are negative.

aCL and/or anti-β2GP1 antibody assays should be repeated, if positive, on two or more occasions at at least 12 weeks apart. This is to rule out a transient increase of levels in the setting of viral or other infections.

SAMPLE AND TEST PREPARATION

Either serum or plasma can be used for testing. If serum is used, heat inactivation at 56°C for 30 minutes should be avoided as this may result in false positive IgM aCL results. Non-haemolysed and non-lipaemic samples should be used. If plasma is used, platelet poor samples (<10 x 10^9/L) are necessary. Serum samples should be stored at 2–8°C if testing is going to be done within two to three days after collection and at -20°C or below if longer storage is required.

ASSAY METHODS

These antibodies are measured with ELISA tests. aCL ELISA tests are able to detect both anti-β2GP1 antibodies as well as antibodies to other PL binding proteins. The use of aCL kits, which is a complex of cardiolipin plus (bovine or human) β2GP1 as antigen, is recommended. However, for anti-β2GP1 ELISAs, whole molecule β2GP1of human origin should be used. The new consensus outlines reliable standards that need to be adhered to by laboratories to ensure standardisation of their assays and appropriate calibration to safeguard the accuracy of tests. The precision of the ELISA test is further emphasised, and is reassuring when the coefficient of variation is ≤10%.

MEASUREMENT AND CUT-OFF

aCL antibodies should be measured using GPL/MPL units. One GPL/MPL unit is defined as the cardiolipin-binding activity of 1ug/ml of affinity-purified IgG or IgM aCL antibody. These are further grouped into low-positive, moderate-positive, or high-positive based on the levels. It is recommended that the cut-offs should be determined using a non-parametric percentile method as autoantibody values do not follow a normal distribution. A cut-off based on the 95th and 99th percentiles should be used by the manufacturers. The current classification criteria define clinically significant titres of aCL as >40 GPL and >40 MPL, or higher than the 99th percentile of the reference range obtained within normal subjects[17]. Nevertheless, these GPL or MPL cut-offs can be different from the 99th percentile value. GPL or MPL cut-offs for low-positive, moderate-positive, and high-positive are not scientifically studied. Budd et al[27] found that the majority of the IgM aCL positive samples in a large population of healthy individuals fell between the 95th and 99th percentiles, but these were not considered to be clinically significant. Therefore, Budd et al[27] recommended that range values between the 95th–99th percentiles be considered as a "grey" zone is appropriate above the 99th percentile in order to prevent over-diagnosis of APS.

There is no universal unit of measurement for anti-β2GP1. Laboratories worldwide have been using different units such as ng/ml, ug/ml, or units/ml. Thus, the development of international units for measurement of anti-β2GP1 antibodies is needed.

ADDITIONAL INFORMATION

Rheumatoid Factor (RF) Interference

The co-existence of medium or high levels of IgG aCL/anti-β2GP1 antibodies and high levels of IgM RF result in false positive IgM aCL/anti-β2GP1 results[28]. This interference appears to be most significant when the IgG aCL antibody level is at least moderately positive. This has resulted in lower clinical specificity of IgM aCL/anti-β2GP1antibodies. This made reporting of IgM RF important when interpreting IgM aCL/anti-β2GP1 results.

CONCLUSION

The LA result should always be interpreted in the context of a full APS profile, which includes aCL and anti-β2GP1 antibodies. The risk of thrombosis is higher if multiple aPL antibodies are present compared to single antibody positivity. However, the current APS classification criteria mandate that the presence of at least one of these antibodies is necessary in making the diagnosis of APS. The aCL antibody is more sensitive than LA and anti-β2GP1 antibodies even though the latter are more specific for APS. Recommendations and consensus statements for testing of these aPL antibodies testing should be closely adhered to by laboratories in order to

reduce variation, enhance accuracy, and improve the reporting process.

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