Antiphospholipid Antibodies Are Directed against Epitopes of Oxidized Phospholipids

Recognition of Cardiolipin by Monoclonal Antibodies to Epitopes of Oxidized Low Density Lipoprotein

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Abstract

The optimal clinical management of patients with antiphospholipid antibody syndrome (APS) is uncertain because of a lack of an underlying hypothesis to explain why antiphospholipid autoantibodies (aPL) form to such ubiquitous compounds as phospholipids (PL). In this paper, we demonstrate that many, if not most, aPL are actually directed at neoepitopes of oxidized PL, or neoepitopes generated by adduct formation between breakdown products of oxidized PL and associated proteins. Each cardiolipin (CL) molecule contains four unsaturated fatty acids and is highly susceptible to oxidation, particularly upon exposure to air. Yet, standard anticardiolipin antibodies (aCL) immunoassays routinely bind CL to microtiter wells by evaporation of the ethanol solvent overnight at 4°C. Using a variety of techniques, we demonstrated that rapid oxidation occurs when CL is plated and exposed to air. Sera from apo E–deficient mice, which have high autoantibody titers to oxidized low density lipoprotein, showed a striking time-dependent increase in binding to CL that was exposed to air for increasing periods of time. Monoclonal antibodies to oxidized LDL, cloned from the apo E–deficient mice, also bound to oxidized CL. Both sera and affinity-purified aCL-IgG from APS patients bound to CL progressively as it was oxidized. However, the monoclonal antibodies from apo E–deficient mice, or sera or aCL-IgG from APS patients did not bind to a reduced CL analog that was unable to undergo peroxidation. These data demonstrate that many aPL are directed at neoepitopes of oxidized phospholipids, and suggest that oxidative events may be important in the pathophysiology of APS. In turn, this suggests new therapeutic strategies, possibly including intensive antioxidant therapy. (*J. Clin. Invest.* 1996. 98:815–825.) **Key words: cardiolipin • antiphospholipid antibody syndrome • oxidized lipoproteins • autoantibodies • atherosclerosis**

Introduction

Over the last decade, the antiphospholipid antibody syndrome (APS)[1] has emerged as a subject of intense clinical and scientific interest. As with other autoimmune conditions, this syndrome is characterized by the presence of circulating autoantibodies that bind in vitro to moieties formed by negatively charged phospholipids such as cardiolipin (anticardiolipin antibodies) or the phospholipid complex in phospholipid-dependent coagulation tests (lupus anticoagulant). Patients with significant levels of antiphospholipid antibodies (aPL) are prone to fetal loss, autoimmune thrombocytopenia, and thrombotic events (1–5). Unlike inherited predispositions to thrombosis, the thrombotic events seen in patients with APS may occur in either the venous or arterial circulation, and so include deep venous thrombosis and pulmonary embolism, as well as stroke and arterial ischemia. Recently, aPL also were found to be independent predictors of myocardial infarction in a large prospective Finnish cohort of middle-aged men (6).

Though the clinical associations with aPL are now widely recognized, there is much controversy over the nature of the pertinent antigen(s) for aPL and the proper aPL titer assays. Indeed, medical progress has been hampered by marked interlaboratory variation of aPL results, even among expert laboratories (7). Perhaps more importantly, optimal clinical management of APS remains uncertain because of the lack of a unifying hypothesis to explain why autoantibodies form against such ubiquitous compounds as phospholipids, and why these autoantibodies occur in a variety of clinical settings. In this manuscript, we demonstrate that many, if not most, "antiphospholipid antibodies" bind to neoepitopes of oxidized phospholipids, or to neoepitopes generated by adduct formation between breakdown products of oxidized phospholipids and associated proteins. A corollary of this hypothesis is that most antiphospholipid autoantibodies do not bind to "native," unmodified phospholipids.

Our laboratory demonstrated that nonenzymatic glycation of autologous LDL renders it immunogenic (8). Subsequently, we demonstrated that many other subtle modifications of au-

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Received for publication 14 March 1996 and accepted in revised form 21 May 1996.

J. Clin. Invest.

0021-9738/96/08/0815/11 $2.00
Volume 98, Number 3, August 1996, 815–825

1. *Abbreviations used in this paper:* aCL, anticardiolipin antibodies; aPL, antiphospholipid antibodies; APS, antiphospholipid antibody syndrome; aPTT, activated partial thromboplastin time; β_2-GPI, β_2-glycoprotein I; BHT, butylated hydroxytoluene; CD, conjugated dienes; GPL units, immunoglobulin G phospholipid binding units; MDA, malondialdehyde; OxLDL, oxidized low density lipoprotein; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid.

tologous LDL also were immunogenic (9). For example, the addition of a methyl group to the epsilon amine of a lysine residue of apo B makes LDL immunogenic (9). During oxidative modification of LDL, there is initiation of peroxidation of the polyunsaturated fatty acids (PUFA) present in phospholipids (10, 11). A variety of highly reactive breakdown products ensue, such as malondialdehyde (MDA), which can then form adducts with lysine residues of intimately associated proteins, such as apo B, or with amine containing headgroups of phospholipids, such as phosphatidylserine (PS) or phosphatidylethanolamine (PE) (Pathway 1, Fig. 1). MDA can also cross-link proteins or proteins and lipids. In addition, aldehydes remaining on the *sn*-2 fatty acid fragment on the glycerol backbone (generated as a result of β-scission), could also form modified phospholipid–protein or phospholipid–lipid adducts (Pathway 2, Fig. 1). Thus, many different structures could occur. We have termed these epitopes "oxidation-specific" epitopes (12) and we (13, 14) and others (15–18) have demonstrated that they exist in vivo in atherosclerotic lesions of the aorta as well as in other tissues in which lipid peroxidation is enhanced (19–21). In addition, these epitopes are immunogenic, inducing both a cellular and humoral immune response (reviewed in 22, 23). The recognition that oxidative modification of lipoproteins can so fundamentally alter "self" as to elicit an immunologic response underlies our hypothesis that many, if not most, aPL are directed at neoepitopes that are generated as the result of the oxidation of phospholipids. In this paper, we demonstrate that monoclonal antibodies, selected for binding to oxidized LDL (OxLDL), as well as reference sera from patients with APS, and anticardiolipin-IgG (aCL-IgG) affinity purified from reference sera, all bind exclusively to neoepitopes generated by oxidation of phospholipids.

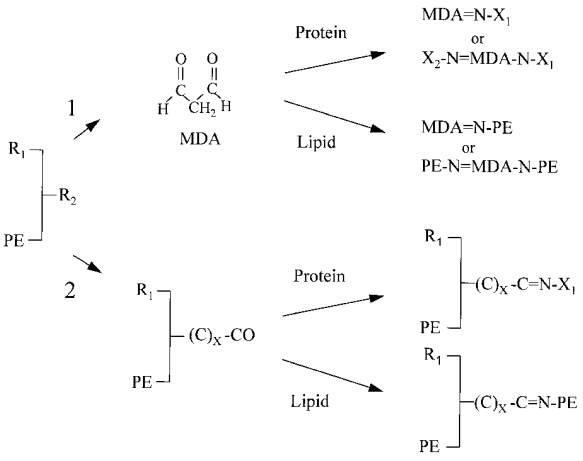

Figure 1. Proposed scheme to explain how oxidation of PUFA of a phospholipid leads to formation of oxidation-derived neoepitopes with associated proteins and lipids. Pathway 1: decomposition of peroxidized PUFA leads to the generation of many reactive compounds, such as MDA. In turn, these can bind to lysine residues of associated proteins, or to amino-phospholipids, such as PE. Pathway 2: as a result of β scission, reactive aldehydes may form and remain bound to the glycerol backbone and further bind to protein or amino-phospholipids. R_1, *sn*-1 fatty acid; R_2, *sn*-2 fatty acid; X_1, protein 1; X_2, protein 2.

Methods

Materials. Cardiolipin (bovine heart) and hydrogenated ("reduced") cardiolipin were obtained from Avanti Polar Lipids (Alabaster, AL). Phosphatidylserine (bovine brain) was obtained from Sigma Chemical Co. (St. Louis, MO). Fatty acid analysis of these phospholipids was performed in our laboratory as described below. The fatty acid content of cardiolipin was 91 to 92%, 18:2; 6%, 18:1; 1%, 18:0; and 2%, 16:0. For the reduced cardiolipin, 91.5% of the fatty acid content was 18:0 and no unsaturated fatty acids were detected. The fatty acid analysis of the brain PS revealed that about 50% of its total fatty acid content was unsaturated: 44.2% was 18:1, 3.4% was 20:1, 0.6% was 20:4, 1.0% was 22:1, 2.4% was 22:5, and 1.8% was 22:6. Luminol was obtained from Sigma Chemical Co. LumiPhos 530 was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Human subjects. Nonfasting sera from 10 women with APS and 10 age-matched female controls were collected at the Department of Obstetrics and Gynecology of the University of Utah Hospital. Patients with APS had one or more of the following clinical features: (*a*) a history of either 1 or more fetal deaths or ≥ 3 consecutive pregnancy losses, (*b*) thrombosis (venous or arterial), or (*c*) autoimmune thrombocytopenia (1, 2, 4). In addition, all patients had both lupus anticoagulant and medium-to-high positive levels of IgG anticardiolipin antibodies. Six patients had high or medium high positive IgM anticardiolipin levels as well. One patient had SLE. Control subjects had no history of fetal death, thrombosis, stroke, or autoimmune disease. Clinical assays for lupus anticoagulant and anticardiolipin antibodies were performed in Dr. Branch's laboratory using standardized techniques. The assay for lupus anticoagulant used a sensitive bovine brain partial thromboplastin time (24–26). The anticardiolipin antibodies were identified in patient's sera using the standard technique described by Harris and co-workers (27, 28). In brief, 50 μg/ml purified cardiolipin (Sigma Chemical Co.) dissolved in ethanol was placed in each well of a 96-well polystyrene microtiter plate and allowed to dry overnight at 4°C. Blocking was accomplished with 10% adult bovine serum in phosphate-buffered saline (10% ABS-PBS). Patient sera were tested in duplicate wells at a 1:50 dilution in 10% ABS-PBS and incubated for 1 h. The wells were then washed and alkaline phosphatase conjugated anti–human IgG or IgM was incubated in each well for 1 h. After washing, color was developed with *p*-nitrophenylphosphate in diethanolamine buffer, and the optical density was read at 405 nm. A standard curve, obtained from a range of standard sera (Antiphospholipid Standardization Laboratory, Louisville, KY), was determined on each microtiter plate (28). Calculated results were expressed in terms of IgG phospholipid binding units (GPL units) or IgM phospholipid binding units (MPL). The cutoff between low positive and medium positive was established at 20 GPL or MPL units (4, 26, 27).

Monoclonal antibodies. "Natural" monoclonal autoantibodies directed against oxidation-specific epitopes of OxLDL were cloned from hybridomas generated from apo E–deficient mice that had not been immunized exogenously. As described in the accompanying manuscript (29), B lymphocytes from the spleens of two apo E–deficient mice were fused with a myeloma cell line. Hybridoma supernatants were screened for binding to OxLDL or to models of OxLDL epitopes: MDA-LDL, 4-hydroxynonenal-LDL, and a mixture of 4 and 16 h copper-oxidized LDL. Selected hybridomas were cloned by limiting dilution and cells were injected intraperitoneally into Pristane (Aldrich Chemical Co., Milwaukee, WI) primed Balb/C mice to produce ascites fluid. After cloning, all 13 monoclonal antibodies, termed "E0 monoclonals," were isotyped as IgM and characterized as described (29). In addition, we used an IgG1a monoclonal antibody termed MDA2, which is specific for MDA-lysine epitopes (12).

Lipoprotein isolation and modification. LDL was isolated from pooled plasma of healthy human donors by sequential ultracentrifugation under conditions to minimize oxidation and proteolysis as previously described (12). MDA-LDL was prepared as described (12).

Determination of autoantibody titers against oxidized LDL. Solid-phase RIA techniques were used to measure autoantibody titers against MDA-LDL as previously described (12). The assays were performed using 96-well polyvinylchloride (Dynatech Laboratories,

Inc., Chantilly, VA) microtiter plates. Plates were coated overnight at 4°C with 50 μl of LDL or MDA-LDL (5 μg/ml) in Tris-buffered saline (TBS) with 0.27 mM EDTA and 20 μM butylated hydroxytoluene (BHT). Nonspecific binding sites were blocked by incubation with 2% BSA for 45 min at room temperature. The wells were washed four times with washing buffer (TBS containing 0.27 mM EDTA, 0.02% NaN_3, 0.05% Tween 20, and 0.001% aprotinin) using an automated plate washer. Serial dilutions of sera diluted in TBS with 3% BSA, 0.27 mM EDTA, and 20 μM BHT (dilution buffer) were added to wells and incubated for 1 h at room temperature. After four washes, the immunoglobulin bound to antigens was detected with affinity-purified mouse anti–human IgM or IgG labeled with ^{125}I using lactoperoxidase (Enzymobeads; Bio-Rad Laboratories, Richmond, CA). Plates were incubated with the secondary antibody (400,000 cpm/well) for 1 h at room temperature. The wells were washed four times and counted in a 1282 Compugamma Universal Gamma Counter (LKB Wallac Inc., Turku, Finland). The serum samples from both APS patients and controls were measured in a single assay in a blinded manner.

Chemiluminescent immunoassay for antibody binding to cardiolipin and phosphatidylserine. The chemiluminescent assay was performed using 96-well white round-bottomed MicroFluor (Dynatech Laboratories, Inc.) microtitration plates. Cardiolipin (or PS) (40 μg/ml in 100% ethanol) was added at 25 μl/well, evaporated to dryness over 10–15 min under a gentle stream of nitrogen, and incubated uncovered at room temperature for various lengths of time as indicated in individual experiments. Absolute ethanol was added to the blank wells. No separate blocking step was performed. Instead, the primary antibodies (e.g., human or mouse sera, or murine monoclonal autoantibodies), were diluted into TBS buffer containing 3% BSA, 0.27 mM EDTA and 20 μM BHT (dilution buffer). This concentration of BSA (30 mg/ml) in the dilution buffer was sufficient to prevent nonspecific binding of either human or mouse immunoglobulin (< 5 μg/ml of immunoglobulin) or monoclonal antibody (10 μg/ml) to microtiter wells. After adding 50 μl/well of solutions containing the primary antibody, the plates were incubated at room temperature for 1 h. The wells were washed four times with washing buffer (see above). In earlier experiments, we used 0.05% Tween 20 in the washing buffer, but this was omitted in later experiments (30). No differences were seen whether Tween 20 was included or not. The amount of antibody bound was measured with alkaline phosphatase–labeled goat anti–mouse IgM or IgG (Sigma Chemical Co.) by incubating for 1 h at room temperature. After four further washes with washing buffer, plates were washed four times with distilled water. 25 μl of a 50% solution of LumiPhos 530 was added to each well, and plates were incubated for 1 h at room temperature in the dark. Luminescence was determined using a Lucy 1 Luminometer and WINLCOM software (Anthos Labtec Instruments, Salzburg, Austria). Results were calculated by subtracting the binding of the secondary antibody to blank wells from the binding to cardiolipin-containing wells.

Measurement of indices of oxidation of cardiolipin. Conjugated dienes (CD) were detected by measurement of absorbance at 233 nm as described (31). CD measurements of cardiolipin (0.5 mg/ml) in ethanol were made at 30°C in quartz microcuvettes using a spectrophotometer (Uvikon 931; Kontron Instruments, Basel, Switzerland). CD measurements also were made of cardiolipin aliquots that were evaporated of solvent and then resolubilized in an equal volume of ethanol after varying times of exposure to air.

Lipid hydroperoxides were measured directly in microtiter wells containing cardiolipin according to a previously published method with minor modifications (32). 10 mg/ml of luminol and 10 mg/ml of BSA were stirred overnight and the solution then heated to 40°C. 1 h before the assay, a solution in PBS was prepared containing 2.7% luminol-albumin, 57% DMSO, and 1% microperoxidase (1 mg/ml). 25 μl of the solution were injected into each well by the automated injector of the Lucy 1 Luminometer and the amount of light generated was measured repeatedly for up to 10 s. Data are expressed as total flashes of light measured over indicated time.

Fatty acids were measured in 50-μg aliquots of cardiolipin, reduced cardiolipin and PS dried under nitrogen, and exposed to air at room temperature for various lengths of time. After incubation, fatty acids were transmethylated and analyzed in a gas chromatograph (3700; Varian, Sugarland, TX) equipped with a column of 10% Silar 5CP on $^{100}/_{120}$ Gas Chrom QII (Alltech Associates Inc., Deerfield, IL) as described (31).

Statistical analysis. Comparisons between anticardiolipin IgM and IgG levels and the amount of autoantibodies against MDA-LDL were made using ANOVA. Scheffes's test was used to identify the groups between which the differences were significant. Correlations between MDA-LDL autoantibodies and lupus anticoagulant were determined by linear regression.

Results

Anticardiolipin antibodies in apo E–deficient mice recognize oxidized phospholipid epitopes. Apo E–deficient mice on high fat diets develop marked hypercholesterolemia and extensive atherosclerosis (14). Immunocytochemistry reveals an abundance of oxidation-specific epitopes in their lesions (14) and

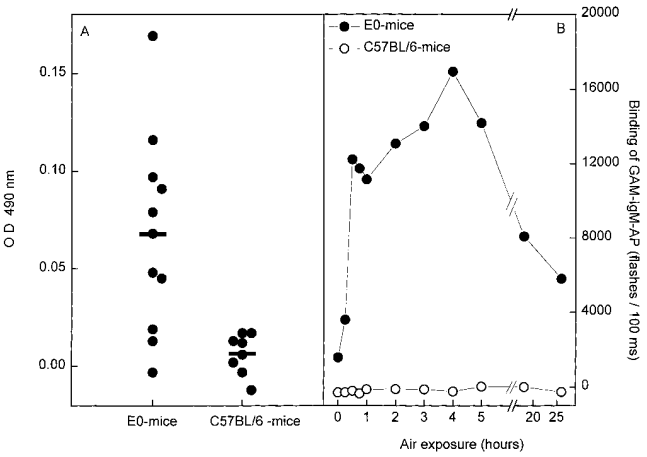

Figure 2. (*A*) The distribution of autoantibodies to cardiolipin in sera of apo E–deficient (E0) mice and C57BL/6 control mice as determined in a standard clinical anticardiolipin assay used at the UCSD Medical Center (except that the secondary antibody was antimouse Ig). In these studies, cardiolipin was evaporated and, after 30 min, murine sera were added for antibody determinations. Results are expressed as OD at 490 nm. The mean OD (±SD) for apo E–deficient mice was 0.0675 (±0.0504) and for C57BL/6 control mice 0.0065 (±0.0103) ($P = 0.005$). (*B*) Chemiluminescent immunoassay of the binding to oxidized cardiolipin of IgM from sera pooled from two apo E–deficient mice or two C57BL/6 control mice. Cardiolipin (40 μg/ml, 25 μl/well) was rapidly dried in microtiter wells under nitrogen until the ethanol was completely evaporated, and then the wells were exposed to air at room temperature for 0 to 26 h. A 1:1,000 dilution of sera (primary antibody) in 3% BSA, 0.27 mM EDTA, and 20 μM BHT was then incubated with each well for 1 h. After washing, the amount of bound antibody was detected by use of alkaline-phosphatase labeled goat anti–mouse IgM (GAM-IgM-AP) and chemiluminescent substrate. Data are expressed as number of flashes (of light) counted per 100 ms. Results were calculated by subtracting the binding of the GAM-IgM in blank wells from the binding to wells containing cardiolipin. Each point represents the mean value of at least six different wells.

very high plasma titers of autoantibodies to epitopes of OxLDL (see Fig. 8 in reference 29). Initially, we measured titers of anticardiolipin antibodies in plasma of 11 apo E–deficient mice and 8 C57BL/6 control mice with the routine clinical assay used for patients at the University of California, San Diego Medical Center (except that anti–mouse Ig was used). Fig. 2 *A* demonstrates that there were significantly higher titers of anticardiolipin antibodies in most of the apo E–deficient mice.

In the usual assay to measure anticardiolipin antibodies, cardiolipin in ethanol is added to microtiter wells and allowed to adhere to the plastic by evaporation of the ethanol. In standardized assays, this is done by "evaporation of ethanol overnight at 4°C" (28). We postulated that if these aPL were directed against oxidation-dependent epitopes, there should be increased binding to the cardiolipin as it was progressively oxidized upon exposure to air. To test this, we developed a sensitive chemiluminescent immunoassay to detect antibody binding to cardiolipin. Binding to cardiolipin was studied as soon as possible after a rapid evaporation of ethanol under nitrogen to establish a "zero time point" and compared to binding to cardiolipin that was exposed to air at room temperature for extended periods of time (analogous to "overnight evaporation" used in standard assays [28]). We determined antibody binding to cardiolipin using a 1:1,000 dilution of pooled plasma obtained from two apo E–deficient mice and two C57BL/6 control mice (Fig. 2 *B*). Whereas the C57BL/6 plasma displayed no binding at any time, there was a striking time-dependent increase in antibody binding for the apo E–deficient plasma. Within 30 min of exposure of the immobilized cardiolipin to air there was a 10-fold increase in IgM binding, which peaked at $\sim$ 4 h in this assay and then decreased with increased air exposure. Similar data were found for IgG binding (data not shown). We interpreted these data to indicate that Ig was binding to epitopes generated by increasing times of oxidation. However, with continued oxidation, some epitopes were subsequently destroyed by the ongoing oxidative changes. Note that there was increased binding of IgM (and IgG) from the apo E-deficient plasma even at the zero-time sample. This is consistent with the presence of some oxidized cardiolipin even in the starting material. Indeed, marked variation in binding to the zero-time samples of different cardiolipin preparations was observed. Consistent with an earlier report (33), we also observed significantly greater binding to older cardiolipin preparations, particularly those that had been previously opened, even if they were stored at −20°C under nitrogen.

To document that the plated cardiolipin was oxidized when exposed to air, we measured several indices of lipid peroxidation (Fig. 3). The absorbance at 233 nm, indicative of CD formation, was measured in an ethanol solution of cardiolipin. An initial measurement was performed on a cardiolipin aliquot that had not been dried under nitrogen (termed "liquid" in Fig. 3). Separate aliquots were dried under nitrogen and then resuspended in an equal volume of ethanol immediately (0 min) or after 35 or 75 min of air exposure. The CD content doubled in the sample exposed to air for even the briefest period of time and increased fourfold by 35 min (Fig. 3 *A*). Lipid hydroperoxides, measured directly in microtiter wells containing cardiolipin that was exposed to air under the exact conditions used in the immunoassays, rose progressively with increasing time of air exposure (Fig. 3 *B*). Furthermore, the fatty acid composition of the cardiolipin changed with exposure to air. Each cardiolipin molecule contains four fatty acids, of

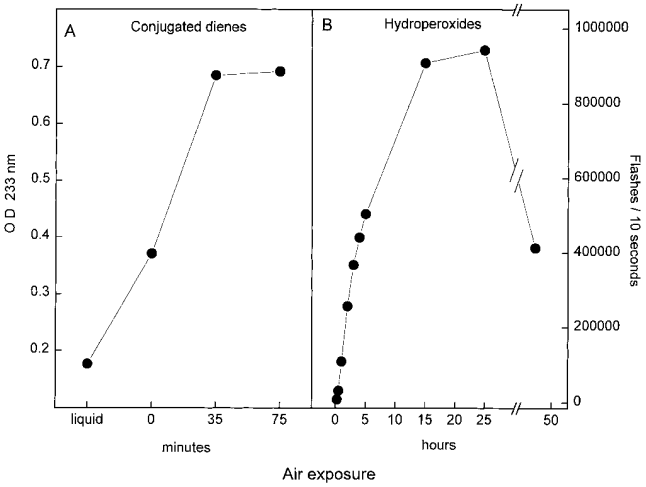

Figure 3. Measurements of oxidation of the cardiolipin. (*A*) Measurement of conjugated dienes in cardiolipin (0.5 mg/ml) in ethanol (liquid), and in identical aliquots of cardiolipin that had been dried under nitrogen and then resolubilized in an equal volume of ethanol after the cardiolipin had been exposed to air at room temperature for 0 to 75 min. (*B*) Formation of lipid hydroperoxides. Cardiolipin in ethanol (40 μg/ml, 25 μl/well) was added to microtiter wells, dried under nitrogen for 15 min until ethanol evaporated and then exposed to air at room temperature for 0 to 48 h. Hydroperoxides were then measured in each well using luminol and microperoxidase as explained in Methods, and the amount of light generated over 10 s quantitated using a microtiter luminometer. Each value is the mean of six determinations. Similar results were seen in four other experiments.

which 91–92% are linoleic acid (18:2). After 5 and 24 h of air exposure, there was a loss of 24 and 98%, respectively, of the initial linoleic acid content (data not shown).

In the accompanying paper (29) we described the cloning of 13 monoclonal antibodies from apo E–deficient mice, each selected for its ability to bind to an epitope of OxLDL. To determine if any of these also recognized cardiolipin, we tested their ability to bind to either freshly opened cardiolipin or to cardiolipin that had been in the freezer for 2 mo and had been opened several times (Fig. 4 *A*). In this assay the plated cardiolipins were exposed to air for only 1 h at room temperature. Antibody E06 showed significant binding to the fresh preparation and even greater binding to the aged one. In a competition assay, cardiolipin was able to significantly compete for the binding of E06 to the older cardiolipin preparation (Fig. 4 *B*). Several other E0 antibodies showed some binding ability, particularly to the older preparation. Of particular note is the marked binding of MDA2 to the aged cardiolipin preparation. MDA2 is a previously cloned monoclonal antibody directed against the model epitope of OxLDL, MDA-lysine (12). Thus, the binding of MDA2 most likely indicates that malondialdehyde, generated from peroxidation of linoleic acid in cardiolipin, formed MDA-lysine adducts with BSA that was used to dilute the primary antibody (Pathway 1, Fig. 1). In these assays, no protein (or gelatin) was added as "post-coat" to block wells after antigen plating. We found that doing so greatly reduced specific antibody binding. We believe this occurred because the added BSA bound not only to the unoccupied sites on the plastic wells, but because of its lipid binding properties, to the plated cardiolipin as well. Therefore, in direct binding

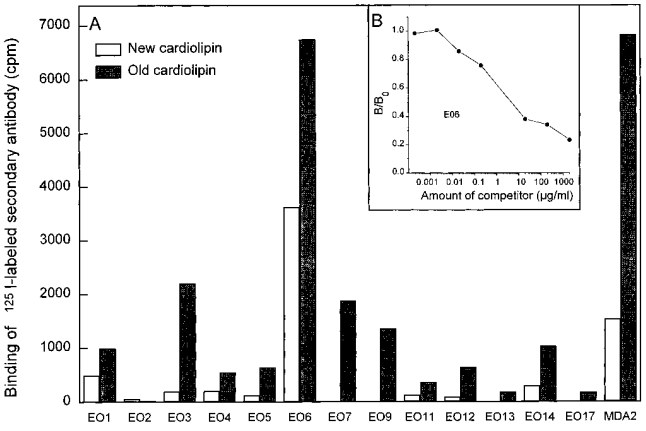

Figure 4. (*A*) RIA of the binding to cardiolipin of 13 natural monoclonal antibodies (E0) cloned from apo E–deficient mice and monoclonal antibody MDA2 (specific for MDA-lysine). The E0 antibodies were selected for their ability to bind to epitopes of OxLDL (29). Two different cardiolipin preparations were used as plated antigen: one was over 2 mo old and had been opened several times (old cardiolipin); and the other was a freshly opened, unused cardiolipin (new cardiolipin). In this assay, cardiolipin was evaporated under nitrogen and then exposed to air at room temperature for 1 h. Each of the purified monoclonal antibodies was diluted to 10 μg/ml and incubated with antigens for 1 h. After washing, the amount of antibody bound was determined using a radiolabeled goat anti–mouse IgM (for E0) or anti–mouse IgG (for MDA2). Results were calculated by subtracting the binding of the secondary antibody to blank wells from the binding to cardiolipin. Each value is the mean of triplicate determinations. (*B*) Competitive solid-phase RIA with monoclonal antibody E06 to oxidized cardiolipin. A fixed dilution of the primary antibody (E06) was added to wells coated with cardiolipin that had been exposed to air for 1 h, in the absence or presence of indicated amounts of cardiolipin competitor. Results were expressed as B/B_0, where B is the binding of E06 to the cardiolipin in the presence of competitor and B_0 is the binding in the absence of competitor.

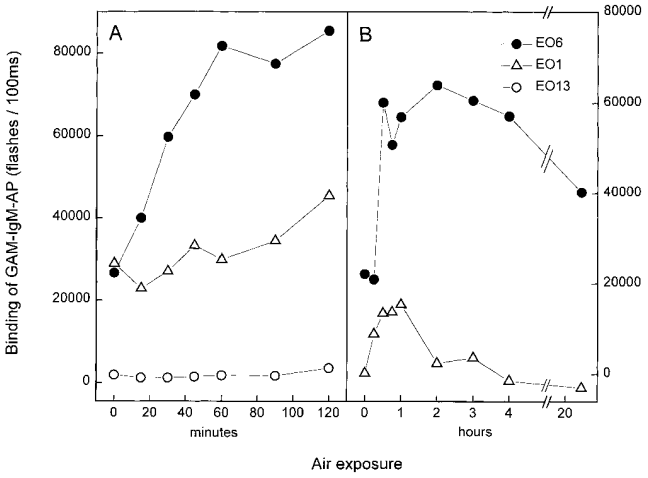

Figure 5. Chemiluminescent immunoassay of the binding of E0 monoclonal antibodies to cardiolipin (*A*) or phosphatidylserine (*B*). Conditions for assay are as explained in legend to Fig. 2. A fixed dilution of each purified monoclonal antibody (10 μg/ml) was used. Results were calculated by subtracting the binding of the secondary antibody to blank wells from the binding to wells with cardiolipin or phosphatidylserine. Each point represents the mean value of triplicate determinations. Note that *A* represents 120 min of air exposure, while *B* represents 22 h.

assays, we diluted the primary antibody in 3% BSA, 0.27 mM EDTA, and 20 μM BHT. The BSA in the dilution buffer was sufficient to prevent nonspecific binding of the primary antibody to the plate and the antioxidants inhibited any further oxidation of the plated cardiolipin.

We also tested the ability of E06 and E01 to bind to a fresh preparation of cardiolipin that was exposed to air for increasing periods of time (Fig. 5 *A*). For comparison we used E013, one of the E0 antibodies that did not bind cardiolipin. There was a marked increase in binding of E06 to cardiolipin with increasing time of air exposure, whereas antibody E01 showed binding that gradually increased over 2 h. In contrast, the binding of E013 was low and did not change with air exposure. Fig. 5 *B* demonstrates that similar results were seen when we tested the binding of E06 and E01 to bovine brain PS, another anionic phospholipid commonly used in aPL assays, which also contains unsaturated fatty acids.

Anticardiolipin antibodies in women with APS recognize oxidized phospholipid. To determine if results similar to those seen with the plasma and monoclonal antibodies of the apo E-deficient mice were found in humans, we performed similar studies with sera from 10 women with APS and 10 age-matched controls, documented in the laboratory of Dr. W. Branch (4, 26). As shown in Fig. 6, women with the highest titer of anticardiolipin antibody determined in Dr. Branch's laboratory also had the highest titer of antibody to MDA-LDL. For the 10 women with APS, all of whom had a positive lupus anticoagulant antibody, there was also a strong correlation between the activated partial thromboplastin time (aPTT) and IgM antibody titer to MDA-LDL. Using a sensitive chemiluminescent immunoassay, the IgG titer to MDA-LDL also correlated with the aPTT ($r = 0.84$, $P < 0.002$) (data not shown). Fig. 7 displays results of IgM (*left*) and IgG binding (*right*) to cardiolipin as a function of time of exposure to air. Each sample was made up of a pool of equal amounts of serum from four women with APS or controls, respectively, and was tested at a 1:100 dilution. Curves very similar to those observed with apo E–deficient mouse sera were obtained with increasing times of oxidation. Note that a laboratory using a 3–5-h evaporation time might get quite different results from a laboratory using the recommended overnight evaporation technique (28). Although absolute binding was much lower, even the non-APS controls showed a threefold rise in binding as a function of oxidation, at least for IgG.

To determine what percentage of anticardiolipin antibodies are binding to oxidation-derived epitopes, we tested the ability of affinity-purified aCL-IgG to bind to cardiolipin exposed to air for varying periods. These aCL-IgG samples were prepared from reference sera in the laboratories of Pierangeli and Harris by immunoabsorption to cardiolipin-containing liposomes (34). After purification, the anticardiolipin binding activity of each aCL-IgG preparation was measured again and the results were expressed as GPL units. Seven of the nine aCL-IgG preparations showed varying degrees of anticardiolipin binding activity (from 68 to 304 GPL units) and two showed no anticardiolipin binding activity (0 GPL units) when retested. Fig. 8 shows the example of the highest titered IgG preparation (GPL = 304) and an IgG sample that had no activity on retesting (GPL = 0). The positive IgG preparation showed virtually no binding to cardiolipin exposed to air < 5

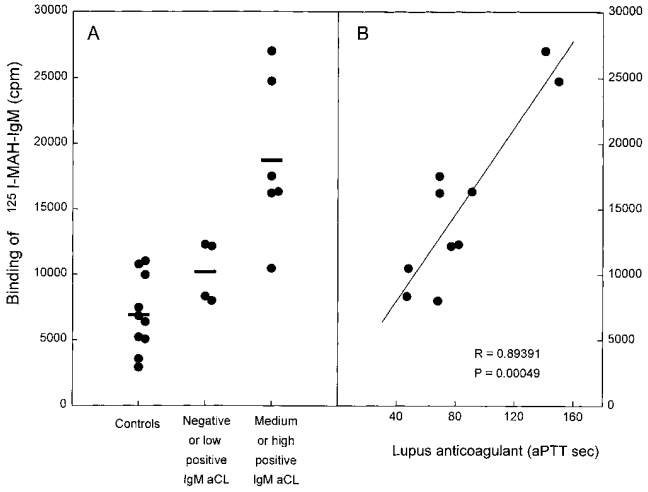

Figure 6. (*A*) RIA showing the binding of IgM autoantibodies from sera of patients with APS and their age-matched controls to malondialdehyde-modified LDL (MDA-LDL). As determined in the standardized laboratory of Dr. Branch (4, 26), four patients had negative or low positive IgM anticardiolipin antibody (*aCL*) levels and six patients had medium or high positive IgM aCL. A fixed dilution of the sera (1:500) was used as primary antibody. Binding to MDA-LDL was calculated by subtracting the binding of labeled mouse anti–human IgM (MAH-IgM) to native LDL from that to MDA-LDL. (*B*) Correlation between the binding of IgM autoantibodies to MDA-LDL and the activated partial thromboplastin time (*aPTT*) in the 10 patients with APS. The upper limit for "normal" in this assay is 48 s. Patients with lupus anticoagulant have a prolonged aPTT.

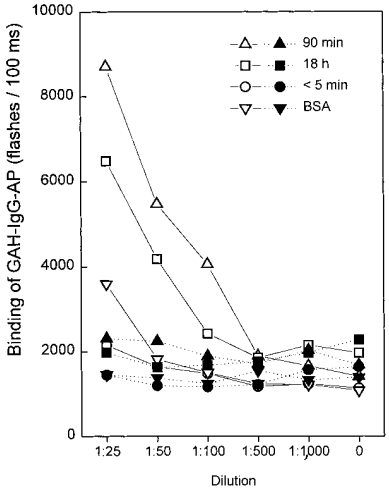

Figure 8. Binding of affinity-purified anticardiolipin IgG to cardiolipin exposed to air for indicated periods of time. IgG from patients with APS were affinity-purified by binding to cardiolipin-liposomes as described (34). After isolation, IgG was retitered in a standardized laboratory (34). The open symbols display binding of a purified IgG fraction with GPL = 304 (protein concentration 0.48 mg/ml), while closed symbols display binding of a purified IgG that showed no anticardiolipin binding activity (protein concentration 0.64 mg/ml). Cardiolipin in ethanol was pipetted into microtiter wells as described in Fig. 2 and ethanol evaporated under nitrogen. The indicated dilutions of the purified IgG fractions were added to wells in which the dried cardiolipin was exposed to air for < 5 min, for 90 min, or for 18 h. Binding to wells coated only with BSA is shown for comparison. The amount of IgG bound was detected by use of alkaline phosphatase–labeled goat anti–human IgG (*GAH-IgG-AP*). Each point is the mean of four replicate determinations.

min, whereas binding to cardiolipin exposed to air for 90 min or 18 h was quite high (i.e., these latter values would be similar to standard assays where antigen was evaporated overnight). The IgG preparation with no binding activity did not bind to any of the wells. Similarly, none of the six other positive aCL-IgG fractions tested showed binding to the cardiolipin sample exposed to air < 5 min, while all six showed marked binding to the cardiolipin exposed to air for 18 h. To the extent that the affinity-purified IgG are representative of all anticardiolipin antibody populations, this experiment suggests that most aPL in these patient's sera are directed against oxidation-dependent epitopes. It should be noted, however, that, although not directly measured, it is likely that the CL-containing liposomes used for isolation contained oxidized CL, since the standard conditions for preparation of such liposomes involves an initial evaporation of the phospholipid on a glass surface (34).

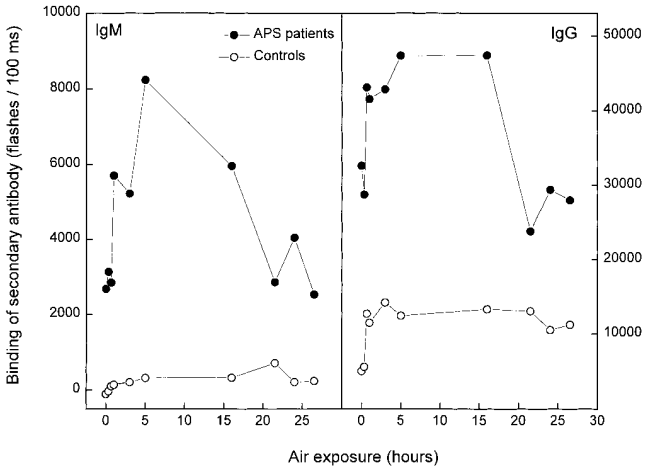

Figure 7. Binding to oxidized cardiolipin of IgM (*left*) and of IgG (*right*) from sera of patients with APS and controls. Equal aliquots of sera from four patients with APS and four age-matched controls were used to make pools of APS and control sera, respectively. Sera were used at 1:100 dilution. Assay conditions were as described in Fig. 2. Each value represents the mean of four replicate determinations.

Binding of APS sera and monoclonals to reduced cardiolipin. The preceding data suggest that the aPL antibodies bind to epitopes of cardiolipin generated as the result of oxidation of the lipid under the conditions of the assay. If this interpretation is correct, then there should be no binding to a cardiolipin analog that is incapable of undergoing lipid peroxidation. To test this, we compared the binding of the APS sera (Fig. 9 *B*), and that of the affinity-purified aCL-IgG (Fig. 9 *A*), to cardiolipin and an analog in which all fatty acid double bonds had been reduced by hydrogenation in the presence of platinum. While there was the expected marked increase in binding of both APS sera and the aCL-IgG to cardiolipin with increased exposure to air, neither preparation bound to the reduced cardiolipin, even after 20 h of air exposure. After 24 h of air exposure, fatty acid analysis of the reduced cardiolipin revealed that there was no change in its fatty acid content (e.g., 91.5% of fatty acids were 18:0) (data not shown). We also demonstrated that murine monoclonal antibody E06 did not bind to the reduced cardiolipin (data not shown). In control experiments, we demonstrated that the expected amount of reduced cardiolipin was indeed solubilized and plated in the microtiter wells.

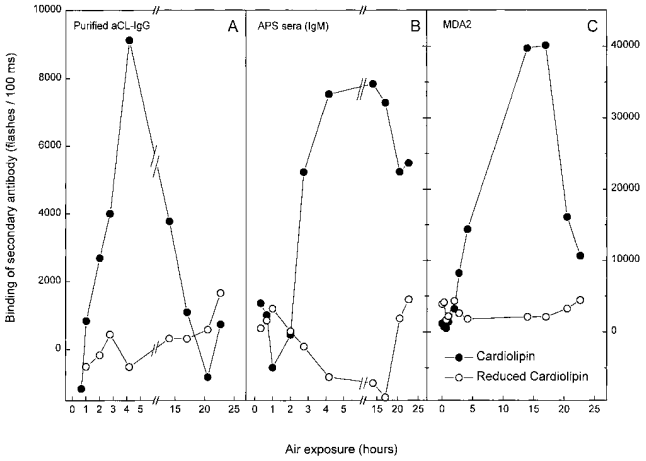

Figure 9. Binding of APS sera, purified anticardiolipin IgG (*aCL-IgG*) and MDA2 to cardiolipin and reduced cardiolipin after exposure to air. In each panel, cardiolipin or reduced cardiolipin was added to microtiter wells in ethanol, dried under nitrogen and then exposed to air for indicated times. (*A*) Binding of a pool of aCL-IgG prepared from equal aliquots from five different purified aCL-IgG fractions known to be positive for binding to cardiolipin. (*B*) Binding of a 1:100 dilution of sera pooled from four APS patients (as in Fig. 7). (*C*) Binding of monoclonal antibody MDA2. In each experiment, binding of primary antibody was detected by use of appropriate alkaline-phosphatase–labeled secondary antibody and the binding of the primary antibody to blank wells was subtracted from binding to antigen-containing wells. Each point is the mean of four replicate determinations. The ordinate for both *B* and *C* is on the right side of the figure.

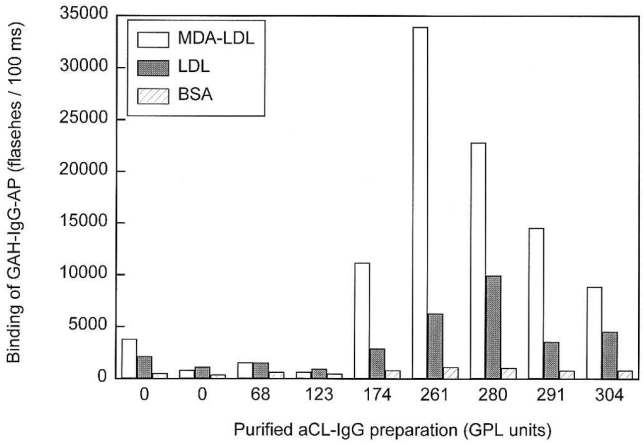

Figure 10. Binding of aCL-IgG to MDA-LDL, LDL, and BSA. IgG fractions were purified from reference sera and anticardiolipin activity was determined (28, 34) and expressed as GPL units. An equal amount of each aCL-IgG (20 μg/ml) was added as primary antibody and the amount of IgG bound was detected by use of alkaline phosphatase–labeled goat anti-human IgG (*GAH-IgG-AP*). Each observation is the mean of three replicate determinations.

In the experiment shown in Fig. 4, it was demonstrated that monoclonal antibody MDA2 also bound to cardiolipin, particularly the older preparation. Because the epitope of this antibody is MDA-lysine, or an immunologically closely related structure, this implied that MDA, generated from decomposition of peroxidized fatty acids in cardiolipin, was forming MDA-lysine adducts with protein (such as BSA) present in the assay. To explore fully the time course of MDA-lysine generation, we tested the binding of MDA2 to cardiolipin as a function of the time of air exposure (Fig. 9 *C*). There was a rapid rise in MDA2 binding, which peaked after 15 h of air exposure; the epitope appears to be destroyed with further degrees of oxidation. In contrast, monoclonal E013 did not bind at any time point (data not shown). To show that generation of the epitope recognized by MDA2 was dependent on oxidation of cardiolipin, we also demonstrated that MDA2 did not bind to the reduced cardiolipin at any time point after exposure to air (Fig. 9 *C*).

These data suggest that one class of epitopes to which anticardiolipin antibodies bind is MDA-lysine, and/or immunologically similar structures, generated when the cardiolipin's fatty acids undergo lipid peroxidation. To test this, we examined the ability of the nine aCL-IgG preparations described above to bind to MDA-LDL, an antigen rich in MDA-lysine epitopes (Fig. 10). In general, the aCL-IgG preparations with high anticardiolipin binding activity bound very well to MDA-LDL, but did not bind to BSA. The five aCL-IgG preparations with the highest GPL titers also bound slightly to native LDL, presumably reflecting minor degrees of oxidation in these LDL samples, despite efforts to inhibit oxidation (12). In contrast, the aCL-IgG preparations with low GPL titers failed to bind to MDA-LDL or native LDL. It should be noted, however, that there was considerable difference in binding to MDA-LDL among the high titered aCL-IgG preparations, suggesting heterogeneity in the antibody populations.

Discussion

For many years our laboratory has been interested in the "oxidation hypothesis" of atherosclerosis (for reviews see references 10, 11, 35, 36). Of relevance to the present studies is the growing recognition that oxidation of LDL results in a profound humoral and cellular autoimmune response (for reviews see references 22, 23). There were two sets of related observations that led us to test the hypothesis that the aPL described in patients with APS were in fact directed against neoepitopes generated when phospholipids containing PUFA underwent lipid peroxidation. The first was our demonstration that women with preeclampsia have increased titers of autoantibodies to MDA-LDL (37). We undertook that study because of the suggestion that there is an enhanced state of lipid peroxidation in women with preeclampsia (38), and because distal portions of the decidual arterioles immediately underlying the placenta show focal accumulation of lipid-laden macrophages in these women (39). The second set of observations was that when LDL undergoes oxidation there is a profound modification of associated phospholipid (40). The initial site of oxidation of LDL (at least when mediated by copper in vitro) occurs in the PUFA of phospholipids, on the surface of LDL (31). Intact phospholipid is lost and a variety of oxidative decomposition products are generated, including phospholipids containing oxidized fatty acid chains in the *sn*-2 position. These oxidized fatty acid fragments are the substrate for a unique phospholipase A_2 activity intrinsic to the LDL (41), which in large part appears to be platelet-activating factor acetyl-hydrolase (42). During this process, many decomposition products

are generated, such as MDA. We reasoned that MDA could modify not only associated amines of lysine residues, but appropriate amino-phospholipids as well (Fig. 1). To model this, we added MDA to PE and generated several MDA-PE products, which were used to immunize guinea pigs. Antisera were generated that recognized MDA-PE when plated on microtiter wells and that immunostained atherosclerotic lesions (S. Socher, S. Parthasarathy, W. Palinski, and J.L. Witztum, unpublished observations). Itabe et al. recently generated a monoclonal antibody to an oxidatively modified phosphatidylcholine epitope, using atherosclerotic plaque as the immunogen, and demonstrated its ability to immunostain fatty streak lesions (43). In addition, elegant studies from several laboratories have now demonstrated that a variety of oxidatively modified phospholipids are generated when LDL undergoes even minimal degrees of lipid peroxidation, and many of these have profound proinflammatory properties. In particular, Watson et al. (44) and Heery et al. (45) have recently demonstrated that platelet-activating factor–like molecules are generated when LDL is mildly oxidized.

Because modified phospholipids were generated when LDL was oxidized, and these could be immunogenic, and because preeclamptic women who had elevated titers of antibodies to MDA-LDL frequently also had aPL, we wondered if the antigens inducing the formation of antibodies against OxLDL might be a source of aPL (or vice versa). Because of our previous experience in working with phospholipids, we were well aware of the extreme susceptibility of cardiolipin and PS to oxidation, particularly when exposed to air. Because the standardized assay for anticardiolipin antibodies, as described by the Anticardiolipin Wet Workshop report (28), recommends plating of cardiolipin by "evaporation of ethanol overnight at 4°C," a condition that would clearly lead to oxidation of the plated phospholipid, we hypothesized that some of the aPL were in fact directed to oxidized phospholipid epitopes. Furthermore, a growing body of evidence suggested a relationship of aPL not only to stroke (4, 46–49), but to coronary artery disease as well (6, 50–52). The studies described in this report validated our hypothesis.

We have provided evidence that reference sera of patients with well-documented APS, as well as affinity-purified aCL-IgG derived from their sera, bind exclusively to cardiolipin that has undergone lipid peroxidation, and not to a cardiolipin analog unable to undergo lipid peroxidation. In addition, we have demonstrated that apo E–deficient mice have high titers of anticardiolipin antibodies, as determined in a routine "clinical" assay. Immunoglobulins from their plasma also bind only to cardiolipin that has been oxidized, but not to a cardiolipin analog unable to be oxidized. Several monoclonal antibodies cloned from these mice, selected for their ability to bind to epitopes of OxLDL, also bind to oxidized cardiolipin and not to the reduced cardiolipin analog. These data clearly support our hypothesis that most "antiphospholipid antibodies" are directed at neoepitopes generated when the plated cardiolipin undergoes lipid peroxidation. Indeed, the very conditions of the assay, by continually generating and destroying epitopes through the unregulated process of oxidation and subsequent rearrangement chemistry, add to variability of results by continually changing the spectrum of epitopes available for antibody binding. The variability due to differing oxidation products is clearly illustrated in Figs. 8–11. This variability is further augmented by the fact that different cardiolipin preparations have undoubtedly undergone differing degrees of oxidation even before plating has begun.

The situation is even more complicated. During the oxidative breakdown of PUFA, a variety of highly reactive compounds are generated (including MDA, 4-hydroxynonenal, acrolein, glyoxal, etc.), which in turn can react with other portions of the phospholipid or with available proteins, as described above for OxLDL (Pathway 1, Fig. 1). Similarly, reactive aldehydes may remain on the oxidized fatty acid fragments attached to the phospholipid molecule after decomposition (Pathway 2, Fig. 1). All of these products may react with lysine residues of proteins (or other groups) creating covalent lipid-protein adducts or modified phospholipid-protein adducts. We have previously demonstrated that such adducts can be highly immunogenic (discussed above). Thus, when any protein is added to the microtiter wells containing oxidized cardiolipin, for example, adduct formation with that protein could occur. This could happen when BSA or adult bovine serum (28) is added to "block" the residual sites on microtiter wells, or even when patient serum itself is added. Proteins with high affinity binding to phospholipids, such as β_2-glycoprotein I (β_2-GPI), would be particularly amenable to such modification. It is also our hypothesis that such adducts between oxidation products of phospholipids and proteins, involving reactive breakdown products that have "separated" from the phospholipid (e.g., MDA, as well as reactive products remaining on the phospholipid molecule) are also epitopes recognized by some aPL (Fig. 1). This hypothesis would be compatible with the data of Matsuura et al. (53) who demonstrated that anticardiolipin antibodies bind to β_2-GPI only when it was bound to a microtiter plate previously irradiated, a condition that introduces oxygen radicals and C-O and C=O moieties in the polystyrene. This binding was competitively inhibited by cardiolipin–β_2-GPI complexes, but not by cardiolipin or β_2-GPI alone.

Our observation that monoclonal antibody MDA2, which specifically recognizes MDA-lysine (12), bound to cardiolipin as it was progressively oxidized (Fig. 4 and Fig. 9 *C*), strongly suggests that MDA-lysine epitopes (or immunologically similar structures) were created between the added protein and MDA released from the oxidized cardiolipin. The failure of MDA2 to bind to the reduced cardiolipin even after extensive exposure to air (Fig. 9 *C*) would also support this hypothesis. The demonstration that the epitopes of many aPL are in fact various lipid-protein adducts, such as MDA-lysine, would explain the well-documented ability of some anticardiolipin antibodies to bind to β_2-GPI when plated in the presence of cardiolipin (54–56); i.e., allowing for such adducts to form between cardiolipin and β_2-GPI. It could also explain why cardiolipin is immunogenic when injected into an animal together with β_2-GPI, but not when injected alone (57, 58). In contrast, when β_2-GPI is the sole immunogen, it has been reported to be capable of inducing "anticardiolipin antibodies." Because of its strong phospholipid binding properties, it is likely that after injection into an animal, the β_2-GPI would associate with endogenous phospholipids, which in turn would become oxidized due to the influence of the strong inflammatory response triggered by the injection of an immunogen, particularly in the presence of adjuvant. The report that immunization of mice with human anticardiolipin IgG leads to endogenous production of anticardiolipin antibodies (58) might be explained on a similar basis. Thus, any protein with strong phospholipid-binding properties might produce a sufficiently close apposition of

protein and lipid, such that when oxidation occurs in the inflammatory setting, oxidation-dependent lipid-protein adducts are generated (in an analogous fashion to generation of MDA-lysine adducts when LDL is oxidized). Indeed, a highly conserved, lysine-rich domain of β_2-GPI has been reported to be responsible for its ability to bind cardiolipin (59). Such highly charged regions are also found in apo B, the protein of LDL.

The pathogenic mechanisms that generate the immunogens in vivo that are responsible for the generation of aPL are unknown. If our hypotheses are correct, many of these antibodies may be induced by neoepitopes formed as the result of proinflammatory conditions that lead to oxidation of PUFA in phospholipids present in cellular membranes or in phospholipids present in lipoproteins. Cardiolipin is also a prominent phospholipid of many bacteria, as well as in the *Treponema pallidum*, the causative agent of syphilis. It is likely that the rise in aPL that occurs with a variety of infections, including syphilis (60), is due to oxidation of the cardiolipin found in the invading pathogen. In fact, aPL are observed in a variety of inflammatory settings, such as those associated with connective tissue diseases, eclampsia, juvenile arthritis, and of course SLE (46, 47), as well as in "active" atherosclerotic lesions (6). It should also follow that some of the autoantibodies generated in these inflammatory settings should recognize oxidation-specific epitopes of OxLDL, and at least some of these should have the characteristics of "aPL." Consistent with this, elevated titers of autoantibodies to various epitopes of OxLDL are found in SLE (61, 62), chronic juvenile arthritis (63), periarteritis nodosa (62), scleroderma (62), and eclampsia (37). For example, Vaarala et al. (61) demonstrated that 80% of 61 SLE subjects had IgG antibodies to an epitope of OxLDL (MDA-LDL) while 40% of patients had elevated IgG anticardiolipin titers, and many of these antisera showed cross-reactivity to OxLDL and cardiolipin. Furthermore, Hashimoto et al. (64) reported that autoimmune-prone $(NZW \times BXSB)F_1$ male mice, which have a systemic lupus-like syndrome, are also a model of APS. These mice have high titers of antibodies to both MDA-LDL and OxLDL, and two monoclonal antibodies cloned from them, on the basis of their binding to cardiolipin, also exhibited enhanced binding to OxLDL (65). Finally, Mironova et al. recently reported that IgG fractions purified from two human subjects by OxLDL affinity chromatography also bound to plated cardiolipin (66).

LDL may well represent a model phospholipid-protein complex that is similar to cardiolipin–β_2-GPI complexes or phospholipid-protein complexes in cells. Our hypothesis would predict that oxidative modification of any such complex should give rise to many neoepitopes, some distinct, but many in common. Antibodies to MDA-lysine adducts are likely to be such a prototype, and indeed antibodies to MDA-LDL were found in many autoimmune conditions noted above. We also suggest that some of the autoantibodies generated against oxidation-specific epitopes in atherosclerotic lesions will recognize similar epitopes in oxidized cardiolipin or oxidized cardiolipin-protein complexes. Since our data suggest that standard anticardiolipin antibody assays measure predominantly antibodies to such oxidation-specific epitopes, it follows that anticardiolipin titers should be increased in individuals with active atherogenesis and ongoing formation of OxLDL. This is consistent with our observation that apo E–deficient mice with active atherogenesis have very high titers of autoantibodies not only to epitopes of OxLDL, but also to cardiolipin. Furthermore, several monoclonal antibodies cloned from these mice on the basis of their ability to bind to OxLDL display classical anticardiolipin and anti–PS binding activity. Our hypothesis would also explain recent data from the Helsinki Heart Study, in which both anticardiolipin titers and anti–MDA-LDL titers were predictors of subsequent myocardial infarction and the respective titers correlated significantly ($r = 0.40$, $P < 0.001$) (6). Of interest, the anticardiolipin titers were significantly higher in smokers, a condition associated with a generalized prooxidant state.

We recognize that there is likely to be heterogeneity among aPL and that, as in most biological issues, complexity and diversity will be the rule. Indeed, reports in the literature of the generation of antibodies (usually in liposomes) to phospholipids containing essentially only saturated fatty acids (67) are hard to reconcile with our working models. However, our hypotheses would not be in conflict with the idea that conformational changes in cardiolipin, due to protein-induced alterations, also lead to immunogenicity (68). It is possible that there are different classes of aPL directed to "oxidation-dependent" and "oxidation-independent" neoepitopes. Our studies have not yet addressed the issue of whether such oxidation-dependent aPL have important biological activities, such as being a lupus anticoagulant. However, the association of elevated aPL to disease states appears to be based almost exclusively on the presence of antibodies to cardiolipin (or PS) determined under conditions that would lead to its oxidation. Knowledge that the immunogens for many anticardiolipin antibodies were generated as a result of prooxidant conditions may give new insights into the underlying pathogenic events responsible for their generation. In turn, this could lead to new forms of therapy, directed at the underlying etiology of the enhanced prooxidant state (e.g., inflammation) as well as more broad-based interventions, such as intensive antioxidant therapy.

For many years, there has been the assumption that antiphospholipid antibodies are directed against native, "self," phospholipids. From a heuristic point of view, we feel it useful to pursue the concept that many antiphospholipid antibodies are not generated against native phospholipid molecules, but are in fact directed against nonself modifications. The trick, of course, is to be able to define the modifications, as well as to obtain the antigen in its true, native state; i.e., can cardiolipin be isolated and used in an assay without any oxidation occurring? Even the presence of only a small percentage of oxidized cardiolipin molecules in a microtiter well could lead to the appearance of antibody binding to "native" cardiolipin. By analogy, are other subtle modifications of native molecules, such as cholesterol or DNA, responsible for binding of anticholesterol (69) and anti–DNA antibodies (70, 71)? Could such common and subtle oxidation-derived modifications be present on both DNA and cardiolipin, for example, and could these epitopes be responsible for the reported ability of some anticardiolipin antibodies to bind to DNA? Further study of this interesting problem may give new insights into the etiology of many so-called "autoantibodies."

Acknowledgments

The authors thank Dr. D. Steinberg for advice and support, Michael Jacobson for help in the initiation of these studies and Florencia Casanada and Richard Elam for technical assistance.

These studies were supported by the National Heart, Lung, and Blood Institute grant 14197 (SCOR, La Jolla, CA) the Emil Aaltonen

Foundation, the Academy of Finland, the Finnish Foundation For Cardiovascular Research, and a gift from the Diagnostic Division of Chugai Pharmaceutical, Ltd., Tokyo.

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