Cross-reactivity between anti-cardiolipin, anti-high-density lipoprotein and anti-apolipoprotein A-I IgG antibodies in patients with systemic lupus erythematosus and primary antiphospholipid syndrome

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Objectives. Atherosclerosis is an important complication of patients with systemic lupus erythematosus (SLE) and primary antiphospholipid syndrome (APS). One suggested mechanism may be the action of autoantibodies directed against plasma lipoproteins. We studied the presence and patterns of cross-reactivity between antibodies directed against cardiolipin, high-density lipoprotein (HDL) and apolipoprotein A-I (Apo A-I) in patients with SLE and APS.

Methods. Sera from 50 patients (25 SLE and 25 APS) and 10 healthy controls together with three human immunoglobulin G anti-cardiolipin (CL) monoclonal antibodies (IS4, CL1 and CL24) were assessed for the presence of autoantibodies binding cardiolipin, HDL and Apo A-I. Classical inhibition assays were performed to study interference by HDL and Apo A-I in the binding of the human monoclonal antibody to CL. To determine the cross-reactivity patterns between these autoantibodies, sera from 12 patients were incubated on ELISA plates coated with the three different molecules and the captured antibodies were then tested for their activity towards each of the other antigens.

Results. All three monoclonals bound to CL. IS4 and CL1 also bound to HDL but only IS4 bound to Apo A-I. Anti-cardiolipin titres were higher in patients with APS than SLE and in healthy controls ($P < 0.03$ and $P < 0.009$ respectively). Titres of antibodies to HDL were higher in patients with SLE and APS than in controls ($P < 0.009$ and $P < 0.03$ respectively). There were no significant differences with respect to the presence of antibodies binding to Apo A-I. In the SLE population, anti-HDL antibody titres correlated with anti-Apo A-I ($r = 0.563$, $P < 0.004$), but not in patients with APS. In the cross-reactive assay, 11/12 (91.7%) of the samples containing isolated anti-CL antibodies reacted to HDL and 2/12 (16.7%) to Apo A-I. Samples containing isolated anti-HDL antibodies also reacted with CL and Apo A-I (7/12 and 3/12 respectively). All samples collected after incubation with Apo A-I bound to HDL and 6/12 (50%) to CL. There were no differences in the cross-reactivity patterns between patients with SLE and APS.

Conclusions. Patients with SLE and APS have antibodies directed against HDL and Apo A-I. A high percentage of these antibodies cross-react with CL, suggesting the presence of different groups of antibodies with different targets. The study of the interaction between the immune response and the lipoprotein components and
Systemic lupus erythematosus (SLE) is a chronic autoimmune rheumatic disease of multifactorial aetiology, characterized by the potential involvement of virtually every organ and/or system of the body. Most aspects of the immune response are altered, with particular emphasis on the production of multiple autoantibodies, of which anti-DNA is the most characteristic [1].

The antiphospholipid syndrome (APS) is a clinical entity characterized by venous and arterial thromboses and recurrent miscarriages in subjects who are persistent carriers of antibodies to negatively charged phospholipids and/or coagulation related proteins [2]. Although the initial autoantigens considered were negatively charged phospholipids [3], subsequent studies have shown a much broader range of targets, including α2-glycoprotein I (α2-GP1) [4, 5] and clotting and complement factors [6, 7]. Furthermore, cross-reactivity between antiphospholipid antibodies and other proteins and cell components has been widely documented, supporting the systemic character of these diseases [8–10].

In the last decade, atherosclerosis has been identified as a significant feature of patients with SLE and those with APS. Although data regarding the objective assessment of atheroma progression in these patients are scarce, an increased risk of atherosclerosis-related events has been reported in different studies [11, 12]. These clinical findings have led to the pursuit of better understanding of the mechanisms and risk factors involved. In particular, attention has focused on the study of plasma oxidative status [13] and the lipoprotein profile in these patients [14].

In patients with SLE, a proatherogenic lipid profile has been found in relation to steroid intake [15]. However, some of the changes found in the plasma lipid content of these patients suggest the existence of other factors ultimately related to the disease itself [16]. Another interesting aspect relates to the interaction between the immune response and plasma lipids, with the recent identification of antibodies against plasma lipoproteins and their constituents [17, 18]. However, most of the studies have looked only at the presence of antibodies against low-density lipoprotein (LDL), suggesting that this activity is a cross-reaction with antiphospholipid (aCL) antibodies [19]. The identification of apolipoprotein A-I (Apo A-I) [a major constituent of the high-density lipoprotein (HDL) complex] as a target for the immune response in patients with SLE [18] suggests that HDL may also be an important player in this context. The presence of cardiolipin in both lipoprotein complexes [20] raises the possibility of cross-reactivity with aCL antibodies. This aspect might not be easy to assess in view of the affinity between the lipid content of the antigens. In our study, we looked for the presence of antibodies to cardiolipin, HDL and Apo A-I and at the different patterns of cross-reactivity between these different antibodies. Classical inhibition assays have been unable to address this issue because of the affinity between cardiolipin and the HDL complex. We therefore developed a novel method to study the cross-reactivity of these antibodies reliably. We demonstrate for the first time that two distinct autoantibody populations exist. One population cross-reacts with both cardiolipin and HDL whilst the other is relatively specific for HDL. In the light of these studies, we propose a different method of studying the affinity and pattern of cross-reactivity of these antibodies in patients with SLE and APS.

**Patients and methods**

Fifty consecutive patients (25 SLE and 25 primary APS) attending the lupus clinic at the Centre for Rheumatology, Middlesex Hospital, were selected along with 10 age- and sex-matched healthy controls (Table 1). All patients with SLE fulfilled the revised American College of Rheumatology criteria [21] and the patients with primary APS fulfilled the Sapporo criteria [22]. Sera from the patients were collected and stored at −80°C and were tested simultaneously for aCL, anti-HDL (aHDL) and anti-Apo A-I (aApo A-I) immunoglobulin G (IgG) antibodies. From this cohort, 12 patients (six SLE and six APS) were selected for the cross-reactivity studies on the basis of their high titres of aCL and aHDL antibodies. The study was carried out according to the Declaration of Helsinki and the subjects gave informed consent before participation in the study.

Three aCL IgG human monoclonal antibodies (IS4, CL1 and CL24), obtained by culture of human hybridoma cell lines from patients with APS [23] kindly provided by Dr Pojen

| TABLE 1. Demographic data and aCL, aHDL and aApo A-I antibody titres in patients with SLE and APS and in healthy controls |
|---------------------|---------------------|---------------------|---------------------|
|                      | Controls            | SLE patients        | APS patients        |
| Age (yr)             | 29 ± 2.9            | 32.4 ± 4.1          | 31.3 ± 3.8          |
|                      | (n.s.)              | (n.s.)              | (n.s.)              |
| Females/males        | 9/1                 | 23/2                | 21/1                |
| Disease duration (yr) | n.a.               | 5.7 (1–10.3)        | 5.4 (1–8.7)         |
| Steroids             | n.a.               | 20 (80%)            | 22 (88%)            |
| Warfarin             | n.a.               | 8.5 ± 6.0           | 14.1 ± 4.7          |
|                      |                     | (n.s.)              | (n.s.)              |
| aHDL (GPL)           | 8.5 ± 6.0           | 14.1 ± 4.7          | 29.0 ± 7.0 (P = 0.008) |
| aHDL (OD) (% of positive control) | 29.0 ± 10.7 | 68.0 ± 7.9 (P = 0.008) | 61.4 ± 10.1 (P = 0.02) |
| aApo A-I (OD) (% of positive control) | 22.4 ± 3.9 | 31.3 ± 3.8 (n.s.) | 26.6 ± 5.0 (n.s.) |

Values (except numbers of patients) are mean ± S.E.M. P values refer to comparisons between patient groups and controls. aCL antibody titres were also significantly different between patients with SLE and APS (P < 0.03). GPL, IgG phospholipid unit; n.s., not significant; n.a., not applicable; OD, optical density.
IgG aCL ELISA

Half of the 96-well γ-irradiated microtitre plates (Polyserp; Nunc Life Technologies, Paisley, UK) were coated overnight at 4°C with 50 µg/ml cardiolipin (Sigma-Aldrich) in ethanol. Plates were blocked for 1 h at 37°C with 10% fetal calf serum in phosphate-buffered saline (PBS) and 100 µl samples (1:100 dilution) and positive control were added in duplicate for 1 h at 37°C. After washing three times, 100 µl of alkaline phosphatase-conjugated anti-human IgG (1:1000 in the blocking agent) was added for 1 h (37°C). p-Nitrophenyl phosphate, (Sigma-Aldrich; 100 µl in 5 ml diethanolamine buffer, pH 9.8) was added, the reaction was incubated at 37°C for colour development, and the absorbance was read at 405 nm after 1 h.

Assays were standardized with sera calibrated against the appropriate international reference material [24] and the results were reported as anti-phospholipid (GPL) units.

IgG aHDL ELISA

HDL was isolated from healthy subjects as described previously [25]. Ninety-six-well plates (Polyserp) were half-coated overnight at 4°C with 20 µg/ml human HDL in 70% ethanol. Blocking was performed using PBS containing 2% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at room temperature. Samples were diluted 1:100 in PBS containing 2% BSA. Samples and positive controls (100 µl) were added to duplicate wells for 1 h at room temperature. After washing three times, 100 µl of alkaline phosphatase-conjugated anti-human IgG (1:1000 in the blocking agent) was added for 1 h. p-Nitrophenyl phosphate (100 µl; Sigma-Aldrich) in 5 ml diethanolamine buffer (pH 9.8) was added, the reaction was incubated at 37°C for colour development, and the absorbance was read at 405 nm after 1 h. All assays were validated by the inclusion of internal quality control samples of known activity.

The results were expressed as a percentage of the positive control present in each plate after subtraction from the background in the uncoated half of the plate.

IgG aApo A-I ELISA

Ninety-six-well plates (Polyserp) were half-coated overnight at 4°C with 10 µg/ml human Apo A-I (Sigma-Aldrich) in PBS. Blocking was performed using PBS containing 2% BSA (Sigma-Aldrich) for 1 h at 37°C. Serum samples (1:100 dilution in PBS containing 2% BSA) and hybridoma cell culture supernatant containing human IgG aCL monoclonal antibodies (neat) were loaded in duplicate along with a positive control (kindly supplied by Dr Joan Merrill, Oklahoma University, OK, USA) and the plates were incubated for 1 h at 37°C. After washing, 100 µl of alkaline phosphatase-conjugated anti-human IgG (1:1000 in the blocking agent) was added for 1 h. p-Nitrophenyl phosphate (100 µl; Sigma-Aldrich) in 5 ml diethanolamine buffer (pH 9.8) was added, the reaction was incubated at 37°C for colour development, and the absorbance was read at 405 nm after 1 h. All assays were validated by the inclusion of internal quality control samples of known activity. The results were expressed as the percentage of the positive control value for each plate after subtraction from the background in the uncoated half of the plate.

Inhibition assays

HDL and Apo A-I inhibition of the binding of IS4 to cardiolipin. IS4 was incubated in the fluid phase, at concentrations ranging from 0.8 to 0.004 µg/ml, with human HDL (obtained from a healthy control) at 400–3.125 µg/ml and with Apo A-I (Sigma-Aldrich) at 40–0.3 µg/ml for 1 h at 37°C.

Ninety-six-well Polysorp plates were half-coated with cardiolipin (Sigma-Aldrich) at 50 µg/ml in ethanol, and the samples (IS4 + HDL and IS4 + Apo A-I) were tested using the aCL ELISA assay described above.

IS4 binding to HDL and HDL + CL in inhibition assay

Ninety-six-well Polysorp plates were half-coated with HDL in ethanol at 5 µg/ml, 100 µl well, overnight at 4°C. Blocking was performed using PBS containing 2% BSA (Sigma-Aldrich) for 1 h at room temperature. Plates were then washed once using PBS. To half of the wells coated with HDL, cardiolipin in decreasing concentrations (125 to 0.5 µg/ml in ethanol) was added for 1 h at 37°C. After five washes with PBS, IS4 (0.8 µg/ml) in PBS was added to all the wells. Alkaline phosphatase-conjugated anti-human IgG (100 µl, 1:1000) was added for 1 h. p-Nitrophenyl phosphate (100 µl; Sigma-Aldrich) in 5 ml diethanolamine buffer (pH 9.8) was added, the reaction was incubated at 37°C for colour development, and the absorbance was read at 405 nm after 30 min. The results were expressed as optical density (OD) after subtraction of the background in the non-coated half of the plate.

Cross-reactivity assay

A 96-well Polysorp plate was divided into three parts, which were coated with CL (50 µg/ml in ethanol), HDL (20 µg/ml in ethanol) and Apo A-I (10 µg/ml in PBS), respectively, at 200 µl/well, overnight at 4°C. Plates were blocked with 10% fetal calf serum in PBS (for CL) and 2% BSA/PBS for HDL and Apo A-I. IS4, CL1 and CL24 IgG human monoclonals (neat) and sera from 12 patients (1:20) were added in quadruplicate. After washing five times with PBS, 250 µl/well of glycine buffer (pH 2.0) was added for 5 min to elute antibodies that remained bound to each of the coating antigens. The contents of the four wells of each sample were then collected and the pH was normalized by adding Tris buffer (1 M, pH 9.0, 20 µl). Each sample was then added to a second ELISA plate, previously coated with CL (50 µg/ml in ethanol), HDL (20 µg/ml in ethanol), Apo A-I (10 µg/ml in PBS) and anti-human IgG (2.5 µl/ml) (Sigma-Aldrich) (100 µl/well in duplicate for each antigen), and blocked as described before. Human IgG was used as a positive control, to assess the presence of antibody after elution from the first plate with glycine buffer. After washing, 100 µl of alkaline phosphatase-conjugated anti-human IgG (1:1000) was added for 1 h. p-Nitrophenyl phosphate (100 µl; Sigma-Aldrich) in 5 ml diethanolamine buffer (pH 9.8) was added, the reaction was incubated at 37°C for colour development, and the absorbance was read at 405 nm after 1 h. Results were expressed as the ratio between binding to each antigen and binding to total IgG for each plate after subtraction of the background value in the uncoated half of the plate. Samples were considered positive when the referred ratio (antibody binding/free total IgG binding) was above 1.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA). Non-parametric tests were employed to compare differences between groups (Kruskal–Wallis test) and to evaluate associations between variables (Spearman’s rank correlation coefficient).
Stepwise multiple regression analysis was used to test the independence of the associations detected by univariate analysis.

Results

Demographic data for patients and controls are shown in Table 1.

**Binding of IS4, CL1, CL24 and patient sera to CL, HDL and Apo A-I**

All monoclonal antibodies bound to cardiolipin as expected. IS4 also bound strongly to HDL and Apo A-I (OD 1.68 and 1.88 respectively) after 30 min. CL1 reacted strongly with Apo A-I (OD 2.27 after 30 min) but not as strongly with HDL (OD 0.84, 30 min). CL24 had little activity against either HDL or Apo A-I, but not as strongly with HDL (OD 0.84, 30 min). CL24 reacted strongly with Apo A-I (OD 2.27 after 30 min) and Apo A-I (OD 1.68 and 1.88 respectively) after 30 min. CL1 and IS4 also bound strongly to HDL and Apo A-I. Whilst aCL antibodies showed activity towards HDL, IS4 did not inhibit the binding of this antibody to CL.

**Relationship between antibody titres**

IgG aHDL antibodies were higher in the samples of patients with APS when compared with patients with SLE and healthy controls (P < 0.03 and P < 0.009 respectively). However, aHDL titres were higher in both SLE and APS when compared with healthy controls (P < 0.009 and P < 0.03 respectively), but there was no difference between the two patient groups. Although a-Apo A-I titres were generally higher in the samples of patients with SLE, the difference did not reach statistical significance when compared with patients with APS (P = 0.3) or healthy controls (P = 0.1) (Table 1).

**Multiple regression model**

To determine which antibody was most closely associated with aHDL titre, stepwise multiple regression was employed. The IgG aHDL titre was the dependent variable and aCL, a-Apo A-I and total HDL cholesterol were the independent variables, and were corrected for age, gender and steroids and warfarin intake. In this model, a-Apo A-I was the only independent predictor of aHDL antibodies (r = 3.715, P = 0.001).

**Inhibition assays**

**HDL and Apo A-I inhibition of binding of IS4 to cardiolipin.** When increasing concentrations of HDL were preincubated with IS4 (0.4 μg/ml), the HDL concentration (3.125–400 μg/ml) was strongly correlated with binding to CL (t = 10.777, R² = 0.95, P < 0.0001) (Fig. 2A). Preincubation of Apo A-I (0.3–40 μg/ml) with IS4 did not inhibit the binding of this antibody to CL.

**IS4 binding to HDL and HDL+CL in inhibition assay.** IS4 bound to both HDL-coated wells and to HDL+CL wells. Binding (OD) directly correlated with the concentration of CL added to the plate (t = 8.723, P < 0.0001). There were no changes in OD in the wells coated with a constant amount of HDL when no CL was added (Fig. 2B).

**Cross-reactivity assay**

All three monoclonal antibodies showed activity (although at different levels) towards CL, HDL and Apo A-I in the second plate following elution from plate 1 and after incubation with the same antigens (Fig. 3). All samples were positive for human IgG in the second plate, and there were no significant differences between the different antigens used in plate 1. After removal of samples from plate 1, an ELISA for aCL was performed to assess the amount of antibody remaining in the plate. The percentage of removal (based on OD reduction) was approximately 50%, with no significant differences for the different antigens used (data not shown).

Sera from 12 patients were incubated in ELISA plates with CL, HDL and Apo A-I. After washing and elution of the binding antibodies, a second ELISA assay was performed for each antigen. Eleven samples (91.7%) containing aCL antibodies showed activity towards HDL and 2/12 (16.7%) were positive for a-Apo A-I antibodies. When the samples were first tested for HDL, 7/12 (58.3%) were positive for CL in a second assay and 3/12 (25.0%) were positive for Apo A-I. After incubation with Apo A-I as first antigen, 6/12 (50.0%) had activity towards CL in the second assay. All the samples collected after incubation with Apo A-I had reactivity towards HDL. There were no differences in the cross-reactivity patterns between patients with SLE and APS (Fig. 4).

**Discussion**

Patients with SLE and APS have antibodies directed against CL, HDL and Apo A-I. Whilst aCL antibodies are frequently present in patients with SLE and are the
hallmark of the phospholipid syndrome, there are few reports examining the presence of apo A-I antibodies in patients with SLE and there is only one previous study of which we are aware showing the presence of apo HDL antibodies in SLE and APS. Even though it has been recognized that a relationship could exist between aCL and apo A-I in SLE patients and that apo HDL antibodies showed a pattern of disease distribution different from aCL, the possibility of cross-reactivity among these antibodies has not been addressed before. So far, antibody activity against lipoproteins has been considered only in relation to LDL, particularly oxidized LDL (oxLDL) and cross-reactivity between aCL and anti-oxLDL antibodies has been shown [17]. However, aCL antibodies isolated from patients with SLE showed different binding patterns to oxLDL and β2-GPI, suggesting that different subtypes might exist [19].

Our initial studies of three monoclonal human IgG aCL antibodies revealed that they all bind to HDL and apo A-I, suggesting that cross-reactivity could occur in human sera. Monoclonal antibodies were used in this study for two main reasons: (i) to standardize the assay; and (ii) to demonstrate that the identified cross-reactivity was not a consequence of generalized polyclonal antibody production.

In our cohort, antibodies against apo A-I correlated with apo HDL titres, and were found to be an independent
component of the immune complex. This hypothesis bind to CL used as antigen on the plate, via the HDL HDL) following their coincubation, which would in turn is that IS4 and HDL formed immune complexes (IS4–HDL actually increased the binding of IS4 to CL instead with the concentration of HDL used as an inhibitor. A possible explanation for this finding ever, the binding of IS4 to CL was directly correlated evaluated following its incubation with HDL and Apo A-I. There was no inhibition in any of the cases; how- to CL and Apo A-I after elution from HDL-coated plates. CL and Apo A-I after elution from HDL-coated plates. (C) Binding of patient sera to CL and HDL after elution from Apo A-I coated plate. Total human IgG was used as the positive control in all plates. Results (y axis) are ratios between binding to each antigen and binding to total IgG.

predictor of the latter, even when corrected for aCL titres, total HDL cholesterol, age, gender and treatment. This observation reinforces the concept that cross-reactivity may be the main mechanism underlying these binding patterns. This finding was not unexpected as Apo A-I is present in the HDL complex. However, we could not find a correlation between the aCL and aHDL titres, even though HDL carries negatively charged phospholipids, including cardiolipin [20]. This finding suggests that aCL and aHDL belong to different families of autoantibodies. In order to address this possibility, we performed inhibition studies with IS4, a human IgG aCL monoclonal antibody. Binding of IS4 to CL was evaluated following its incubation with HDL and Apo A-I. There was no inhibition in any of the cases; however, the binding of IS4 to CL was directly correlated with the concentration of HDL used as an inhibitor. HDL actually increased the binding of IS4 to CL instead of inhibiting it. A possible explanation for this finding is that IS4 and HDL formed immune complexes (IS4–HDL) following their coincubation, which would in turn bind to CL used as antigen on the plate, via the HDL component of the immune complex. This hypothesis was tested in the second part of the experiment, which suggested that CL was absorbed into the HDL complex coated on the plate, as shown by the strong correlation between the binding of IS4 and the concentration of CL in the system. The affinity between CL and HDL could thus have hindered the study of cross-reactivity of the antibodies against these two antigens.

To overcome this problem, we used a different method for the characterization of the cross-reactivity patterns of these antibodies. Autoantibodies were first isolated from serum by incubation with CL, HDL and APO A-I on a polystyrene ELISA plate. The binding of one autoantigen-specific eluted antibody was then tested against that of the other antigens. Our data showed cross-reaction between aCL and aHDL antibodies in the majority of the patients. Only a few patients demonstrated cross-reactivity between CL and Apo A-I, suggesting that these are a different subset of antibodies. Interestingly, antibodies selected for their binding to HDL on the first plate cross-reacted with CL (7/12) and Apo A-I (3/12), but when Apo A-I was used as an initial antigen all the selected antibodies cross-reacted with HDL. This suggests that aHDL antibodies (as described previously [26] and confirmed in this study) may take part in at least two different types of cross-reactivity: one with CL and the other with a different antigen, probably Apo A-I. Finally, there is also a group of antibodies reacting against both CL and Apo A-I, in a similar way to the independent activity against β2-GP1 found in some aCL antibodies [5].

The importance of these findings lies in the increased recognition of the protective role of HDL and Apo A-I against atherosclerosis. HDL has been shown to prevent the oxidation of LDL and its consequent uptake by monocytes, in this way preventing the formation of ‘foam cells’, one of the most important steps in athero-genesis [28]. This anti-oxidant mechanism is due mainly to the presence in the HDL particle of an antioxidant enzyme called paraoxonase. This enzyme is stabilized by the presence of Apo A-I, contributing to its optimal activity [29]. A significant reduction in paraoxonase activity has been reported in patients with SLE and APS, reinforcing the importance of this enzyme in these conditions [26]. Cross-reactivity between aCL antibodies and HDL or Apo A-I, by tampering with any of these structures, may contribute to the increased atherogenesis found in these conditions.

In this study we confirmed the presence of antibodies against both HDL and Apo A-I in patients with SLE and APS, and we propose a different method of studying the cross-reactivity between these antibodies in order to avoid possible misinterpretation due to the affinity between HDL and CL. The full characterization of these autoantibodies might uncover different antibody sub-types that relate to particular clinical features and/or pathological aspects of these conditions.

Further large-scale epidemiological studies should be put in place to determine the importance of these specific immune markers in relation to the progression of atherosclerosis.
Conflict of interest

The authors have declared no conflicts of interest.

References