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Detection and importance of anticardiolipin antibodies

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Introduction

Confirmatory evidence that antiphospholipid antibodies (the lupus anticoagulant or anticardiolipin antibodies) are associated with an increased risk for arterial and venous thrombosis, recurrent spontaneous abortions, and fetal loss has led to increased laboratory requests for identification of these antibodies. These antibodies were first identified because of prolonged phospholipid dependent coagulation tests (the lupus anticoagulant test) attributable, presumably, to the presence of phospholipid binding antibodies. The antiphospholipid test was introduced in 1983 with the aim of finding a more sensitive, specific, and quantitative method of identifying patients with clinical disorders related to antiphospholipid.

Enzyme linked immunosorbent assays (ELISA) are now increasingly being applied to the detection of antibodies in a variety of biological substances. Their potential as reliable, sensitive, reproducible and rapid assays is well established, and ELISAs have been used since 1985 for the measurement of antiphospholipid antibodies by large numbers of laboratories worldwide. These assays have clearly defined a subset of patients with a strong risk of thrombosis.

An international effort was made to standardise the antiphospholipid test in 1986. Criteria were established to define the validity of the assay and to compare various assay methods available. Units of measurement of IgG (G phospholipid (GPL) units) and IgM (M phospholipid (MPL) units) positivity were established. Recently, a second international standardisation workshop, designed to determine the comparability of the antiphospholipid test, showed an excellent interlaboratory agreement.

Clinical relevance of antiphospholipid antibodies

Antiphospholipid antibodies encompass a spectrum of antibody specificities associated with an expanding range of clinical and laboratory manifestations (table 1).

In 1987 Harris et al proposed that the combination of clinical features including both venous and arterial occlusive events, recurrent spontaneous abortions, and thrombocytopenia with antiphospholipid antibodies (identified as moderate to high titres of IgG or IgM antiphospholipid antibody or the lupus anticoagulant), should be termed the “antiphospholipid syndrome”.

The consensus of opinion is that antiphospholipid antibodies have a pathogenic role in the vasculopathy of the antiphospholipid syndrome, but the mechanism is unknown. Proposed mechanisms to explain the thrombotic diathesis include increased platelet aggregation due to decreased production of prostacyclin through the effect of antiphospholipid antibodies on the vascular endothelium. Other groups have proposed inhibition of different plasma or platelet factors involved in the process of coagulation thrombosis. The demonstration that a subset of antiphospholipid antibodies reacts with the complex of phospholipid and the serum protein, B2 glycoprotein I (which inhibits factor XII activation, platelet activation, and prothrombinase activity) suggests a potent way in which antiphospholipid antibodies might predispose to a prothrombotic diathesis.

Over the past decade, the antiphospholipid test has become a critical factor in identifying patients with the antiphospholipid syndrome.

Table 1 Clinical and laboratory features associated with antiphospholipid antibodies

<table>
<thead>
<tr>
<th>Major features</th>
<th>Moderate to high titres of IgG and/or IgM antiphospholipid antibody</th>
<th>Lupus anticoagulant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venous and arterial occlusions</td>
<td>Thrombocytopenia</td>
<td></td>
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<tr>
<td>Recurrent spontaneous abortion</td>
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</tbody>
</table>

Possible features:

- Livedo reticularis
- Heart valve lesions
- Chorea
- Epilepsy
- Transverse myelitis
- Immune thrombocytopenic purpura
- Guillain-Barré syndrome
- Pulmonary hypertension
- Haemolytic anaemia

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Most of these patients have persistent, moderate to high IgG antiphospholipid titres. A few, however, are only IgM positive. The unrelated behaviour of lupus anticoagulant and antiphospholipid antibodies in the course of disease and in individual patients indicates that both assays are required if all cases with the antiphospholipid syndrome are to be picked up.13

Anticardiolipin assay

The assay described below is an ELISA for the separate determination of IgG and IgM antibodies to cardiolipin using antigen coated microwells as a solid phase. The equipments and reagents required to perform the assay are summarised in tables 2 and 3.

PRINCIPLE

This method consists of coating wells of microtitre plates with pure cardiolipin (diphasphatidyl glycerol) from samples of bovine heart. After washing, the non-specific binding sites are blocked and diluted test samples and standard control sera are added, allowing any anticardiolipin antibody present to bind to the plate surface. After washing to remove unbound materials alkaline phosphatase conjugated goat anti-human IgG or IgM is then added, which binds to any of the IgG or IgM antibody bound to the plate. Unbound conjugated antibody is removed by washing. Detection of the degree of binding is achieved by the addition of an enzyme substrate which, if positive, turns yellow in colour. The degree of colouration is proportional to the quantity of cardiolipin antibody in the original serum. The enzymatic reaction is blocked by the addition of 3N sodium hydroxide to each well, and the optical absorbance of each well is read at 405 nm with a spectrophotometer.

Estimation of the concentration of anticardiolipin antibodies present in the test sera or plasma can then be made quantitatively, once a calibration curve has been established, by using a standard serum in each assay. Values of unknown samples can be determined from the curve and reported in GPL or MPL units.

SPECIMEN COLLECTION AND PREPARATION

Plasma or serum samples may be used. If not tested immediately, the specimens should be stored at 4°C. If specimens are to be stored for more than 72 hours they should be stored frozen at −20°C or preferably −70°C. If plasma is used, centrifugation at 2000 × g for 10 minutes to minimize platelet contamination is recommended. Decomplementation by heating serum to 56°C gives rise to false positive assay results and should be avoided. Freeze-thawing samples may result in a decrease of anticardiolipin binding activity. Haemolysed samples may produce erratic results.

REAGENT PREPARATION (TABLE 3)

1 Antigen preparation:

Prepare working cardiolipin antigen solution by diluting stock cardiolipin with 95% ethanol to make a 50 µg/ml solution for coating the ELISA plates. Cardiolipin antigen solution (30 µl) is applied to each well of the ELISA plates at room temperature. Ideally the organic solvent should be evaporated under nitrogen and dried under vacuum to prevent oxidation of the antigen, but this can be achieved by leaving the plates open in the refrigerator (4°C) for between 12 and 24 hours.

Warning: Warming plates to 37°C to evaporate the organic solvent appears to yield unreliable results.

2 Phosphate buffered saline (PBS) solution:

Dissolve 20 tablets of PBS in 2 litres of distilled water and mix thoroughly. The pH of the final solution should be 7.4 × 0.1. Store at 4°C and use within 15 days.

3 10% ABS in PBS:

Prepare 10% ABS (adult bovine serum) in PBS by adding 25 ml of ABS to 225 ml of PBS; 10% ABS-PBS should be made up freshly every week and stored at 4°C ready for use.

4 Conjugate working dilution:

Prepare conjugate working dilution (1 in 1000) by adding 5 µl of alkaline phosphatase conjugated anti-human IgG or IgM to 5 ml of 10% ABS-PBS and mix thoroughly. This preparation should be made up not more than 30 minutes before use.

5 Diethanolamine buffer:

Using containers known to be free of metal and glassware washed in acid, prepare diethanolamine buffer by adding 97 ml diethanolamine, 0.1 g magnesium chloride hexahydrate, and 0.2 g sodium azide to 900 ml of distilled water. Mix thoroughly and

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**Table 2** Equipment required for ELISA measurement of antiphospholipid antibodies

| 1 | Microtiter polystyrene multiwell plate (96 flat bottom wells) |
| 2 | 8 channel adjustable volume pipettor (50–200 µl) |
| 3 | Other single channel adjustable volume pipettors to dispense 10, 50, 100 and 150 µl are needed to make appropriate dilutions |
| 4 | Disposable micropipette tips |
| 5 | Plastic disposable tubes of 3-4 ml capacity for dilution of test sample sera |
| 6 | 30 ml glass pipettor reservoir |
| 7 | Dark chamber |
| 8 | 37°C incubator |
| 9 | MicroELISA plate washer (optional) |
| 10 | Aspirator or vacuum pump (optional) |

**Table 3** Reagents required for ELISA measurement of antiphospholipid antibodies

| 1 | Cardiolipin from bovine heart (Sigma Chemicals, St Louis, Missouri, catalogue No: C-1649); store at –20°C. |
| 2 | Ethanol 95% |
| 3 | Distilled water |
| 4 | Phosphate buffered saline (PBS) |
| 5 | Adult bovine serum (ABS) Make 25 ml aliquots and store at –20°C. |
| 6 | Alkaline phosphatase conjugated goat anti-human IgM or IgG catalogue No: A-3275 and IgG catalogue No: A-5403 (Sigma Chemicals, store at 4°C. |
| 7 | p-Nitrophenyl phosphate disodium hexahydrate tablets (Sigma Chemicals, No: 104–105); store at 20°C. Do not use after expiration date |
| 8 | Analar grade diethanolamine, magnesium chloride hexahydrate, sodium hydroxide, and sodium azide. |
adjust pH to 9.8 using 1M hydrochloric acid or sodium hydroxide as required. Store at 4°C.

**Warning:** It is vitally important that the pH of the diethanolamine buffer is exactly 9.8. If the pH is too far off this value, +0.2 pH units, there is incomplete (or no) development of the colour reaction.

**6 p-Nitrophenyl phosphate substrate solution:**

Using a clean pipette, transfer 5 ml of diethanolamine buffer into suitable container (10 ml conical, screw-cap centrifuge tube). To obtain the final concentration of 1 mg/ml, one tablet (5 mg) of p-nitrophenyl phosphate is required for each 5 ml of diethanolamine buffer. The preparation of substrate solution must be carried out immediately before use. Solution (5 ml) is required for each plate. Ensure that tablets are completely dissolved before use. The substrate solution should be clear when dispensed. Discard any substrate solution which has become overly yellow before use.

**Warning:** Use plastic forceps to handle p-nitrophenyl phosphate tablets. Never allow this reagent to come into contact with mucous membranes.

**PREPARATION OF SERUM SAMPLES AND CONTROL DILUTIONS**

Bring the serum samples to room temperature and mix well before using. Prepare test samples at 1 in 50 dilutions by adding 10 μl of serum or plasma to 0.5 ml of 10% ABS-PBS. Diluted samples are usually run in duplicate wells and the mean of these results determined. Prepare for each plate eight serial dilutions (1 in 50 to 1 in 6400) of a standard positive sample (IgG or IgM) in 10% ABS-PBS. These dilutions cover the full sensitivity range of the assay and will allow a standard curve for each run to be derived. Anticardiolipin titres of unknown samples can be determined with respect to this curve (standard sera are available from United Kingdom National External Quality Assessment Scheme or UKNEQAS). To exclude false positive anticardiolipin antibody results caused by non-specific binding to the wells, it is very important to subtract the absorbance values obtained from wells coated with ethanol alone.

**ASSAY PROCEDURE**

1. Using the eight channel pipette, add 30 μl of each 50 μg/ml cardiolipin in ethanol. Leave the plates uncovered overnight at 4°C to allow the organic solvent to evaporate.

2. The following day wash the plates three times with PBS, 100 μl per well, using the microELISA plate washer or the eight channel pipette.

3. Block the unoccupied sites in the wells by adding 75 μl per well of 10% ABS-PBS. Seal and incubate for one hour at room temperature on a horizontal surface.

4. At the end of the incubation period, empty the wells using a disposable plastic pipette connected to a vacuum pump, or by rapid inversion and flicking of the plates over a laboratory sink. Wash the plates once with PBS.

5. Add 50 μl of test samples at 1 in 50 dilution to each of the duplicate wells. Reserve the first two rows of the plates for the standard positive curve. Add 50 μl of the eight serial dilutions of a standard positive sample (IgG or IgM) to each of the duplicate wells. Label the plates accordingly. Leave two wells of each plate empty to serve as blanks. Seal the plates and incubate for three hours at room temperature.

6. About 15 minutes before the end of the incubation period, prepare the appropriate conjugates according to instructions in "reagents preparation" section.

7. At the end of the incubation period, empty the wells and wash three times with PBS as in step 4.

8. Using the eight channel pipette add 50 μl of the goat anti-human IgG or goat anti-human IgM conjugates to the wells. Seal the plates and incubate for 90 minutes at room temperature.

9. About 20 minutes before the end of incubation prepare substrate solution according to instructions in "reagents preparation" section.

10. At the end of incubation period, conjugates are discarded and plates washed three times with PBS as in step 4.

11. Use the eight channel pipette to add 50 μl of p-nitrophenyl phosphate substrate solution to all wells as quickly as possible. Place the plates in a dark chamber and allow the reaction to develop in an incubator at 37°C for 45 minutes.

12. Stop the enzyme reaction by adding 50 μl 3N sodium hydroxide to each well.

13. Read the optical absorbance at 405 nm using a suitable multiscan. Use the blank wells to zero the microELISA reader, then read and record the absorbance of each well.

**CALCULATION OF RESULTS**

Each plate should be run with eight dilutions of a standard positive sample which together span the full sensitivity range of the ELISA, and where the concentration of anticardiolipin antibody at each dilution has been pre-determined. Thus for each plate a standard curve can be constructed and the concentration of each test sample determined. The level of positivity of the test samples can be determined with respect to a standard curve obtained by plotting logarithm optical absorbance against logarithm anticardiolipin concentrations of the eight standard dilutions run on the same plate.

**REPORTING OF RESULTS**

In an effort to standardise our ELISA Harris et al created standards and units for the quantitative reporting of both IgG and IgM anticardiolipin antibody titres. IgG and IgM titres are reported in GPL and MPL units, respectively. One GPL or MPL unit is defined as the cardiolipin binding activity of 1 μg/ml of an affinity purified IgG or IgM anticardiolipin preparation from standard
serum. Reference serum 90/656 (UKNEQAS) is assigned values for IgG anticardiolipin antibody in relation to this material.

The results can be reported as GPL and MPL units or as high, medium, low positive, or negative. We consider an IgG anticardiolipin value above 80 GPL units as high positive, the range 15-80 GPL units medium positive, and below 15 GPL units a low positive. For IgM anticardiolipin, titres above 50 MPL units are considered high positive, those between 6-50 MPL units medium positive, and less than 6 MPL units low positive. The cutoff for IgG is less than 5 GPL units and for IgM it is less than 3 MPL units (these cutoff levels were determined using the mean plus five standard deviations of 40 normal control sera). Patients with low positive test results should have a repeat measurement several weeks later because many revert to negative values.

QUALITY ASSURANCE
Each assay run (plate) should include at least one replicate pair of control samples. The results of these samples in successive assays should be subjected to standard statistical analysis for the assessment of interassay variability. If only one control serum is used this should be selected to give a value in the 12-15 GPL range.

Results for the control serum more than 2 SD deviant from the mean or expected value should call for rejection of the batch and repeat assay. If the standard curve has a poor slope, the $r^2$ value is less than 0.95, or if there are high non-specific binding values, the assay must be repeated.

EXTERNAL QUALITY ASSESSMENT
It is recommended that all laboratories performing this assay participate in an External Quality Assessment Scheme. The assay of IgG anticardiolipin antibody is surveyed by UKNEQAS for autoimmune serology, details of which can be obtained on application to the Organiser.