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Anticardiolipin antibodies: isotype distribution and phospholipid specificity

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SUMMARY  Quantitative isotype specific enzyme linked immunosorbent assay (ELISA) was used to determine the distribution of immunoglobulin isotypes and phospholipid specificities of anticardiolipin (anti-CL) antibodies in 40 patients with one or more of the following 'antiphospholipid (anti-PL) antibody associated clinical complications'—namely, thrombosis, fetal loss, thrombocytopenia. Twelve of 40 patients had IgG, IgM, and IgA anti-CL antibodies. Ten patients had IgG and IgM, five patients had IgG and IgA, and three patients had IgM and IgA anti-CL antibodies. There was no statistical association between any single isotype or any group of isotypes with thrombosis, fetal loss, or thrombocytopenia. The presence of IgG anti-CL antibodies in 36 of the 40 patients suggests that this isotype may be most important in determining clinical complications, but there were four patients without IgG anti-CL antibodies who also appeared susceptible to thrombosis, fetal loss, and thrombocytopenia. IgG, IgM, and IgA anti-CL antibodies bound the negatively charged phospholipids, phosphatidylserine and phosphatidylinositol, but not the zwitterionic phospholipid, phosphatidylcholine. There was no significant difference between binding to cardiolipin and binding to other negatively charged phospholipids, suggesting that the specificity of these antibodies is for negatively charged phospholipids in general rather than for cardiolipin in particular.

Key words: antiphospholipid antibodies, thrombosis, recurrent fetal loss, thrombocytopenia.

Many recent studies have shown associations of the anticardiolipin (anti-CL) antibody and the closely related 'lupus anticoagulant'2 with thrombosis3–9 recurrent fetal loss10–12 and thrombocytopenia.13 14 Other studies have shown that anti-CL antibody, like the lupus anticoagulant, may bind phospholipids other than cardiolipin,2 15–16 and both groups of antibodies should together be termed antiphospholipid (anti-PL) antibodies. Since phospholipids such as phosphatidylcholine and phosphatidylserine are much more widely distributed in the body than cardiolipin, the determination of differences in the binding of these antibodies to various phospholipids may be important in understanding their role in the disorders with which they are associated. Furthermore, no study has yet addressed itself to determining the distribution of anti-CL antibody isotypes in patients with these clinical disorders.

To study some of these questions we have investigated the distribution of anti-CL antibody isotypes and differences in phospholipid binding activity in 60 patients with 'anti-PL antibody associated clinical disorders'—namely, thrombosis, fetal loss, and thrombocytopenia. To perform this study we devised a new quantitative ELISA technique that has proved to be both sensitive and reproducible in determining antibody binding to cardiolipin and other phospholipids.

Patients and methods

PATIENTS AND CONTROLS
Forty patients were studied with anti-PL antibodies detected either by the lupus anticoagulant test or by solid phase radioimmunoassay for anti-CL antibodies. These 40 patients had one or more of the following 'anti-PL antibody associated clinical features': thrombosis, recurrent fetal loss, thrombocytopenia.17 18 Sera from 20 patients with systemic lupus erythematosus without antiphospholipid antibodies and 40 normal controls were also studied.

REAGENTS
Cardiolipin (CL), phosphatidylinositol (PI), phos-
phatidylserine (PS), phosphatidylcholine (PC), alkaline phosphatase conjugated goat antihuman IgG, IgM, and IgA, alkaline phosphatase substrate (p-nitrophenyl phosphate disodium hexahydrate), and bovine serum albumin (BSA) were purchased from Sigma Chemicals Ltd, UK. Bovine serum (BS) and fetal calf serum (FCS) were purchased from Imperial Laboratories, UK. Microtitre plates were obtained from Flow Laboratories Ltd, Irvine, Scotland.

DEVELOPMENT OF ELISA ASSAY FOR ANTIPHOSPHOLIPID ANTIBODIES

Antigen concentration
The optimal antigen concentration was determined by checker board experiments where wells were coated with CL, PI, PS, PC at varying concentrations—namely, 250, 50, 10, 2, 0.5 µg/ml, and by organic solvent alone. The binding of serial dilutions of a patient serum known to have a high anti-CL antibody titre and of a negative control serum to the various antigens at varying concentrations listed above was studied. Once the optimal antigen concentration was determined, this was used throughout the study.

Protein containing diluent
To determine the best diluent for test samples cardiolipin binding activity of a positive sample and of a negative control sample diluted in the various diluents—namely, 1% and 2% BSA; 5%, 10% and 20% FCS; and 5%, 10% and 20% adult bovine serum (ABS) in phosphate buffered saline (PBS) pH 7.3, were compared. The diluent at a concentration that gave the best discrimination between the positive and negative sera was selected and used throughout the study.

Choice of incubation times
Various time intervals were studied to determine the optimal times for blocking plates and for each incubation step that gave best discrimination between the positive and negative control sera.

ELISA protocol
Microtitre plates were coated with 30 µl/well of 50 µg/ml phospholipid in organic solvent (ethanol for CL and chloroform:methanol 1:3 v/v for the other phospholipids). The plates were left open overnight at 4°C to allow the organic solvent to evaporate. On the following day the plates were washed three times with PBS 100 µl/well and blocked with 75 µl of 10% BS per well for one hour at room temperature. After blocking the plates were washed once with PBS and 50 µl aliquots of test samples at 1/50 dilution in 10% ABS were added to each of duplicate wells and incubated for three hours at room temperature. After incubation the plates were washed three times with PBS and 50 µl aliquots of alkaline phosphatase conjugated goat antihuman IgG, IgM, or IgA in 10% ABS were added. The working dilution for anti-IgG and anti-IgM was 1:1000 and for anti-IgA was 1:500. The plates were then incubated for 90 minutes at room temperature, after which they were washed three times with PBS. After washing, 50 µl of 1 mg/ml substrate (p-nitrophenyl phosphate) in diethanolamine buffer (containing 97 g diethanolamine; 0.1 g MgCl₂·6H₂O; 0.2 g sodium azide/l) pH 9.8 was added to each well. The reaction was allowed to take place at 37°C in a dark chamber for 45 minutes and then stopped by adding 50 µl aliquots of 3 M sodium hydroxide to each well. Absorbance was read at 405 nm with a Titretek multiscanner (Flow Laboratories, UK).

Each plate was run with eight dilutions of a standard positive sample where the concentration of anti-CL antibody at each dilution had been predetermined using affinity purified anti-CL antibodies of the given isotype. Thus for each plate a standard curve could be constructed and the concentration of each test sample in ng/ml could be determined. Test samples were reported as having raised anti-CL antibody levels when their values exceeded that of the mean plus four standard deviations of 40 normal control sera.

Affinity purified anticardiolipin antibodies
IgG, IgM, and IgA anti-CL antibodies were affinity purified as previously described. Affinity purified samples were characterised by double immunodiffusion (Ouchterlony technique) and by immunoelectrophoresis (IEP) against anti-whole human serum (Unipath, UK), as well as antihuman IgG, IgM, IgA antiserum, and measured by single radial immunodiffusion (Mancini).

Results

OPTIMAL ANTIGEN CONCENTRATION
The binding activity of a serum positive for anti-CL antibody to the phospholipids studied at varying antigen concentrations was compared with that of a negative serum (Fig. 1). The best distinction between positive and negative sera was found at about 50 µg/ml for three of the four phospholipids studied—namely, CL, PS, and PI. There was little or no binding to plates coated with PC and plates coated with organic solvent alone.

OPTIMAL PROTEIN CONTAINING DILUENT
All three diluents—namely, ABS, FCS, BSA, gave adequate distinction between positive and negative
Anticardiolipin antibodies

Fig. 1 Binding activities of an IgG anti-CL antibody positive serum and normal serum to plates coated with varying concentrations of cardiolipin and phosphatidylcholine (PC).

Inhibition studies
To demonstrate that the assay for each phospholipid was specific a series of inhibition studies was performed. A serum positive for anti-CL antibody was preincubated with varying concentrations of phospholipid liposomes, and the inhibition of binding activity to plates coated with the phospholipids determined (Fig. 2).

Sensitivity of the assay
Serial dilutions of affinity purified IgG, IgM, and IgA anti-CL antibodies were tested for cardiolipin binding activity (Fig. 3). The assay was able to detect IgG anti-CL antibodies down to a concentration of 20 ng/ml, IgM anti-CL antibodies to a concentration of 30 ng/ml, and IgA anti-CL antibodies to a concentration of 40 ng/ml.

Phospholipid specificity
All 60 patient sera and 40 control sera were tested for binding to three phospholipids other than cardiolipin—namely, phosphatidylserine, phosphatidylinositol, and phosphatidylcholine. There were strong correlations between binding to cardiolipin, phosphatidylserine, and phosphatidylinositol (Fig. 4), all three being negatively charged phospholipids. There was no binding to the zwitterionic phospholipid, phosphatidylcholine.

Fig. 2 Inhibition of IgG and anti-CL antibody activity by increasing concentrations of cardiolipin liposomes.
IgG, IgM, and IgA anti-CL antibodies were present to varying extents in the sera of the 40 patients with 'anti-PL antibody associated clinical features' (Table 1). Twelve patients had IgG, IgM, and IgA anti-CL antibodies. There were 10 patients with IgG and IgM anti-CL antibodies, five with IgG and IgA anti-CL antibodies, and three patients with IgM and IgA anti-CL antibodies. Nine patients had IgG anti-CL antibodies alone, one had IgA anti-CL antibodies alone, but none had IgM anti-CL antibodies alone.

One or more of the following—namely, thrombosis, fetal loss, thrombocytopenia, occurred in patients with IgG anti-CL antibodies alone, and in other patients with IgG in combination with IgM or IgA anti-CL antibodies, or both. Of the three patients with IgM and IgA anti-CL antibodies, one had a history of recurrent fetal loss and thrombocytopenia, and two had histories of thrombosis and thrombocytopenia. The single patient with IgA anti-CL antibodies had a history of cerebrovascular accident and of thrombocytopenia. There was no correlation between IgG and IgM anti-CL antibody
levels ($r=0.28$) or between IgG and IgA anti-CL antibody levels ($r=0.29$). There was a strong correlation, however, between IgM and IgA anti-CL antibody levels ($r=0.84$, $p<0.001$).

**Discussion**

Relatively little is known about antiphospholipid antibodies, although these antibodies were first detected several decades ago. These antibodies have assumed importance clinically because of reported associations with thrombosis, recurrent fetal loss, thrombocytopenia, and Coombs’ positivity. Until three years ago anti-PL antibodies were detected primarily by agglutination (Venereal Disease Research Laboratory test), complement fixation test (Wasserman), or by the ‘lupus anticoagulant’ reaction, which uses the ability of some of these antibodies to prolong clotting tests such as the partial thromboplastin time. None of these tests enable easy determination of isotype distribution or phospholipid specificity of the anti-PL antibodies. Both questions may be important in terms of determining the nature of their relation with the clinical disorders with which they are associated. The introduction of solid phase assay techniques to measure anticardiolipin antibodies has enabled better characterisation of these antibodies. In the present study we have described a quantitative isotype specific ELISA technique able to determine the binding of IgG, IgM, and IgA antibodies to cardiolipin and other phospholipids. We have found that the use of adult bovine serum to block non-specific binding to plastic microtitre plates and as diluent of serum samples has enabled a more sensitive determination of anti-CL antibodies than was possible using gelatin, bovine serum albumin, or fetal calf serum. Using affinity purified IgG, IgM, and IgA anti-CL antibodies, we have shown that this assay method enables measurement of IgG anti-CL antibody down to a concentration of 20 ng/ml, IgM anti-CL antibody to a concentration of 30 ng/ml, and IgA anti-CL antibody to a concentration of 40 ng/ml. The relation between optical absorbance readings and IgG anti-CL antibody concentration appears to be linear over the range 1000 ng/ml to 100 ng/ml, and this probably represents the most effective range over which these antibodies can be reproducibly measured.

This study also describes methods for the determination of binding anti-PL antibodies to phosphatidylserine and phosphatidylinositol. There was little or no binding to phosphatidylcholine. In general, we found that the binding of antiphospholipid antibodies to cardiolipin correlated strongly with binding to phosphatidylserine and phosphatidylinositol, confirming previous findings that these antibodies bind the phosphodiester group of negatively charged phospholipids. On the basis of these findings, there seems to be little advantage to be gained by the use of phosphatidylerine or phosphatidylinositol in preference to cardiolipin in solid phase anti-PL antibody assay systems.

This study shows that patients with anti-CL antibody related clinical disorders often have more than one class of anti-CL antibodies. It was noteworthy that 36 of the 40 patients with ‘anti-PL antibody associated clinical disorders’ had IgG anti-CL antibodies and nine of the 36 had IgG anti-CL antibodies alone. On the other hand, there were four patients without IgG anti-CL antibodies who were also subject to anti-PL antibody related clinical disorders. Three of these four patients had IgM and IgA anti-CL antibodies, and one had IgA anti-CL antibodies alone. None of the patients studied with ‘anti-PL antibody related clinical features’ had IgM anti-CL antibodies alone. Other studies have shown that IgM anti-CL antibodies may occur alone in some clinical disorders, an example of which is phenothiazine induced autoimmune disorders. Our own and other studies have suggested that patients with phenothiazine induced

### Table 1

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Anticardiolipin antibodies
anti-PL antibodies, whether measured by the lupus anticoagulant test\textsuperscript{6} 23 24 or the anti-PL test,\textsuperscript{22} are not usually subject to "anti-PL antibody associated clinical disorders". This might indicate that clinical complications occur only after the 'class switch' from IgM to IgG or IgA has occurred.

Although there was no correlation between IgM and IgG anti-CL antibody levels, we found that there was good correlation between IgM and IgA anti-CL antibody levels. This correlation could not be explained by cross reactions of the enzyme labelled antihuman IgM and IgA anti-CL antibodies used in this assay method. We believe that our identification of patients with 'anti-PL antibody related clinical disorders' who have IgM together with IgA, but not IgG anti-CL antibodies, provides some justification for measurement of both IgM and IgA anti-CL antibodies when screening patient sera.

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