Anticardiolipin Antibodies From Patients With the Antiphospholipid Antibody Syndrome Recognize Epitopes in Both β2-Glycoprotein 1 and Oxidized Low-Density Lipoprotein

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Background—We recently suggested that many anticardiolipin antibodies bind only to oxidized cardiolipin (OxCL) and/or to OxCL–β2-glycoprotein 1 (β2GP1) adducts but not to a “reduced” cardiolipin that is unable to undergo oxidation. To test this hypothesis, we investigated 24 sera, 4 protein A–purified IgG fractions, and 3 human monoclonal antibodies that were all isolated from patients with antiphospholipid antibody syndrome (APS); testing was also performed in 7 controls. Two monoclonal antibodies (IS3 and IS4) were selected for binding to CL and one was selected for binding to β2GP1 (LJB8).

Methods and Results—By chemiluminescent immunoassay, all APS sera samples bound only to OxCL and not to reduced CL, and the binding was inhibited >95% by OxCL but not reduced CL. All purified IgG fractions bound to β2GP1 but only when the β2GP1 was plated on microtiter wells coated with OxCL. All 3 monoclonal antibodies bound only to OxCL. On Western blots, IS4 and LJB8 bound to β2GP1 as well as to delipidated apoB of oxidized LDL but not to native apoB. IS3 also bound to oxidized apoB on Western blot. Covalent modification of β2GP1 with oxidation products of CL made it more antigenic for APS serum samples, for purified IgG fractions, and for the monoclonal antibodies.

Conclusions—These data support the hypothesis that oxidation of CL is needed to generate epitopes for many anticardiolipin antibodies and that some of these epitopes are covalent adducts of OxCL with β2GP1 or apoB. (Circulation. 2001;103:941-946.)

Key Words: antibodies, antiphospholipid lipoproteins autoimmunity

Antiphospholipid antibodies (aPL) are heterogeneous autoantibodies detected in vitro by solid-phase immunoassays as antibodies binding to phospholipids such as cardiolipin (CL) or to complexes of phospholipid and β2-glycoprotein 1 (β2GP1).1–6 Patients with high levels of aPL are prone to fetal loss, autoimmune thrombocytopenia, and thrombotic events in either the venous or arterial circulation.4–7 Elevated levels of aPL in combination with one or more clinical features has been termed the antiphospholipid antibody syndrome (APS).

The exact nature of the epitope(s) for anticardiolipin antibodies (aCL) has been controversial.4–6 We recently demonstrated that CL is rapidly oxidized when plated on microtiter wells and exposed to air—as is done in conventional solid-phase aCL immunoassays.5 We also showed that a few selected reference sera and affinity-purified aCL-IgG from APS patients progressively bound to CL as it was oxidized but not to a “reduced” CL (CLred) analogue that was unable to undergo lipid peroxidation (all 4 unsaturated fatty acids in CLred are hydrogenated to saturated fatty acids). We proposed that aCL bind to epitopes generated when CL undergoes oxidation.5,9 β2GP1 is a phospholipid-binding apolipoprotein (also called apolipoprotein H) that seems to be necessary for the binding of some aCL.2–3 It has been proposed that, as a result of noncovalent protein-lipid interactions, novel, conformational epitopes are created on the plated CL, on β2GP1, or on an admixture of these two, or that β2GP1 alone is the target antigen.5–10,11 We demonstrated that some aPL bind to proteins like β2GP1 only as a consequence of covalent adduct formation between oxidized phospholipids (OxPL) and the protein.9 The formation of neoepitopes between OxPL and associated proteins would be analogous to LDL oxidation, which generates immunogenic neoepitopes.8,12,13 Autoantibodies to OxLDL are present in the sera of animals and humans and are increased in those with increased atherosclerosis.14,15 Indeed, aCL in patients with systemic lupus erythematosus cross-react with oxidized LDL (OxLDL).16
In the present article, we demonstrate that oxidized CL (OxCL) and covalent adducts of OxCL with β2GP1 are epitopes for many aCL. In addition, we show that “native” β2GP1 and OxLDL share common epitopes recognized by monoclonal antibodies cloned from APS patients.

Methods

Materials

CL (diphasphatidylglycerol, bovine heart) containing 4 unsaturated fatty acids and hydrogenated CL (CLred) containing 4 saturated fatty acids were obtained from Avanti Polar Lipids. Fatty acid analysis confirmed that linoleic acid accounted for >92% of the fatty acids of CL, whereas all fatty acids in CLred were saturated (18:0). CL was oxidized by air exposure to generate various decomposition products, as previously described.8 Human β2GP1 was purified as previously described.17

Human Subjects

Serum samples from 21 women and 1 man with APS and from 7 healthy controls were collected at the Department of Obstetrics and Gynecology of the University of Utah Hospital. Two patients had donated a serum sample at 2 different times, 7 and 8 years apart, for a total of 24 serum samples from the APS patients. APS patients had ≥1 of the following clinical features: (1) a history of either ≥1 fetal deaths or ≥3 consecutive pregnancy losses, (2) venous or arterial thrombosis, or (3) autoimmune thrombocytopenia. Six patients had systemic lupus erythematosus. All patients had aCL IgG, as measured by a standardized assay18 in Dr Branch’s laboratory (19 samples were >100 IgG phospholipid-binding units and 6 samples were 30, 40, 44, 47, 62, and 75 IgG phospholipid-binding units, respectively).

Chemiluminescent Immunoassay for Antibody Binding

CL or CLred in 100% ethanol was added at 25 µg/mL into white, round-bottomed High Binding Microfluor (Dynex) microtitration plates and exposed to air for the indicated time at room temperature to induce oxidation. Absolute ethanol was added to blank wells. The wells were washed with PBS buffer containing 0.27 mmol/L EDTA and blocked with 10% fetal bovine serum, 1% bovine serum albumin (BSA), or 0.25% gelatin in indicated experiments. The primary antibodies were incubated for 1 hour, and the amount of antibody bound was measured with alkaline phosphatase-labeled goat anti-human IgG (Sigma) using LumiPhos 530 (Lumigen) as the substrate. Chemiluminescence was measured in relative light units (RLU) with a Dynex Luminometer (Dynex Technologies).8,9 Each point in each of the figures is the mean of triplicate determinations.

Preparation of OxCL-β2GP1

CL was dried and exposed to air for 3 hours. Purified human β2GP1 (in PBS and 20 µmol/L EDTA) and NaCNBH3 (10 mmol/L) were added and incubated at 37°C for 6 hours. After incubation, 80 mmol/L octylglucoside was added and dialyzed against PBS to remove the unbound CL.

Protein A Purification of IgG

Whole IgG fractions were purified using ImmunoPure Plus Immobilized Protein A IgG Purification Kit (Pierce). The absence of β2GP1 in the IgG fractions was verified by a capture assay. Samples were incubated in wells coated with polyclonal anti-human β2GP1 antibody (10 µg/mL; Enzyme Research Laboratories) and by detecting the amount of β2GP1 captured with the biotinylated anti-human β2GP1 antibody and alkaline phosphatase–labeled avidin (Pierce).

Results

Antibody Binding to OxCL and CLred

Figure 1 demonstrates that IgG binding was substantially higher to OxCL than to CLred for each of the APS serum samples. Control samples had low binding to both antigens. When the assay was repeated in the absence of 10% bovine serum, virtually identical results were observed (data not shown).

Because the aCL binding has been suggested to depend exclusively on the presence of β2GP1, we tested whether β2GP1 binds to both OxCL and CLred to the same extent. Figure 2A shows an example in which there is increased IgG binding from one APS serum sample to the OxCL but no binding to CLred. In the same experiment, we used an anti-β2GP1 antibody to demonstrate that substantial amounts of human β2GP1 (from the added serum) bound to both OxCL and CLred when measured in parallel wells under identical conditions (Figure 2A). This suggests that the binding of aCL to phospholipids does not depend exclusively on the presence of β2GP1.
Specificity of aCL IgG Binding

The serum samples were preincubated with OxCL or CLred, and the supernatants were then tested for binding to OxCL. Figure 2B shows that the preincubation of 1 APS serum sample with OxCL but not CLred removed 95% of the original aCL binding. Using a capture assay (see Methods) we found that 20% of the total β2 GP1 content was absorbed from the 1:50 dilution of serum during the preincubation with OxCL or CLred (Figure 2B).

Figure 3 demonstrates that preincubation of all APS serum samples with OxCL removed >95% of the IgG binding to OxCL. A control incubation for each sample without phospholipid did not remove any IgG binding to OxCL (data not shown). To examine whether a population of antibodies binding to OxCL could also bind to another OxPL epitope, we tested the IgG binding to copper-oxidized LDL (CuOx-LDL). Figure 3 also shows that preincubation with OxCL absorbed >40% of the binding to CuOx-LDL. Preincubation with CLred did not remove any IgG binding to either OxCL or CuOx-LDL. Using similar competition assays, we demonstrated that even the slight degree of binding to CLred observed with a few of the APS samples (Figure 1) was nonspecific (data not shown).

Binding of Human Monoclonal IgG aCL Antibodies

Figure 4 shows that monoclonal antibodies IS4 (selected for binding to CL) and LJB8 (selected for binding to β2GP1) had high binding to OxCL but not to CLred. Both of these monoclonals also bound to CuOx-LDL and to another model epitope, malondialdehyde-modified LDL (MDA-LDL) but not native LDL. Monoclonal antibody IS3 (selected for binding to CL) also showed identical results (data not shown). To test if these monoclonals specifically recognized lipid-protein adducts, we performed Western blot analyses. Figure 5A shows that IS4 bound to the protein of both MDA-LDL (lane E) and CuOx-LDL (lane F), but not to native LDL (lane D) or BSA (lane B). In addition, IS4 showed strong binding to β2GP1 (lane C). Figure 5A also demonstrates the absence of human β2GP1 on native-LDL (lane I) or CuOx-LDL (lane J). Figure 5B shows that LJB8 bound to β2GP1 (lane B), MDA-LDL (lane D), and CuOx-LDL (lane E) but not to native-LDL (lane C) or BSA (lane F). IS3 (Figure 5B) also showed binding to MDA-LDL (lane I) and CuOx-LDL (lane...
but not to native LDL (lane H) or to β₂GP1 (lane G). These data clearly demonstrate that IS4 and LJB8 recognize similar oxidatively-modified protein moieties of CuOx-LDL, MDA-LDL, and β₂GP1 but that IS3 seems to recognize a different epitope.

Antibody Binding to Oxidatively Modified Human β₂GP1

To test whether OxCL on microtiter wells could modify β₂GP1 and create epitopes for aCL antibodies, we first dried OxCL, CLred, or solvent only (ethanol) in microtiter wells before adding β₂GP1. Figure 6 demonstrates that most of the APS serum samples showed increased binding to the native unmodified β₂GP1 compared with BSA (mean, 30 776 versus 6445 RLU/100 ms, respectively; P<0.001 when using Student’s paired t test), the binding to OxCL-β₂GP1 (mean, 138 907 RLU/100 ms) increased 4-fold (P<0.001). The control samples showed very little binding to either native β₂GP1 or OxCL-β₂GP1 (Figure 7). There was a positive correlation between the measurements of IgG binding to OxCL and to OxCL-β₂GP1 among the APS samples (r=0.84, P<0.0001, linear regression analysis). The protein A–purified IgG fractions IS4 and LJB8 also had increased binding to OxCL-β₂GP1 compared with the native β₂GP1 (data not shown).

Figure 6. Immunoassay of protein A–purified IgG fractions from sera of 4 APS patients (APS-IgG #1 through #4) and 1 control subject (human IgG). Wells were coated with OxCL or CLred (25 μg/mL) or ethanol only. After 4 washes, wells were incubated with β₂GP1 (10 μg/mL in PBS buffer) or 1% BSA-PBS buffer for 1 hour at room temperature, and amount of IgG or β₂GP1 bound to wells was measured with appropriate antibodies.
Discussion

On the basis of previous observations, we proposed that some aCL bind to neoepitopes of OxPL or to neoepitopes generated by adduct formation between reactive breakdown products of OxPL and associated proteins. We now demonstrate that most aCL in the tested APS sera require CL oxidation for the CL to be an antigen. Furthermore, our data using purified IgG fractions indicate that CL oxidation is required even in the presence of $\beta_2$GP1 (ie, $\beta_2$GP1 alone or bound to CLred does not yield epitopes). Two monoclonal antibodies from two APS patients (IS4 and LBJ8) bound to both $\beta_2$GP1 and to the apoB of OxLDL but not to native apoB, implying similar oxidized lipid-protein epitopes on these different proteins. Antibody IS3 bound to both OxCL and oxidized apoB but not to $\beta_2$GP1, implying variations in oxidized lipid-protein epitopes. We postulate that a large number of different lipid-protein and even lipid-lipid adducts could form when phospholipids undergo oxidation. This would be analogous to the adduct formation between oxidized lipids and apoB that occurs during LDL oxidation. Indeed, the recent observation that CL is found in the circulation suggests that OxCL-apoB adducts form on OxLDL.

$\beta_2$GP1 has been reported to be the primary serum “cofactor” or target antigen for many aCL. We and others have reported that other proteins, such as polylysine, LDL, and apoAI, also have cofactor activity. $\beta_2$GP1 seems to be an excellent cofactor because of its high avidity to phospholipids and its ability to form adducts with OxCL (and possibly other phospholipids). To test this idea, we demonstrated that the binding of the purified IgG fractions to $\beta_2$GP1 occurred only when the $\beta_2$GP1 was plated with OxCL (Figure 6). Also, we demonstrated that APS sera, purified IgG fractions, and human mononuclears all showed strong binding to the OxCL-$\beta_2$GP1 adduct. Furthermore, among all APS sera, the binding to OxCL-$\beta_2$GP1 correlated well with the binding to OxCL. These data suggest that not only does $\beta_2$GP1 readily form adducts with OxPL in the microtiter wells, but it may already contain some oxidized lipid-protein epitopes. In support of this, we demonstrated that monoclonal antibodies cloned from APS patients recognized epitopes not only on native $\beta_2$GP1 but also on oxidatively modified apoB on Western blots. These data strongly indicate that these epitopes are covalently oxidized lipid-protein adducts on $\beta_2$GP1 and apoB. In the case of $\beta_2$GP1, this may occur in plasma in vivo or during the isolation procedure ($\beta_2$GP1 is often isolated with a method involving perchloric acid precipitation that generates a strong pro-oxidant condition).

There has been considerable difficulty in generating reliable and reproducible clinical assays for measuring aCL, which we believe is partly because of the oxidation of CL. In fact, there is variability not only between different preparations but even in the same CL preparation depending on its “age” (even if stored at $-70^\circ$C under argon). Because the rate of CL oxidation is extremely difficult to control or standardize, an alternative approach might be to use an adduct between OxCL and $\beta_2$GP1 (or other protein) as an antigen. If many aPL are, in fact, directed against oxidation-dependent epitopes and because many OxPL products can form, it is likely that some aPL are against unique oxidation-specific structures whereas other aPL are against more common oxidation-dependent structures. These data imply heterogeneity even among aPL to OxCL epitopes. Moreover, our data do not address the observations that there are many oxidation-independent antibodies that bind exclusively to conformational changes or even primary sequences of $\beta_2$GP1, independent of any bound lipid or lipid-$\beta_2$GP1 adducts. Furthermore, there may also be antibodies against conformational changes in either CL, $\beta_2$GP1, or prothrombin.

There is controversy over which type of antibodies are best associated with various aspects of clinical disease. Knowledge that many aPL can be oxidation-dependent may give insight into some of the pathogenic events underlying the clinical manifestations of APS. These data suggest that inflammatory conditions and an attendant pro-oxidant state are associated with the generation of epitopes to many aPL.

References


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