Oxidized Low-Density Lipoprotein/β_2-Glycoprotein I Complexes and Autoantibodies to oxLig-1/β_2-Glycoprotein I in Patients With Systemic Lupus Erythematosus and Antiphospholipid Syndrome

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Abstract

Oxidized low-density lipoprotein (oxLDL) interacts with β_2-glycoprotein I (β_2-GPI) via oxLDL-derived specific ligands (oxLig-1) forming complexes. The prevalence and significance of oxLDL/β_2-GPI complexes and antibodies to oxLig-1/β_2-GPI were evaluated in patients with systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS). The oxLDL/β_2-GPI complex was 69% positive (above mean + 3 SD of control subjects) in 97 consecutive patients with SLE, 62% in 40 patients with SLE with secondary APS, and 60% in 50 control patients with SLE without APS. IgG anti–oxLig-1/β_2-GPI antibody was positive in 31 (32%) of 97 consecutive patients with SLE, in 26 (65%) of 40 patients with SLE with secondary APS, and in 6 (19%) of 32 control patients with SLE. Anti–oxLig-1/β_2-GPI antibodies were 93.7% specific with a positive predictive value of 90.0% for APS, better than anticardiolipin antibodies (80.0% specific, 71.4% predictive value). These results confirm that oxLDL/β_2-GPI complexes are common in SLE and suggest a possible immunogenic role in APS. In contrast, IgG anti–oxLig-1/β_2-GPI antibodies not only are associated with but also are clinically useful risk factors for APS.

Vascular thromboembolic events, pregnancy morbidity (miscarriages and fetal loss), and thrombocytopenia in association with the presence of elevated serum levels of antiphospholipid antibodies are common clinical features of the antiphospholipid syndrome (APS). APS is classified as primary if there is no coexisting autoimmune disease or secondary when present in the context of an autoimmune disorder. There is considerable evidence to suggest a pathogenic role of antiphospholipid antibodies in the development of these clinical features.[1-3] Antiphospholipid antibodies are a heterogeneous group of autoantibodies characterized by their reactivity to anionic phospholipids, phospholipid/protein complexes, and certain proteins presented on suitable surfaces in the absence of phospholipids, ie, activated cell membranes and oxygenated polystyrene.[4-6]

Several plasma proteins that participate in coagulation and interact with anionic phospholipids have been reported to function as antiphospholipid cofactors, eg, β_2-glycoprotein I (β_2-GPI), prothrombin, protein C, protein S, and annexin V. β_2-GPI is the most extensively studied of the cofactors and has been shown to be a relevant antigenic target for antiphospholipid antibodies.[7,8] β_2-GPI is a 50-kd, single-chain polypeptide composed of 326 amino acid residues, arranged in 5 homologous repeats known as complement control protein domains. β_2-GPI's fifth domain contains a patch of positively charged amino acids that likely represents the binding region for phospholipids.[9-11]

Oxidized low-density lipoprotein (oxLDL) has an important pathogenic role in early events leading to atherosclerosis,[12,13] and oxLDL has been shown to be a proinflammatory chemotactic agent for macrophages and T lymphocytes, cells with a central role in atherogenesis.[14]

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During the 1980s, oxLDL was localized in atherosclerotic lesions of rabbit and man,[15] and it can be immunogenic, as antibodies to oxLDL have been demonstrated in patients with autoimmune disorders such as systemic lupus erythematosus (SLE) and APS.[16,17] More recently, the premature (or accelerated) development of atherosclerosis has been recognized in patients with autoimmune diseases.[18-20] The traditional risk factors for atherosclerosis failed to account for these changes,[21] and alternative mechanisms have been proposed such as increased levels of autoantibodies to Lp(a), oxLDL, and phospholipids, as well as certain biochemical and genetic abnormalities.[22] An immune component in the pathogenesis of atherosclerosis is becoming apparent, and antiphospholipid antibodies might be possible participants.[23]

β_2-GPI also has been localized in human atherosclerotic lesions by immunohistochemical staining,[24] which suggests a role for β_2-GPI (and antiphospholipid antibodies) in atherosclerosis. Hasunuma et al[25] reported that Cu^{2+}-oxidized LDL, unlike native LDL, binds to β_2-GPI. In vitro macrophage uptake of oxLDL was partly but significantly decreased when β_2-GPI was added compared with oxLDL alone. This uptake was inhibited by polyinosinic acid, a scavenger receptor blocker. The addition of an antiphospholipid antibody, either β_2-GPI-dependent anticardiolipin (aCL) or anti-β_2-GPI, resulted in a significant increase of complex uptake by macrophages. Because the increased uptake was not affected by polyinosinic acid, it was suggested that macrophage Fcγ receptors were involved. This mechanism might be relevant to the development of atherosclerosis in patients with APS. Kobayashi et al[26] recently demonstrated a covalent interaction between oxLDL and β_2-GPI that resulted in a "stable" (or nondissociable) oxLDL/β_2-GPI complex. The ligand on the oxLDL molecule (oxLig-1, 7-ketocholesteryl-9-carboxynonanoate) responsible for the interaction with β_2-GPI has been identified and isolated. Increased in vitro macrophage uptake also has been reported when oxLig-1/β_2-GPI antibody complexes were used.[27,28] In addition, high serum levels of oxLDL/β_2-GPI complexes and autoantibodies to these complexes have been associated with venous and arterial thrombosis in patients with APS.[26,29]

The objectives of the present study were to determine the presence of oxLDL/β_2-GPI complexes and IgG autoantibodies to oxLig-1/β_2-GPI and to further investigate their association with APS in patients with autoimmune disease. Two SLE populations, one consisting of consecutive patients with SLE to assess the prevalence of the complexes and antibodies and another of selected patients with SLE (with and without APS) were studied to evaluate their association with APS. Our results show that oxidation of LDL and its interaction with β_2-GPI to form stable circulating complexes are common in SLE and APS but not in rheumatoid arthritis (RA) and that serum levels of these complexes fluctuate widely with time. Autoantibodies to oxLig-1/β_2-GPI were present in some consecutive patients with SLE and frequent in selected patients with SLE with secondary APS. These autoantibodies seem to correlate with measures of disease activity (using the SLE disease activity index [DAI]), suggesting a pathogenic role in the development of APS and atherosclerosis in patients with SLE.

Materials and Methods

Subjects

Two separate populations of patients with autoimmune diseases were studied. To study the first population, we used serum samples from 97 consecutive patients with SLE and 120 with RA. The patients with SLE attended the Rheumatology Clinic, Western General Hospital, Guadalajara, Mexico, from January to October 2001, and the patients with RA from April to August 2002. The samples were stored at –20°C until tested. The diagnoses of SLE and RA were established according to American College of Rheumatology Classification Criteria.[30] Control samples consisted of 20 serum samples from patients with syphilis and 34 from healthy blood bank donors. Six (30%) of the syphilis samples were positive for IgG aCL antibodies and none for anti-β_2-GPI antibodies.

Of the patients with SLE, 93 (96%) were women, and of the patients with RA, 102 (85.0%) were women. The mean age for patients with SLE was 31 years (range, 18-82 years) and for patients with RA was 49 years (range, 19-80 years). Informed consent was obtained from all patients and institutional review board approval from Western General Hospital.

For 6 patients with SLE, at least 4 serum samples were obtained at different intervals during a follow-up period of 12 months. The hospital records were reviewed, and clinical and serologic measures of disease activity corresponding to the time of the samples were recorded. Scoring for the SLE-DAI was as follows: 0, inactive; 1 to 5, minimal; 6 to 10, moderate; more than 10, high.[31] SLE-DAI scores were generated by the attending rheumatologist from 24 weighted clinical and serologic descriptors of disease activity. Anti–double-stranded DNA antibodies, C3 and C4 serum levels, and erythrocyte sedimentation rate were also recorded.

To study the second population, we used serum samples from 90 selected patients with SLE classified into 2 subgroups: 50 without APS and no clinical history of antiphospholipid antibodies and 40 with secondary APS. The clinical diagnosis was established according to the Sapporo criteria for the classification of APS.[32] Of the patients with SLE, 82 were females and 8 were males. The mean age was 38.7 years (range, 17-74 years). A separate

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group of 60 serum samples from healthy blood donors were used as control samples.

Monoclonal Antibodies

The following monoclonal antibodies were used to develop the enzyme-linked immunosorbent assay (ELISA) tests for measuring oxLDL/β_2-GPI complexes and anti–oxLig-1/β_2-GPI antibodies: WB-CAL-1 monoclonal antibody reactive to β_2-GPI (IgG2a, κ) derived from a NZW x BXSB F1 mouse, a model of spontaneous APS,[33] and EY2C9 monoclonal anti-β_2-GPI antibody (IgM) established from peripheral blood lymphocytes of patients with APS.[34] Both monoclonal antibodies bind only to β_2-GPI/negatively charged phospholipid (or oxLDL) complexes and not with monomeric (free) β_2-GPI in solution. 1D2 is an IgG murine monoclonal antibody specific to human apolipoprotein B-100. 1D2 equally reacts with native and oxLDL.

Purification of Human β_2-GPI

Human β_2-GPI was purified from fresh normal plasma as previously described[35] with slight modifications. Briefly, human plasma was precipitated with 70% perchloric acid, extensively dialyzed against tris(hydroxymethyl)aminomethane–sodium chloride buffer (pH 8.0), and concentrated before loading into a heparin column (Amersham Biosciences, Piscataway, NJ). Pooled β_2-GPI fractions again were dialyzed against sodium acetate–sodium chloride buffer (pH 4.8) and concentrated. This preparation was then loaded into a carboxymethylcellulose column (Sigma-Aldrich, St Louis, MO), and β_2-GPI fractions were pooled, dialyzed against sodium acetate–sodium chloride buffer, concentrated at approximately 1 mg/mL, and stored at –70°C until used. The β_2-GPI preparation contained more than 95% of protein, and a 50-kd, single, diffuse band was demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In addition, the immunoreactivity of the purified β_2-GPI was checked by using an anti-β_2-GPI ELISA procedure before use.

LDL Purification and Oxidation

LDL was isolated by ultracentrifugation of fresh normal human plasma in EDTA–potassium bromide solutions as described.[36] LDL (d = 1.019-1.063 g/mL) was adjusted to a concentration of 100 µg/mL based on the protein concentration. The LDL fraction was oxidized with a 5-µmol/L concentration of copper sulfate in a 10-mmol/L concentration of Hepes buffer and a 150-mmol/L concentration of sodium chloride, pH 7.4 (Hepes buffer), at 37°C for 12 hours. Oxidation was terminated by the addition of EDTA (at a final concentration of 1 mmol/L), and oxLDL was dialyzed extensively against Hepes buffer containing EDTA. The degree of oxidation was measured using the thiobarbituric acid reactive substance procedure.[37]

ELISA Procedure for oxLDL/β_2-GPI Complexes

Monoclonal antibody against β_2-GPI (WB-CAL-1) was coated onto Immulon 2HB microplates (Dynex Technologies, Chantilly, VA) by incubating 50 µL per well of 5 µg/mL of WB-CAL-1 in phosphate-buffered saline (PBS), pH 7.4, overnight at 2°C to 4°C. WB-CAL-1 is an IgG murine monoclonal antibody against human β_2-GPI used in this assay to capture oxLDL/β_2-GPI complexes via its reactivity with β_2-GPI.

The plate was blocked with PBS–1% nonfat dry milk for 1 hour. Then, 100 µL of serum samples diluted 1:25 in PBS–nonfat dry milk containing a 10-mmol/L concentration of magnesium chloride were added to the appropriate microwells and incubated for 2 hours at room temperature. Magnesium chloride dissociates intermediate oxLDL/β_2-GPI complexes (ie, electrostatically bound), permitting the specific detection of nondissociable and covalently bound complexes present in serum samples.[26] The microwells were washed 4 times with PBS–0.05% polysorbate 20 between each step. Biotinylated 1D2 (antihuman apolipoprotein B-100) antibody diluted in PBS–nonfat dry milk was added to the microwells and incubated for 1 hour at room temperature, followed by horseradish peroxidase–streptavidin. Color was developed with tetramethylbenzidine–hydrogen peroxide, and the reaction was stopped with 0.36N sulfuric acid. Optical density was read at a wavelength of 450 nm (650 nm reference).

The intra-assay precision (coefficient of variation percentage) ranged from 7.2% to 12.3% for weakly and 4.5% to 8.9% for moderately and strongly reactive samples. The serum oxLDL/β_2-GPI complex concentration (expressed in units per milliliter) was calculated against a reference curve built with 2-fold serial dilutions of oxLDL/β_2-GPI complex solution. The complexes were prepared in advance by incubating equal amounts of Cu^{2+}-oxLDL and purified human β_2-GPI, pH 7.4, for 12 hours at 37°C. The unit value was derived arbitrarily from the protein concentration of the oxLDL/β_2-GPI complex used in the reference curve. A normal cutoff value for the assay was established by testing serum samples from healthy blood donors (mean + 3 SD). **Figure 1** shows that only oxidized (not native) LDL reacted with exogenous human β_2-GPI forming oxLDL/β_2-GPI complexes.

ELISA for IgG Anti–oxLig-1/β_2-GPI Antibodies

The ELISA procedure described by Kobayashi et al[27] was used with slight modifications. We coated 50 µL of 100 µg/mL of oxLig-1 (7-ketocholesteryl-9-carboxynonanoate) in ethanol onto Immulon 2HB microplates by evaporation. The synthesis and characterization of oxLig-1 has been reported.[28]

The plate was blocked with 1% bovine serum albumin (BSA) for 1 hour at room temperature and washed. Then 50 µL of 30 µg/mL of β_2-GPI in PBS–3%

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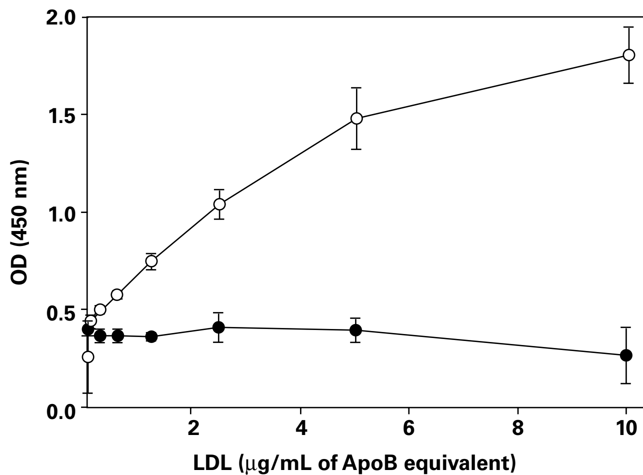

Figure 1 Complex formation between oxidized low-density lipoprotein (oxLDL) and β_2-glycoprotein I (β_2-GPI). Increasing concentrations of oxLDL (open circles) or native LDL (closed circles) were incubated together with purified human β_2-GPI in Immulon 2HB microwells coated with anti-β_2-GPI monoclonal antibody (WB-CAL-1). Bound LDL was detected with biotinylated human apolipoprotein B-100 monoclonal antibody (1D2) and avidin–horseradish peroxidase. Results are expressed as the mean optical density (OD) of triplicate testing ± SD (error bars). For proprietary information, see the text.

BSA was added to oxLig-1–coated microwells to permit complex formation. These oxLig-1/β_2-GPI complexes served as antigenic substrates to capture patients' antibodies. Subsequently, 50 µL of serum or plasma samples diluted 1:100 in PBS–3% BSA were added to the microwells and incubated for 1 hour at room temperature. The microwells were washed 4 times with PBS–0.05% polysorbate 20 between steps. Diluted horseradish peroxidase–conjugated antihuman IgG antibody was added to the microwells and incubated for 1 hour. Color was developed with tetramethylbenzidine–hydrogen peroxide, and the reaction was stopped with 0.36N sulfuric acid. Optical density was read at a wavelength of 450 nm (650 nm reference).

The intra-assay precision (coefficient of variation percentage) ranged from 7.4% to 12.6% for weakly and 5.5% to 9.9% for moderately and strongly reactive samples. EY2C9 is an IgM monoclonal antibody against β_2-GPI (derived from a patient with APS), and it was used in this assay only as a reference human antibody reacting to the β_2-GPI of the antigenic substrate, to select strongly reactive samples to be used as control samples. However, the IgG oxLig-1/β_2-GPI antibody concentration of patients' samples (expressed in units per milliliter as stated earlier) was calculated against a standard curve prepared with a selected positive sample. A normal cutoff value for the assay was established by testing serum samples from healthy blood donors (mean + 3 SD).

ELISA for aCL and Anti-β_2-GPI Antibodies

All APS samples were tested for IgG aCL and anti-β_2-GPI antibodies by using commercially available ELISA test kits (Corgenix, Westminster, CO) according to the manufacturer's instructions.[38] The IgG aCL ELISA test requires exogenous bovine β_2-GPI, thus measuring β_2-GPI-dependent antiphospholipid antibodies. The anti-β_2-GPI ELISA uses purified human β_2-GPI as the antigen and detects anti-β_2-GPI antibodies in the absence of exogenous phospholipids.

Statistical Analysis

Statistical analysis was performed with a SigmaStat program (SPSS Science, Chicago, IL). The Student *t* test was performed to compare the results between different groups and the Fisher exact test to assess the relationship between antibodies and clinical manifestations. Sensitivity, specificity, positive predictive value (PPV), and odds ratio of anti–oxLig-1/β_2-GPI antibodies were calculated by 2 × 2 contingency table analysis. We also calculated 95% confidence intervals for the odds ratios. The Pearson product moment correlation was performed to assess the association of individual values between variables. A *P* value of .05 or less was considered significant.

Results

Serum oxLDL/β_2-GPI Complexes in Consecutive Patients With SLE

Of 97 consecutive patients with SLE, 67 (69%) had positive serum levels of oxLDL/β_2-GPI complex with a mean level of 73.2 ± 78.1 U/mL, which was significantly higher than the mean level for healthy control subjects (1.3 ± 0.4 U/mL; $P = 7.2 \times 10^{-15}$). Only 3 (2.5%) of 120 consecutive patients with RA had positive results for oxLDL/β_2-GPI with a mean of 1.4 ± 0.7 U/mL, which was statistically different from that for healthy control subjects (*P* = .09). In contrast with the RA group, 13 (65%) of 20 patients with syphilis had positive results for oxLDL/β_2-GPI with a mean of 56.2 ± 71.5 U/mL, which was significantly higher than the level for the healthy control subjects (*P* = .001). The mean level for the patients with syphilis was lower than for patients with SLE, but this difference was not statistically significant (*P* = .174) **Figure 2A**. These results indicate that oxidation of LDL and its complex formation with β_2-GPI are common in SLE and syphilis. Although RA is a systemic autoimmune disease, oxLDL/β_2-GPI complexes were not detected in our RA patient population.

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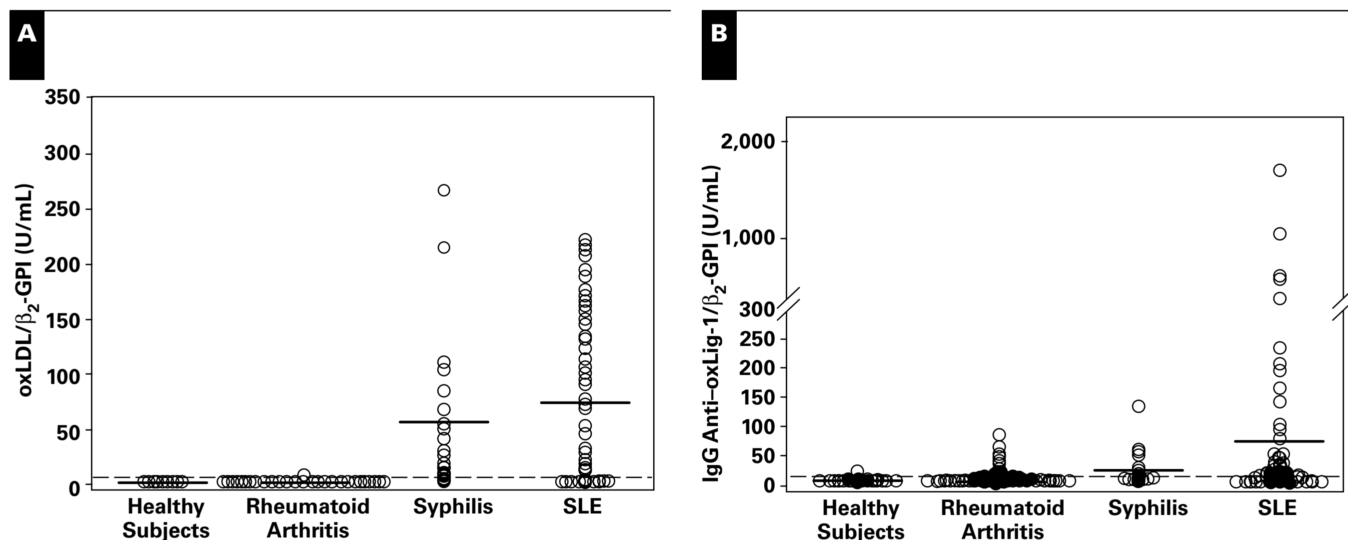

Figure 2 Serum levels of (**A**) oxidized low-density lipoprotein (oxLDL)/β_2-glycoprotein I (β_2-GPI) complexes and (**B**) IgG anti–oxLDL–derived specific ligand (oxLig-1)/β_2-GPI antibodies in healthy control subjects (n = 34) and consecutive patients with rheumatoid arthritis (n = 120), syphilis (n = 20), and systemic lupus erythematosus (SLE; n = 97). The cutoff (horizontal broken line) was established by testing 34 healthy subjects (mean + 3 SD). The horizontal solid line indicates the mean of each group. **A**, Healthy subjects vs patients with SLE, $P = 7.2 \times 10^{-15}$; healthy subjects vs patients with syphilis, $P = .001$; patients with syphilis vs patients with SLE, $P = .174$. **B**, Healthy subjects vs patients with SLE, $P = .002$; patients with rheumatoid arthritis vs patients with SLE, $P = .003$; patients with syphilis vs patients with SLE, $P = .02$.

Serum IgG Anti–oxLig-1/β_2-GPI Antibodies in Consecutive Patients With SLE

Of 97 consecutive patients with SLE, 31 (32%) had positive serum levels of IgG anti–oxLig-1/β_2-GPI antibodies (mean, 72.3 ± 215.2 U/mL; median, 13.4 U/mL with 25% and 75% quartile ranges of 7.6 and 30.9 U/mL, respectively) that were significantly higher (P = .002) than the mean level for healthy control subjects (8.9 ± 4.4 U/mL; median, 7.8 U/mL with 25% and 75% quartile ranges of 6.5 and 8.5 U/mL, respectively). Of 120 consecutive patients with RA, 7 (5.8%) had results positive for IgG anti–oxLig-1/β_2-GPI antibodies (mean, 11.8 ± 11.3 U/mL; median, 8.9 U/mL with 25% and 75% quartile ranges of 6.7 and 11.4 U/mL, respectively; P = .02 vs healthy control subjects), and 5 (25%) of 20 patients with syphilis had positive results (mean, 25.4 ± 30.3 U/mL; median, 11.6 U/mL with 25% and 75% quartile ranges of 9.6 and 24.8 U/mL, respectively; P = .01 vs healthy control subjects). The mean antibody level for the patients with syphilis was significantly lower than that for patients with SLE (P = .02) **Figure 2B**. The results indicate a higher prevalence of IgG anti–oxLig-1/β_2-GPI antibodies in patients with SLE than in patients with RA or syphilis and in healthy control subjects.

Time Course Studies of Serum oxLDL/β_2-GPI Complexes and IgG Anti–oxLig-1/β_2-GPI Antibodies

For 6 patients with SLE, at least 4 serum samples were obtained at different intervals within a 12-month follow-up period. The oxLDL/β_2-GPI complexes and IgG anti–oxLig-1/β_2-GPI antibodies were measured in these samples.

Table 1 summarizes the relationship of IgG anti–oxLig-1/β_2-GPI antibodies with various clinical and serologic measures of disease activity in these patients. Four patients were classified as having moderate or high disease activity (by the SLE-DAI) during the follow-up period, 3 had elevated levels of IgG anti–oxLig-1/β_2-GPI antibodies, and all 4 had a positive anti–double-stranded DNA antibody titer and a low C4 level. A high erythrocyte sedimentation rate was found in 3 of 4 patients with active disease and in 1 with inactive disease. Serum levels of oxLDL/β_2-GPI complexes for each patient were mostly positive, while IgG aCL and anti-β_2-GPI antibody levels were normal or borderline. Except for the anti–oxLig-1/β_2-GPI antibodies, oxLDL/β_2-GPI complexes and antiphospholipid antibodies (aCL and anti-β_2-GPI) showed no relationship with disease activity. These results suggest a possible association of IgG anti–oxLig-1/β_2-GPI antibody levels with disease activity in patients with SLE. **Figure 3** depicts results for 4 of 6 patients with SLE, 2 with active disease and 2 with inactive disease as representative cases. Serum levels of oxLDL/β_2-GPI complexes fluctuated widely without a partic-

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Table 1
Relationship Between IgG Anti–oxLig-1/β_2-GPI Antibodies and Disease Activity in Patients With SLE*

Patient No.	Mean IgG Anti–oxLig-1/ β_2-GPI (U/mL)	SLE-DAI†	Anti-dsDNA‡	C3 Level	C4 Level	ESR	Mean IgG aCL Level (GPL U)	Mean IgG Anti-β_2-GPI Level (U)
1	9.8	Inactive	Negative	NA	NA	**High**	6.5	0.9
2	**234.5**	**Moderate**	**Positive**	N	**Low**	N	**23.2**	2.6
3	**38.3**	**High**	**Positive**	N	**Low**	**High**	11.5	2.8
4	9.9	**Moderate**	**Positive**	**Low**	**Low**	**High**	4.4	0.8
5	**24.5**	**Moderate**	**Positive**	N	**Low**	**High**	7.4	0.7
6	9.1	Inactive	Negative	N	N	N	5.7	0.6

aCL, anticardiolipin; β_2-GPI, β_2-glycoprotein I; DAI, disease activity index; dsDNA, double-stranded DNA; ESR, erythrocyte sedimentation rate; GPL, IgG phospholipid units; N, normal; NA, not available; oxLig-1, oxidized low-density lipoprotein–derived specific ligand; SLE, systemic lupus erythematosus.
* Bold type indicates an abnormal value. Reference ranges are as follows: C3, 60-220 mg%; C4, 20-40 mg%; ESR (Westergren method), <20 mm/h; IgG aCL, <23 GPL U; IgG anti-β_2-GPI, <20 U.
† Scoring for the SLE-DAI is as follows: 0, inactive; 1-5, minimal; 6-10, moderate; >10, high.
‡ By immunofluorescence crithidia assay; a positive titer was defined as >1:10.

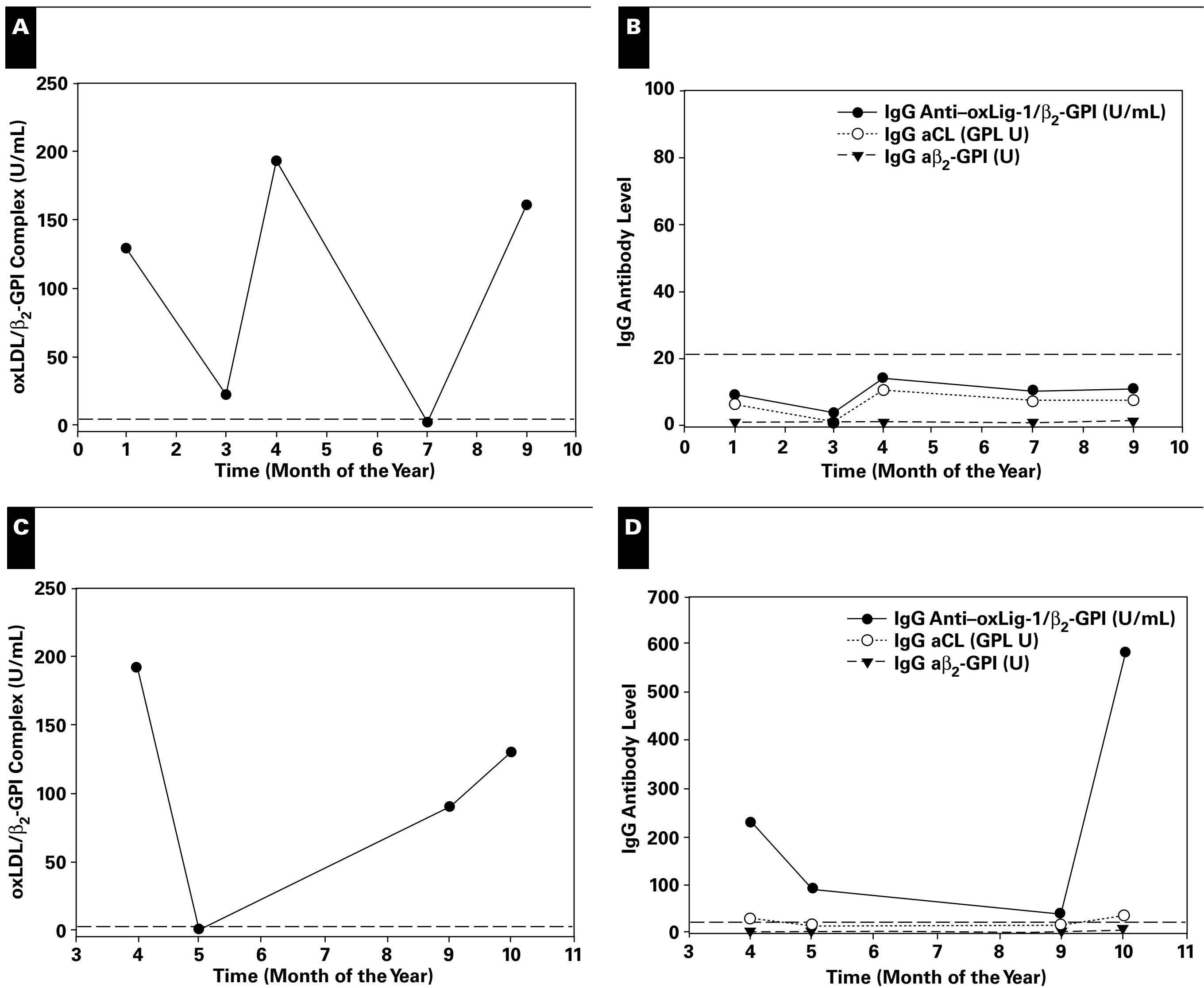

Figure 3 Serum levels of oxidized low-density lipoprotein (oxLDL)/β_2-glycoprotein I (β_2-GPI) complexes (**A**, **C**, **E**, **G**) with corresponding IgG anti–oxLDL–derived specific ligand (anti–oxLig-1)/β_2-GPI antibodies (**B**, **D**, **F**, **H**) in 4 of 6 patients with systemic lupus erythematosus (SLE) tested over time. According to the SLE disease activity index (see text), patients 1 (**A** and **B**) and 6 (**G** and **H**) had inactive disease and patients 2 (**C** and **D**) and 3 (**E** and **F**), active disease. (cont next page)

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ular pattern regardless of the disease activity of the patient. In contrast, IgG anti–oxLig-1/β_2-GPI antibody levels were positive only in patients with active disease.

Serum oxLDL/β_2-GPI Complexes in Selected Patients With APS

Of 40 selected patients with SLE with APS, 25 (63%) had positive serum levels of oxLDL/β_2-GPI complexes with a mean of 13.5 ± 24.4 U/mL, which was significantly higher than the mean for 60 healthy control subjects (0.7 ± 0.1 U/mL; $P < .001$). Of 50 selected patients with SLE without APS, 30 (60%) had positive levels with a mean of 10.6 ± 19.8 U/mL, which also was significantly higher than the mean for healthy control subjects ($P = .001$). The mean level for patients with SLE with APS was not statistically different ($P = .279$) from that for patients with SLE without APS **Figure 4A**. These results indicate that oxidation of LDL and its complex formation with β_2-GPI is common in patients with SLE, regardless of the presence of APS.

Serum IgG Anti–oxLig-1/β_2-GPI Antibodies in Selected Patients With APS

Of 40 selected patients with SLE with APS, 26 (65%) had positive serum levels of IgG anti–oxLig-1/β_2-GPI antibodies compared with 6 (19%) of 32 selected patients with SLE without APS. The mean level for patients with SLE

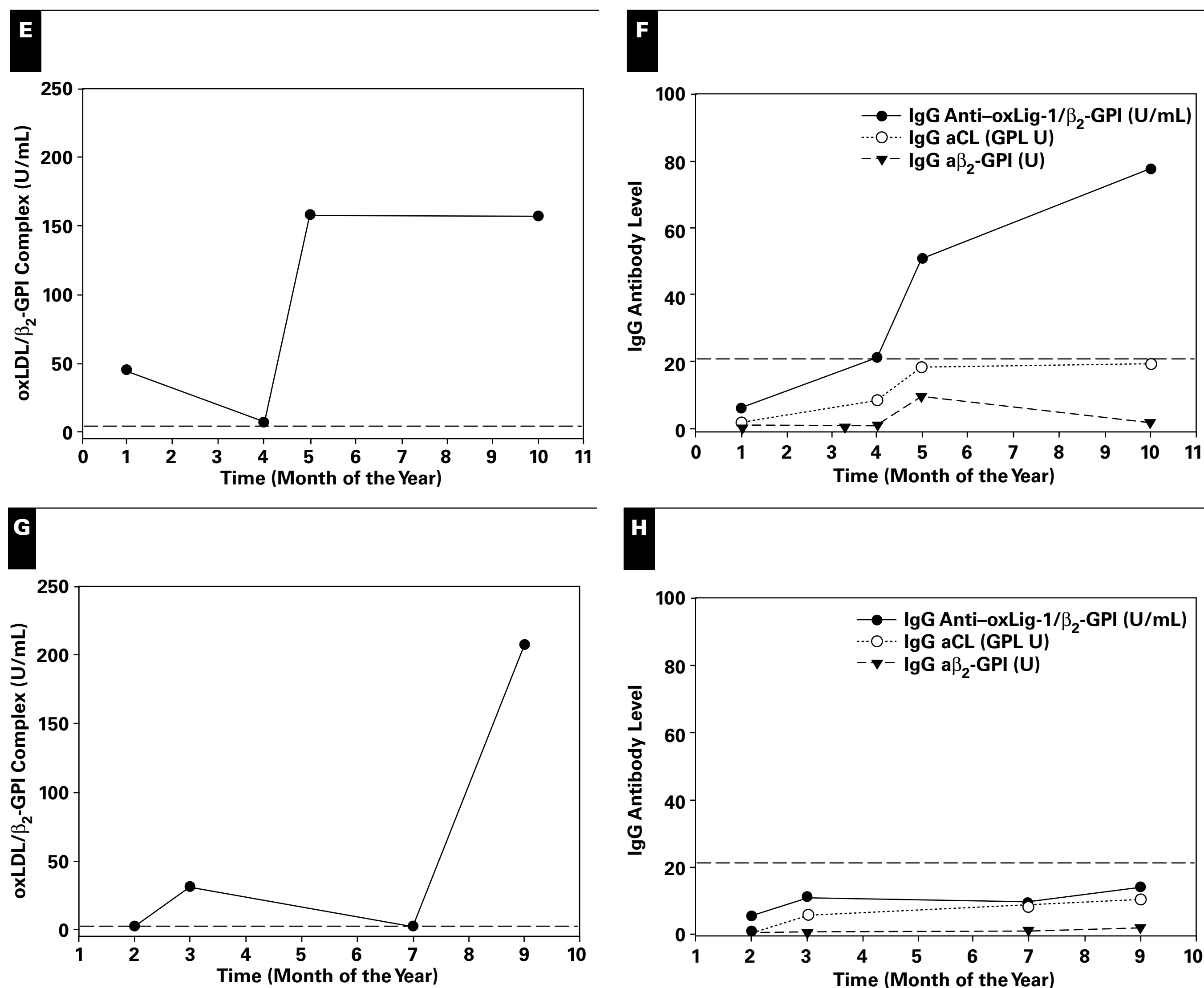

Figure 3 (cont) The numbers on the x-axis represent the month of the year when the samples were collected. All samples were tested simultaneously in duplicate for oxLDL/β_2-GPI complexes and for anti–oxLig-1/β_2-GPI, anticardiolipin (aCL), and anti-β_2-GPI antibodies. The oxLDL/β_2-GPI complex and anti–oxLig-1/β_2-GPI antibody cutoff values (horizontal broken line) were established by testing healthy subjects (mean + 3 SD). The cutoff values for IgG aCL and anti-β_2-GPI antibodies had been established by the kit manufacturer at 23 GPL U and 20 U/mL, respectively.

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with APS (24.4 ± 28.4 U/mL) was significantly higher than the mean for SLE control patients without APS (9.1 ± 5.1 U/mL; P = .0008) and the mean for 43 healthy control subjects (5.7 ± 1.4 U/mL; $P = 8.6 \times 10^{-5}$) **Figure 4B**. The results indicate a higher prevalence and serum levels of IgG anti–oxLig-1/β_2-GPI antibodies in patients with SLE with APS patients compared with patients with SLE without a history of APS, which suggests a possible pathogenic role for these antibodies in APS.

Relationship of IgG Anti–oxLig-1/β_2-GPI With aCL and With Anti-β_2-GPI Antibodies

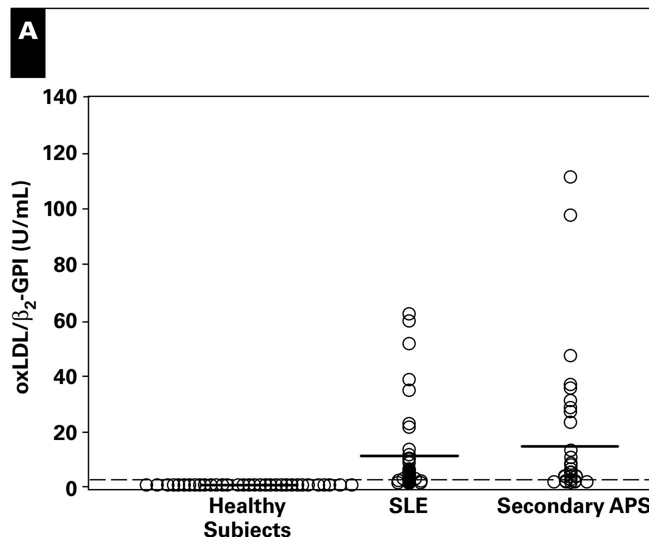
Owing to the prominent presence of β_2-GPI in the antigenic mixture used to detect IgG anti–oxLig-1/β_2-GPI antibodies, the relationship of these antibodies with β_2-GPI-dependent aCL and anti-β_2-GPI antibodies was evaluated in 40 selected patients with SLE with secondary APS. **Figure 5** shows the relationship of IgG anti–oxLig-1/β_2-GPI antibodies with IgG aCL and with anti-β_2-GPI antibodies. The distribution of IgG anti–oxLig-1/β_2-GPI vs anti-β_2-GPI antibodies followed a pattern different from that of aCL antibodies. This pattern suggests the presence of antibodies with at least 2 different reactivities: to β_2-GPI and to oxLig-1/β_2-GPI. Although preliminary, these results might indicate that IgG anti–oxLig-1/β_2-GPI antibodies represent a distinct subset of antiphospholipid antibodies.

Comparative Clinical Performance

The clinical performance (relative sensitivity, specificity, and PPV) of IgG anti–oxLig-1/β_2-GPI antibodies for clinical manifestations of APS in 90 selected patients with SLE was evaluated by 2 × 2 contingency table analysis. IgG anti–oxLig-1/β_2-GPI antibodies were 45% sensitive and 93.7% specific, with a PPV of 90% for APS (P < .001). **Table 2** shows the comparison of the results of IgG anti–oxLig-1/β_2-GPI antibodies with those of IgG aCL and anti-β_2-GPI antibodies. IgG aCL antibodies were 62.5% sensitive, compared with 35% for anti-β_2-GPI and 45% for anti–oxLig-1/β_2-GPI antibodies. This is due to more false-positive results observed in the SLE control group as evidenced by the lower specificity and PPV for IgG aCL antibodies. Thus, IgG anti–oxLig-1/β_2-GPI antibodies are better predictors of APS.

Discussion

Oxidation of LDL occurs in vivo and has an important pathogenic role in atherogenesis.[12,13] More than 60% of the patients with SLE in the present study had elevated serum levels of oxLDL/β_2-GPI complexes as compared with 2.5% in RA and 0% in healthy control subjects (Figure 2A). However, the prevalence and the mean level of oxLDL/β_2-GPI complexes in patients with SLE without a history of APS were similar to those for patients with APS (Figure 4A). The chronic inflammation of the vasculature frequently seen in



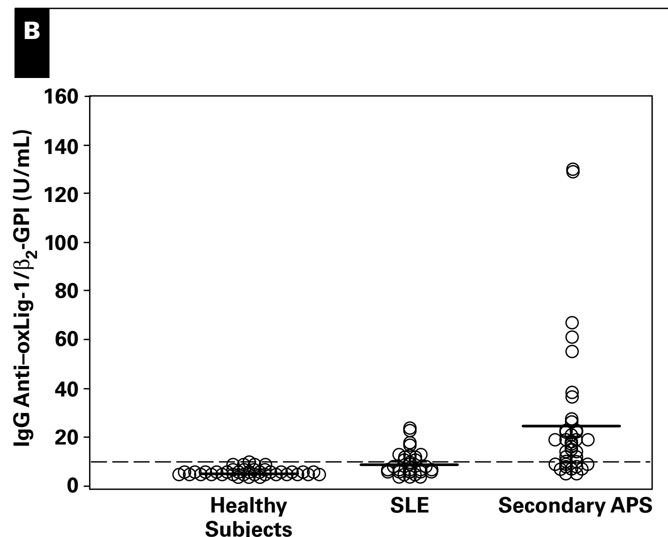


Figure 4 Serum levels of (**A**) oxidized low-density lipoprotein (oxLDL)/β_2-glycoprotein I (β_2-GPI) complexes and (**B**) IgG anti–oxLDL–derived specific ligand (anti–oxLig-1)/β_2-GPI antibodies in healthy control subjects (**A**, n = 60; **B**, n = 43), selected patients with systemic lupus erythematosus (SLE) (without antiphospholipid syndrome [APS]; **A**, n = 50; **B**, n = 32) and with secondary APS (**A** and **B**, n = 40). The cutoff (horizontal broken line) was established by testing 60 healthy subjects (mean + 3 SD). The horizontal solid lines indicate the mean for each group. **A**, Healthy subjects vs patients with SLE and secondary APS, P = .001; patients with SLE and no APS vs patients with SLE and secondary APS, P = .279. **B**, Healthy subjects vs patients with SLE and secondary APS, $P = 8.6 \times 10^{-5}$; patients with SLE and no APS vs patients with SLE and secondary APS, P = .0008.

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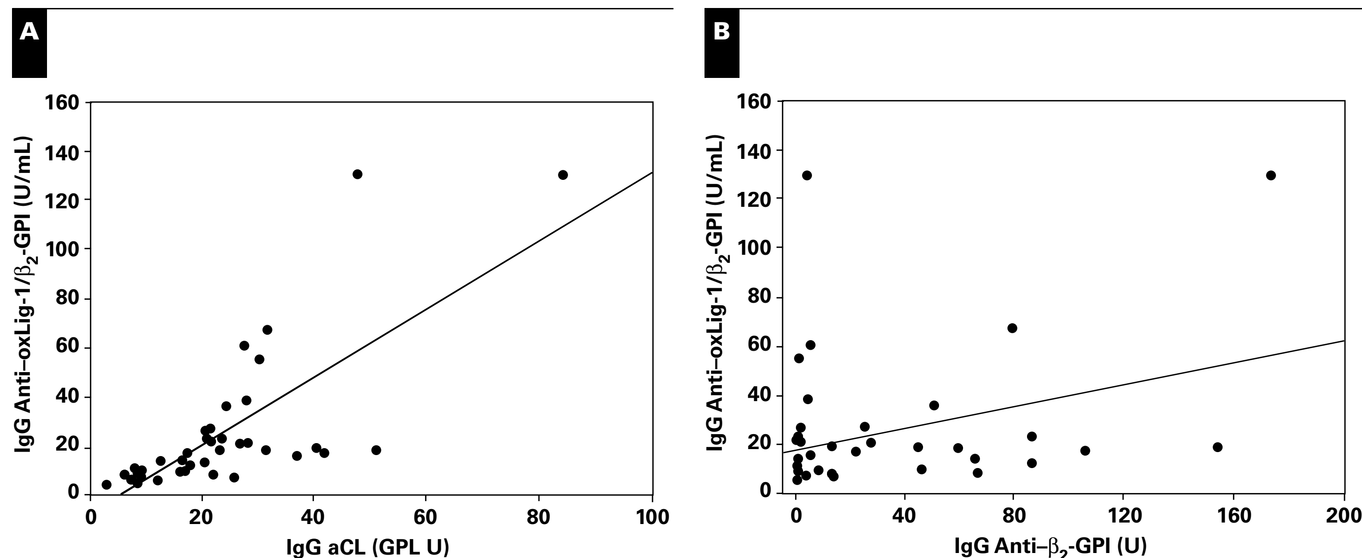

Figure 5 Correlation between IgG anti–oxLDL–derived specific ligand (anti–oxLig-1)/β_2-glycoprotein I (β_2-GPI) antibodies and antiphospholipid antibodies determined by enzyme-linked immunosorbent assay in 40 selected patients with systemic lupus erythematosus with secondary antiphospholipid syndrome. **A**, IgG anti–oxLig-1/β_2-GPI vs IgG anticardiolipin (aCL) ($r = 0.737$; $P = 5.96 \times 10^{-8}$; n = 40). **B**, IgG anti–oxLig-1/β_2-GPI vs IgG anti-β_2-GPI ($r = 0.325$; $P = .04$; n = 40). The straight line represents the best-fit linear regression.

patients with certain autoimmune diseases might account for the increased oxidative modification of LDL and its interaction with β_2-GPI. These results are in agreement with recent reports that oxidation of LDL and interaction with β_2-GPI are common in patients with these diseases.[26,29] Circulating oxLDL/β_2-GPI complexes have been implicated as atherogenic autoantigens, and their presence might represent a risk factor or an indirect but significant contributor to thrombosis and atherosclerosis.[26] The presence of autoantibodies against oxLig-1/β_2-GPI in patients with SLE with secondary APS (Figure 4B), together with the common occurrence of oxLDL/β_2-GPI complexes, might help explain the accelerated (or premature) development of vascular complications, including atherosclerosis, seen in some of these patients.

In contrast with SLE, oxLDL/β_2-GPI complexes were not found in RA (Figure 2A). To our knowledge, autoantibodies against oxLDL have not been reported in this disease. However, a high incidence of atherosclerosis has been described in patients with RA.[18] The lack of circulating oxLDL/β_2-GPI complexes might help to explain the low prevalence of autoantibodies to the complex (Figure 2B) and the low incidence of thromboembolic complications seen in RA. It is possible that other mechanism(s), different from oxidation of LDL, might contribute to the development of atherosclerosis in RA. A recent review proposed several potential proatherogenic mechanisms, including dyslipidemias such as Lp(a), medications (steroids, methotrexate), proinflammatory cytokines (interleukins 1 and 6, tumor necrosis factor α), acute phase reactants (C-reactive protein, serum amyloid A), and newer risk factors such as hyperhomocysteinemia.[18]

The oxLig-1/β_2-GPI complexes (Figure 2A) were detected in 13 (65%) of a small group (n = 20) of patients with syphilis. The presence of these complexes suggests that the infectious process also might result in vascular inflammation leading to oxidation of LDL and β_2-GPI complex formation, perhaps similar to SLE. About 25% of the patients with

Table 2
Association Between IgG Anti–oxLig-1/β_2-GPI Antibodies and Clinical Manifestations of APS in Patients With SLE

Antibody	Sensitivity (%)	Specificity (%)	PPV (%)	P^*	OR (95% CI)
IgG anti–oxLig-1/β_2-GPI	45.0	93.7	90.0	<.001	12.3 (2.6-58.6)
IgG aCL	62.5	80.0	71.4	<.001	6.7 (2.6-17.1)
IgG anti-β_2-GPI	35.0	98.0	93.3	<.001	26.4 (3.3-212.1)

aCL, anticardiolipin; APS, antiphospholipid syndrome; β_2-GPI, β_2-glycoprotein I; CI, confidence interval; OR, odds ratio; oxLig-1, oxidized low-density lipoprotein–derived specific ligand; PPV, positive predictive value; SLE, systemic lupus erythematosus.
* Fisher exact test.

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syphilis showed low positive levels of IgG anti–oxLig-1/β_2-GPI antibodies (Figure 2B). These antibody titers had a stronger correlation with IgG aCL (r = 0.889) compared with anti-β_2-GPI (r = 0.229) antibodies (data not shown). These results not only show that anti–oxLig-1/β_2-GPI antibodies might be different from anti-β_2-GPI antibodies, but also suggest that oxLDL/β_2-GPI complexes can induce the production of low levels of "infectious" anti–oxLig-1/β_2-GPI antibodies. Unlike patients with APS, no "autoimmune" anti-β_2-GPI antibodies were detected in this group of patients with syphilis. The exact mechanism(s) and possible pathogenic effect of these antibodies in thrombosis and atherosclerosis remain to be elucidated.

Kobayashi et al[26] recently demonstrated that magnesium chloride dissociates intermediate forms of oxLDL/β_2-GPI complexes bound by electrostatic interactions. Our ELISA system included magnesium chloride in the sample diluent to permit the detection of "stable" nondissociable oxLDL/β_2-GPI complexes bound by covalent interactions. It can be hypothesized that these stable complexes might be clinically relevant. The wide fluctuation in serum levels of oxLDL/β_2-GPI complexes in some patients with SLE (Figure 3) suggests that oxidation of LDL and/or the formation of complexes with β_2-GPI in circulation is an active process. It is possible that a dysfunction of some regulatory mechanism(s) might account for these findings. For example, the HDL-associated enzyme paraoxonase (PON) has antioxidant activity that protects LDL from oxidation.[39] Decreased PON activity has been reported in patients with aCL antibodies.[40] Furthermore, IgG anti-β_2-GPI antibodies were associated with reduced PON activity in patients with SLE and patients with primary APS.[41] Vascular injury (biochemical or immunologic) as seen in patients with autoimmune disease might affect PON activity or any other antioxidant mechanism, triggering the oxidative aforementioned changes. In addition, it is possible that patients with APS have circulating immune complexes (antibody/oxLig-1/β_2-GPI), as suggested by the coexistence of oxLDL/β_2-GPI and autoantibodies. These immune complexes might be removed from circulation by macrophages via Fcγ receptors, a mechanism also relevant in atherogenesis.[26]

High serum levels of IgG anti–oxLig-1/β_2-GPI antibodies were demonstrated in 31 (32%) of 97 consecutive SLE patients (Figure 2B). The APS status of the consecutive patients with SLE with IgG anti–oxLig-1/β_2-GPI antibodies was not known; however, the 32% prevalence corresponds with the expected general prevalence or risk of APS in SLE.[3-5] High serum levels of these antibodies were found in 4 patients with SLE with persistent clinical and serologic measures of disease activity (Figure 3 and Table 1). Although this is a small group of patients, these results suggest that IgG anti–oxLig-1/β_2-GPI antibodies might serve as indicators of disease activity in patients with SLE and might contribute to the pathogenesis of APS. Taken together, these findings provide additional support to the hypothesis that oxidative stress has an important role in antiphospholipid antibody production and the development of APS.

We found no correlation between serum levels of oxLDL/β_2-GPI complexes and IgG anti–oxLig-1/β_2-GPI antibodies in patients with SLE with APS (data not shown). It is possible that high complex levels might result in increased antibody binding and immune complex formation, depleting "free" autoantibodies. However, the possible relationship between oxLDL/β_2-GPI complexes and the autoantibodies is illustrated by the presence of IgG anti–oxLig-1/β_2-GPI antibodies only in patients with SLE with a history of APS and the report of a stronger association of anti–oxLig-1/β_2-GPI antibodies with thrombosis in patients with high levels vs low levels of oxLDL/β_2-GPI complexes.[26]

Serum levels of IgG anti–oxLig-1/β_2-GPI antibodies showed a stronger correlation with IgG aCL (r = 0.737) than with anti-β_2-GPI (r = 0.325) antibodies in patients with SLE with APS (Figure 5). On the other hand, the PPVs for APS of IgG anti–oxLig-1/β_2-GPI (90%) and anti-β_2-GPI (93.3%) antibodies were better than IgG aCL (71.4%) antibodies in our study population (Table 2). These results suggest that IgG anti–oxLig-1/β_2-GPI antibodies might represent a subset of antiphospholipid antibodies that coexist with other autoantibodies. Additional studies using purified antibodies and/or inhibition experiments are needed to further characterize these antibodies. The results of this study suggest that measuring oxLDL/β_2-GPI complexes and IgG anti–oxLig-1/β_2-GPI antibodies by ELISA might be useful in the serologic evaluation of APS and that they are possible contributors to the development of atherosclerosis in some patients with autoimmune diseases such as SLE.

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