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Journal

Journal of Biological Chemistry, 277(9)

ISSN

0021-9258

Authors

Friedman, Peter
Hörkkö, Sohvi
Steinberg, Daniel
et al.

Publication Date

2002-03-01

DOI

10.1074/jbc.m108860200

Peer reviewed

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 9, Issue of March 1, pp. 7010–7020, 2002

Printed in U.S.A.

Correlation of Antiphospholipid Antibody Recognition with the Structure of Synthetic Oxidized Phospholipids

IMPORTANCE OF SCHIFF BASE FORMATION AND ALDOL CONDENSATION*

Received for publication, September 13, 2001, and in revised form, December 13, 2001
Published, JBC Papers in Press, December 14, 2001, DOI 10.1074/jbc.M108860200

Peter Friedman‡§, Sohvi Hörkkö§¶, Daniel Steinberg¶, Joseph L. Witztum¶, and Edward A. Dennis‡‖

From the Departments of ‡Chemistry and Biochemistry and ¶Medicine, University of California San Diego, La Jolla, California 92093-0601

The oxidation of low density lipoproteins (LDL) has been correlated with atherogenesis through a variety of pathways. The process involves nonspecific fragmentation, oxidative breakdown, and modification of the lipids and protein of LDL. The process yields a variety of bioactive products, including aldehyde-containing phospholipids, which can cross-react with primary amines (*i.e.* peptides or phospholipid head groups) to yield Schiff base products. We also demonstrate that such oxidized phospholipid products may further react through a post-oxidation chemical pathway involving aldol condensation. EO6, an IgM monoclonal autoantibody to oxidized phospholipids, blocks the uptake of oxidized LDL (OxLDL) by macrophages. Because the epitope(s) of EO6 also blocks the uptake of OxLDL, a series of oxidized phospholipids, their peptide complexes, and their aldol condensates have been synthesized and characterized, and their antigenicity has been determined. This study defines structural motifs of oxidized phospholipids responsible for antigenicity for EO6. Certain monomeric phospholipids containing short chain fatty acids were antigenic whether oxidized or not in the sn-2 position. However, oxidized phospholipids containing sn-1 long chain fatty acids were not antigenic unless the sn-2 oxidized fatty acid contained an aldehyde that first reacted with a peptide yielding a Schiff base or the sn-2 oxidized fatty acid underwent an aldol type self-condensation. Our data indicate that the phosphorylcholine head group is essential for antigenicity, but its availability depends on the oxidized phospholipid conformation. We suggest that upon oxidation, similar reactions occur in phospholipids on the surface of LDL, generating ligands for macrophage recognition. Synthetic imine adducts of oxidized phospholipids of this type are capable of blocking the uptake of OxLDL.

Oxidation of LDL[1] is thought to play an important role in atherogenesis. Indeed there is considerable experimental evidence that oxidized LDL (OxLDL) exists *in vivo* in the artery wall and contributes to the initiation and progression of atherosclerotic lesion formation (1–6). When LDL undergoes oxidation, a wide variety of oxidatively modified phospholipids can be formed that are biologically active (7–11), as reviewed elsewhere (12, 13). Specifically, polyunsaturated fatty acids are converted to fatty acid hydroperoxides, which decompose to form highly reactive breakdown products such as malondialdehyde and 4-hydroxynonenal (14). Such reactive aldehydes can then form covalent Schiff base and Michael-type adducts with lysine residues of apoB, the protein moiety of LDL (15). In addition, the sn-2 fatty acid fragments remaining attached via the ester bonds in the phosphatidylcholine may also contain terminal reactive aldehydes (8, 13, 16–20). For example, in the case of the common phospholipid 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC), a near terminal oxidation product would yield an aldehyde at the ϵ-carbon of the sn-2 oxidized arachidonic acid, yielding the reactive compound POVPC (1-palmitoyl-2-(5′-oxo)valeroyl-*sn*-glycero-3-phosphorylcholine). In turn, this reactive phospholipid, also termed a "phospholipid core aldehyde" (21–23), could also form Schiff base adducts with lysine residues of apoB and presumably other proteins and also with amine-containing phospholipids such as phosphatidylethanolamine and phosphatidylserine. Obviously, many other oxidized lipid-protein and oxidized lipid-lipid adducts could occur.

We have previously demonstrated that when LDL undergoes oxidative modifications, a variety of neoepitopes are formed that render the modified LDL highly immunogenic (24–26). Indeed, there are a variety of autoantibodies to "oxidation-specific" epitopes of OxLDL in animals and humans, the titers of which appear to be increased in subjects with clinical and morphological measures of atherosclerosis (27–30). In particular, the natural immune response was so robust in cholesterol-fed apoE-deficient mice (EO) that it enabled us to prepare hybridomas from the spleens of these mice and to clone a panel of autoantibodies to epitopes of OxLDL (26). All of the autoan-

* This work was supported by National Institutes of Health Grants HL57505 (to J. L. W.), HL56989 (to La Jolla Specialized Center of Research in Molecular Medicine and Atherosclerosis), and GM20501 (to E. A. D), Tobacco Related Disease Research Program Grant 0KT-0220 (to P. F.), and an American Heart Association Western States Affiliates postdoctoral fellowship (to P. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work, as did the laboratories of Drs. Witztum and Dennis.

‖ To whom correspondence and reprint requests should be addressed. Fax: 858-534-7390; E-mail: edennis@ucsd.edu.

[1] The abbreviations used are: LDL, low density lipoprotein; OxLDL, oxidized LDL; PC, phosphorylcholine; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine; POVPC, 1-palmitoyl-2-(5′-oxo)valeroyl-*sn*-glycero-3-phosphorylcholine; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; PONPC, 1-palmitoyl-2-(9′-oxo)nonanoyl-sn-glycero-3-phosphorylcholine; C_6OVPC, 1-caproyl-2-(5′-oxo)valeroyl-*sn*-glycero-3-phosphorylcholine; PAF, platelet-activating factor; PAPE, 1-palmitoyl-2-arachidonoyl phosphatidylethanolamine; PLPS, 1-palmitoyl-2-linoleoyl phosphatidylserine.

tibodies were of the IgM isotype and were termed "EO" autoantibodies. An initial characterization revealed that some of them bound to OxLDL, prepared by copper-induced oxidation, and bound also both to the isolated lipid as well as to the delipidated apoB of OxLDL (OxapoB). None bound to the lipid or apoB of native LDL. In subsequent studies, it was shown that these EO antibodies, as exemplified by EO6, specifically bound to POVPC preparations plated on microtiter wells and to $NaCNBH_3$-reduced POVPC-bovine serum albumin (BSA) adducts. On Western blots, antibody EO6 immunostained OxapoB but not native apoB (31). Furthermore, if the OxapoB was first subjected to saponification to remove the covalently bound phospholipid moiety at the sn-2 ester bond, then EO6 binding to OxapoB was abolished (21). These data strongly suggested that EO6 recognized the oxidized phospholipid either as the nonconjugated lipid or as a covalent adduct with lysine or lysine residues of apoB.

The EO autoantibodies to oxidized phospholipid, such as EO6, and oxidized phospholipid-apoB adducts were shown to have important biological activities. For example, they effectively blocked the binding and uptake of OxLDL by mouse peritoneal macrophages (31). In addition, they bound to the surface of apoptotic cells and blocked in part their phagocytosis by macrophages (32). Furthermore, the POVPC-BSA adduct was also able to compete effectively for OxLDL and apoptotic cell uptake by macrophages (31, 32). These data strongly support the hypothesis that the epitope(s) recognized by EO6 and related autoantibodies are important biological ligands.

As part of these studies we cloned the variable regions of the heavy (V_H) and light (V_L) chains of the EO antibodies that bind to POVPC adducts, such as EO6, and discovered that the V_H/V_L regions were identical to an antibody first described more than 30 years ago, the T15 natural IgA antibody (33). The T15 is of germ-line origin, which confers optimal protection to mice against lethal infection with Gram-positive bacteria such as *Streptococcus pneumoniae*. T15 is known to be an "anti-phosphorylcholine antibody" and binds to this moiety in the capsular polysaccharide (C-PS) present in the cell wall of many pathogens. Indeed, both T15 and EO6 have similar binding properties, and in particular, phosphorylcholine (PC) is able to fully compete for the binding of EO6 and T15 to OxLDL (33). Because the head group PC is present in both PAPC as well as in POVPC, it was not clear why EO6 and T15 would bind only to the oxidized phospholipid. Because the epitope(s) recognized by EO6 is an important biological ligand, we therefore undertook a careful analysis of the structural requirements of an oxidized phospholipid to make it antigenic for the EO antibodies, using EO6 as a model antibody.

In the present study, we demonstrate that both Schiff base formation and aldol condensation are important reactions for the conversion of phospholipids containing reactive aldehydes into immunogenic products. During the aldol condensation process, the α-carbon of one aldehyde containing phospholipid adds to the carbonyl carbon of another aldehyde containing phospholipid molecule, yielding a β-hydroxy aldehyde (aldol) (34) which can further dehydrate, leading to an α,β-unsaturated aldehyde. Thus, the aldol condensation can yield intermolecular reactions between two or more aldehyde-containing phospholipids. Apparently the latter process can even occur with synthetic oxidized phospholipids stored in chloroform at −20 °C. We now examine the conditions for the formation of the aldol condensate, the stability of the oxidized phospholipids, and the effect of structural modifications on the antigenicity for EO6.

EXPERIMENTAL PROCEDURES

Materials

All of the phospholipids were purchased from Avanti Polar Lipids. Arachidonic acid was purchased from Cayman Chemical Co. Keyhole limpet hemocyanin (KLH) was obtained from Biosearch Technologies Inc. Schiff's Reagent (for detection of aldehydes on thin layer chromatography), Sigma spray reagent molybdenum blue (for detection of phosphates on thin layer chromatography), BSA, and phosphate buffer saline (PBS) were purchased from Sigma. Methyl sulfide, nonanedioic (azelaic) acid, 1,3-diisopropylcarbodiimide, 1,3-dicyclohexyl carbodiimide, 4-(dimethylamino)pyridine, 1-hydroxybenzotriazole, 2-methyl-2-butene, sodium cyanoborohydride, and sodium borohydride were obtained from Aldrich. Both preparative and thin layer chromatography plates were purchased from Merck (EM Science). Optima grade solvents were obtained from Fisher. Deuterated chloroform was purchased from Cambridge Isotope Laboratories.

Antibody

EO6 is a monoclonal IgM antibody that recognizes and binds to epitopes of OxLDL (25, 26, 31, 33). It was cloned from apoE-deficient mice (EO autoantibody) and purified as described (33).

Antigenicity

To define the structural motifs of various phospholipids that are the basis for their antigenicity toward EO6, we compared the ability of various phospholipid analogues to compete with EO6 for binding to OxLDL plated on a microtiter well using competition immunoassays as described (31, 33). For these studies, increasing amounts of the indicated compounds were added to siliconized 1.5-ml microcentrifuge tubes. In the case of various lipid analogs, they were added in ethanol, and the solvent was evaporated. To each tube, EO6 in PBS (1 μg/ml) was added and incubated for 18 h at 4 °C. After incubation, the mixture was centrifuged at 15,000 × g for 30 min, and an aliquot of the supernatant was taken to test its binding to OxLDL. For this assay, 50 μl of OxLDL diluted in PBS to 5 μg/ml containing 0.27 mM EDTA was added to 96-well white round-bottomed Micro-Fluor microtiter plates (Dynex Technologies) and left overnight at 4 °C. The wells were washed three times with PBS using an automated plate washer, and unoccupied sites were blocked with PBS containing 1% BSA (PBS+BSA) for 30 min. After further washing, 50 μl of the EO6-containing supernatants were then applied to triplicate wells and incubated for 1 h at room temperature. The wells were then washed three times with PBS. Binding of EO6 was detected with alkaline phosphatase-labeled goat anti-mouse IgM secondary antibody (Sigma) in Tris-buffered saline containing 150 mM NaCl, 50 mM Tris base, 0.27 mM EDTA, and 1% BSA. This was followed by a rinse with water and the addition of 25 μl of 50% Lumi-Phos 530 solution (Lumigen Inc.). Light emissions were measured as relative light units over 100 ms using a DYNEX Luminometer (Dynex Technologies). Data were expressed as the ratio of B/B_0 where B represents the binding of EO6 to OxLDL in the presence, and B_0, in the absence of competitor.

General Method for the Synthesis of Oxidized Phospholipids

The aldehyde-containing phospholipids were synthesized by ozonolysis of the unsaturated, double-bond of the fatty acid of the appropriate phospholipids at −70 °C in dichloromethane for about 20 min followed by the breakup of the ozonide by adding methyl sulfide (16). Typically, ozone was bubbled through a solution of 25 mg of phospholipid dissolved in 3 ml dry dichloromethane at −70 °C for about 20 min followed by removal of excess ozone by bubbling dry nitrogen through the solution. Typically, the ozone-saturated dichloromethane became light blue, a color that could be used as an internal indicator. Then 0.8 ml of methyl sulfide was added, and the solution was shaken and slowly allowed to reach 4 °C over 5 h. The solvent was evaporated under a stream of nitrogen and lyophilized to remove the dimethyl sulfoxide formed. The precipitate was re-suspended in 1 ml of chloroform/methanol (1:1) mixture and separated on preparative layer chromatography. The reduction of the aldehyde to the alcohol was accomplished utilizing an equimolar quantity of sodium borohydride ($NaBH_4$) in isopropanol at room temperature for 3 h followed by a preparative layer chromatography separation.

The further oxidation of the aldehyde to the carboxylic acid was accomplished with sodium chlorite (35). The aldehyde was dissolved in a mixture of *tert*-butanol and 2-methyl-2-butene. An aqueous solution of sodium chlorite and sodium dihydrogen phosphate was added dropwise. The reaction mixture was stirred overnight, followed by drying the mixture under vacuum, re-dissolving it in water, acidifying with HCl to

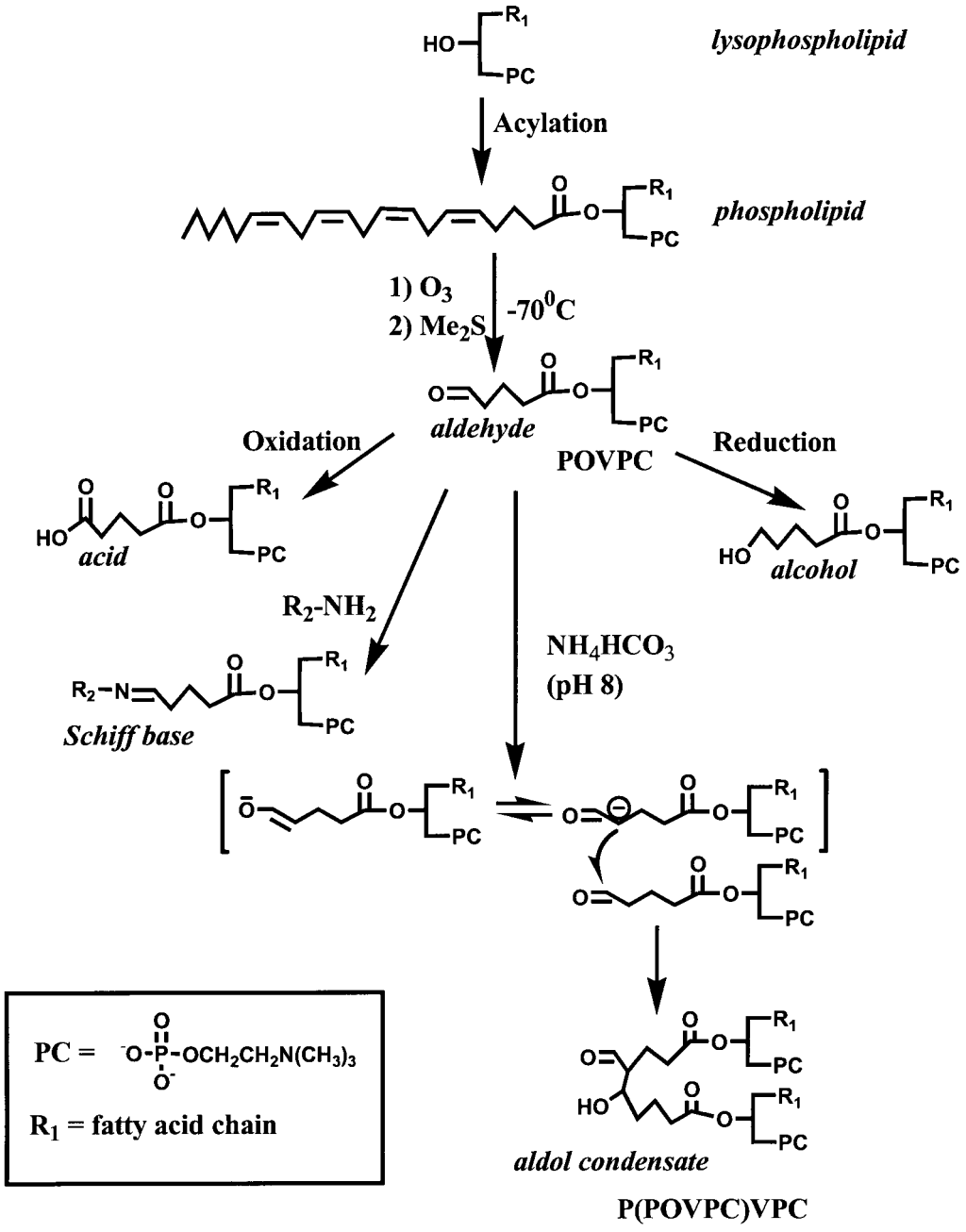

FIG. 1. **Schematic presentation of the synthetic pathway where PC is phosphorylcholine, R_1 is fatty acid chain, and R_2-NH_1 is lysine or lysine-containing peptide/protein.** In the first step, the appropriate phospholipid is prepared if commercially unavailable (such as 1-caproyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine). The polyunsaturated fatty acid of the phospholipid is oxidized to yield an aldehyde. The aldehyde is subjected to further oxidation, reduction, formation of a Schiff base with a free amine containing molecule, or aldol condensation.

pH 5, and extracting with chloroform. The product was isolated after a preparative layer chromatographic separation.

The peptide-lipid adducts were formed by incubating the aldehyde-containing lipids with the appropriate lysine-containing peptide in borate buffer at pH 7.4 to form a Schiff base between the aldehyde and the ϵ-amine of the lysine. The newly formed imine was reduced with sodium cyanoborohydride ($NaCNBH_3$) to yield a secondary amine.

Diacyl phospholipids that were not commercially available were synthesized by the acylation of the appropriate lysophospholipids. Typically an equimolar quantity of the appropriate fatty acid (*i.e.* arachidonic or linoleic acid) was esterified to the sn-2 hydroxyl of lysophospholipid using 1.1 molar eq of 1,3-dicyclohexyl carbodiimide, 0.3 molar eq of 4-(dimethylamino)pyridine, and 0.2 molar eq of 1-hydroxybenzotriazole in dry dichloromethane at 0 °C overnight. Upon discarding the urea salt via filtration, the product was separated on preparative layer chromatography. The synthetic steps are summarized in Fig. 1.

POVPC

POVPC was prepared by oxidizing PAPC as described under "General Method for the Synthesis of Oxidized Phospholipids." The product was separated on a silica-based preparative layer chromatography as described below. The product was identified by positive reactions with molybdenum blue reagent (36) and Schiff's reagent sprays. The Rf of the product was 0.375. The purity of the aldehyde was confirmed by mass spectroscopy (single molecular peak, m/z = 594), ^{13}C NMR, ^1H NMR, and reverse phase high performance liquid chromatography (HPLC) (single peak), which all were consistent with the theoretical prediction as well as previous findings (16). Based on these measurements, the purity of the POVPC has been estimated to be at least 99.5%. The yield of the product was 47%.

P(5′-carboxy)VPC was prepared by further oxidation of POVPC with sodium chlorite as described above. The product was purified on a silica-based preparative layer chromatography as described below. Its

structure and purity were confirmed by NMR and mass spectroscopy (m/z = 610). The yield of the product was 86%.

P(5′hydroxy)VPC was prepared by reducing POVPC with $NaBH_4$ as described above. The product was purified on a silica-based preparative layer chromatography as described below. Its structure and purity were confirmed by NMR and mass spectroscopy (m/z = 596). The yield of the product was 94%.

1-Palmitoyl-2-(9′-oxo)nonanoyl-sn-glycero-3-phosphorylcholine (PONPC)

PONPC was prepared by ozonolysis as described under "Experimental Procedures." from 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphorylcholine. The product was separated by silica-based preparative layer chromatography using chloroform/methanol/water (10:5:1) as the mobile phase. The product was identified by positive reactions with molybdenum blue reagent (36) and Schiff's reagent sprays. The purity of the aldehyde was confirmed by ^{13}C NMR, ^1H NMR, reverse phase HPLC (single peak), and mass spectroscopy, which all were consistent with the prediction. Based on the experimental data, the purity of the PONPC was estimated to be at least 99.5%. The yield of the product was 56%.

POVPC Adducts

POVPC-Lys—POVPC was incubated in methanol with lysine in the molar ratio of POVPC:Lys 10:11 for 3 h at room temperature. This yielded the Schiff base product. Reduction of the formed imine to the appropriate secondary amine was performed by the addition of $NaCNBH_3$, and the mixture was kept overnight at 4 °C. The Schiff base product was used without further purification, whereas the reduced amine was purified by reverse phase HPLC.

POVPC-peptide

The following peptides containing a single lysine residue were used: Ac-Ala-Ala-Lys-Ala-Tyr (Ac-AAKAY) and Ac-Val-Ile-Asp-Ala-Leu-Gln-Tyr-Lys-Leu-Glu-Gly-Thr-Thr, the 3373–3385 fragment from the sequence of the ApoB-100 that is part of the putative LDL receptor binding site (GenBank™ accession number P04114), yielding POVPC-AcAAKAY and POVPC-LDL peptide (the 13-mer above), respectively. The tyrosine was included in the pentapeptide to allow for spectrophotometric detection. POVPC was incubated in methanol with an equimolar amount of peptide for 4 h to give the Schiff base product, which subsequently was reduced to an amine as for POVPC-Lys. POVPC-BSA was synthesized as previously described (31), and PONPC-BSA was generated in a similar manner.

POVPC-PE

POVPC was incubated in methanol with an equimolar amount of dipalmitoyl phosphatidylethanolamine for 4 h. Sodium cyanoborohydride was added to reduce the imine to a secondary amine; the reaction was kept for 4 h at room temperature. The purification involved preparative layer chromatography separation followed by scraping of the appropriate silica lane and extracting the product from the silica as described under "Experimental Procedures."

1-Caproyl 2-(5′-oxo)-valeroyl-sn-glycero-3-phosphorylcholine (C_6OVPC)

1-Caproyl-2-(5′-oxo)valeroyl-*sn*-glycero-3-phosphorylcholine (C_6OVPC) was synthesized in two steps. First, the 1-caproyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lysocaproyl-PC) was esterified with arachidonic acid. The reaction occurred between the hydroxyl group on the sn-2 position of the glycerol backbone of the lysophospholipid and the carboxylate of the arachidonic acid as described under "Experimental Procedures." This step yielded 1-caproyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine, which was further oxidized with ozone as described under "Experimental Procedures" to yield the desired C_6OVPC.

P(POVPC)VPC was prepared by incubating POVPC in ammonium carbonate buffer (50 mM) at pH 8 at room temperature overnight (Fig. 1). The reaction mixture was dried completely (sublimating the salts) and resuspended in $CDCl_3$ for spectral analysis. The product proved to be the aldol condensate (Fig. 1). It did not undergo spontaneous dehydration to an α,β-unsaturated aldehyde, which in turn would have been able to undergo a variety of further reactions (Michael reaction followed by another aldol condensation, polymerization due to the electronegativity of the allylic carbon, etc.). The yield of the product was 76%.

P(5′-P(hydroxy)VPC)VPC (Fig. 2)

The aldehydic residue of P(5′-POVPC)VPC was reduced to an alcohol upon incubation in isopropanol in the presence of $NaCNBH_3$. The product was separated using preparative layer chromatography. ^1H NMR, mass spectroscopy (m/z = 1190), and IR confirmed the structure and the purity of the product. The yield of the product was 88%.

Di-lysoPC-C9 (Fig. 2)

1-Palmitoyl-2-hydroxy-*sn*-glycero-3-phosphorylcholine was reacted with nonanedioic acid (in the ratio of 2:1) in the presence of 1 eq of 1,3-dicyclohexylcarbodiimide, 0.3 eq of 1-hydroxybenzotriazole, and 0.2 eq of 4-(dimethylamino)pyridine in dichloromethane, stirring overnight at 10 °C. The product was purified after preparative layer chromatography using chloroform/methanol/water (10:5:1) as the mobile phase. The appropriate silica spot was scraped, and the product was extracted twice using chloroform/methanol/water (20:50:1) followed by isopropanol/hexane (2:1) once and chloroform/methanol/water (10:5:1) twice. The combined extracts were concentrated and re-dissolved in deuterated chloroform. The structure and the purity of the product were confirmed by ^1H NMR, mass spectroscopy (m/z = 1144), and IR. The yield of the product was 64%.

DiOVPC

1,2-Diarachidonoyl-*sn*-glycero-3-phosphorylcholine was oxidized and worked up as in the synthesis of POVPC in an attempt to synthesize di-1,2-(5′-oxo)-valeroyl-*sn*-glycero-3-phosphorylcholine. Upon structure determination by NMR, it appeared to form a covalent adduct yielding polymeric fractions of dimer, trimer, and tetramer units. The adducts were of the aldol condensation type as described under "Results." The units purity and structure were confirmed by ^1H NMR (the obvious "fingerprint" was the change in the ratio between the aldehyde hydrogen and the hydrogens of the trimethylamine of the head group yielding 1:6 for the dimer and 1:7 for the trimer and tetramer) and mass spectroscopy (m/z = 882 for the dimer, m/z = 1326 for the trimer, and m/z = 1793 for the tetramer).

Chromatography

The HPLC experiments were performed on a Waters system using Keystone Scientific reverse phase Betasil-C18 columns. For the lipid-peptide adducts a gradient separation was used starting with a 2:3 mixture of methanol and 10 mM NH_4HCO_3 buffer at pH 7, and ending with *n*-propanol at a flow of 0.6 ml/min for 100 min. For the pure lipid, an isocratic separation was employed using methanol/acetonitrile/water (95:5:1) in the presence of 10 mM choline chloride as the mobile phase.

The preparative layer chromatography separation was performed with Merck plates coated with 2-mm thick silica gel $60F_{254}$ in chloroform/methanol/water (10:5:1). The appropriate oxidized phospholipid was identified using Schiff's reagent (detects aldehydes) and molybdenum blue (detects phosphates). The appropriate modified phospholipids containing segments of the silica were scraped off, and the lipid was extracted from the silica three times using chloroform/methanol/water (20:60:1) followed by isopropanol/hexane (2:1) and again twice with chloroform/methanol/water (30:50:1). The combined extracts were dried, resuspended in deuterated chloroform, filtered to eliminate traces of silica, and analyzed.

Analysis

A Bruker AMX-500 was used to perform the NMR experiments for structural and purity determination. The IR measurements were performed on a Nicolet Magna-IR 550 series II system. The molecular weights of the synthetic phospholipids were determined by mass spectroscopy using matrix-assisted laser desorption/ionization. Phospholipid quantification was performed by converting the organic phosphate of the phospholipid into inorganic phosphate using sulfuric acid followed by the addition of ammonium molybdate and ascorbic acid to form a phosphomolybdate complex according to the standard phosphate determination protocol (36). The amount of phosphate was quantified measuring its absorption at 660 nm, and the corresponding concentration values were calculated from a standard curve. Qualitative recognition on thin and preparative layer chromatography were performed using commercial Schiff base reagent and molybdate phosphate reagent.

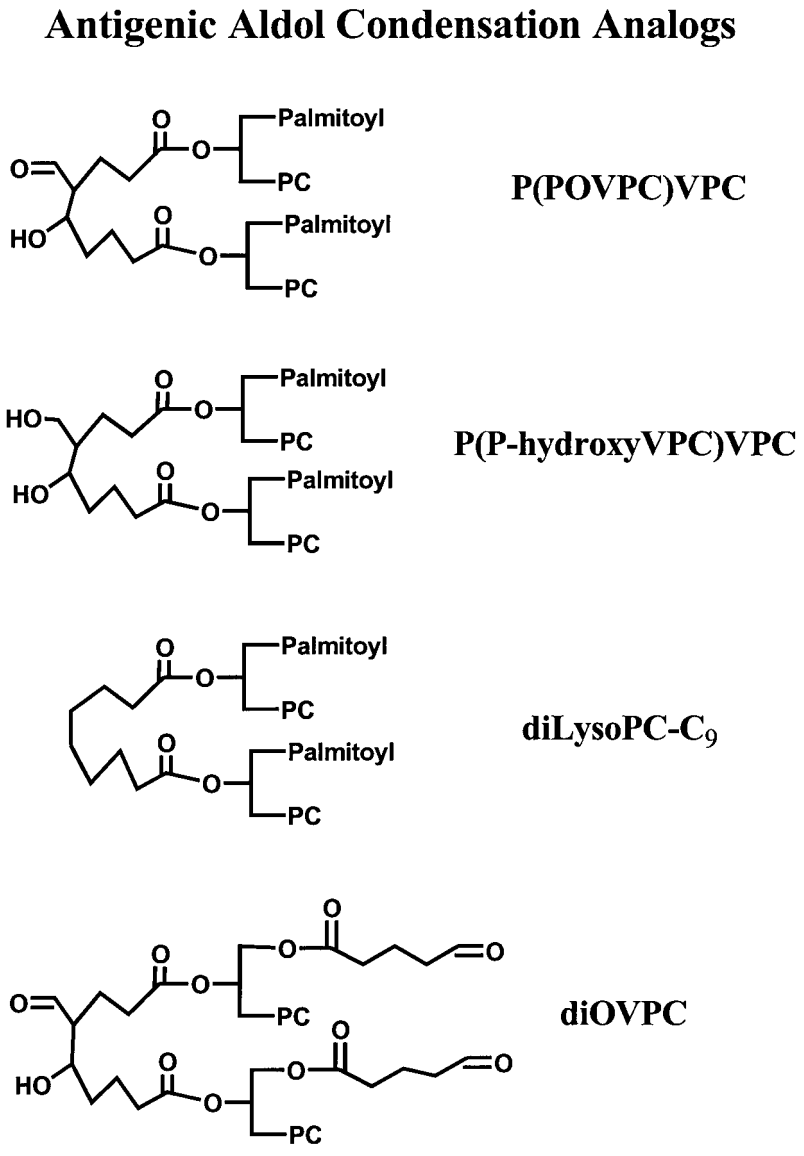

FIG. 2. **Synthetic aldol condensation analogs that are antigenic.**

RESULTS

Small Molecules and Short Fatty Acid Chain Phospholipids—Consistent with the sequence homology between the V_H/V_L region of EO6 and T15, a murine monoclonal IgA antiphosphorylcholine antibody, PC was found to be a very good antigen for monoclonal EO6 (33). Therefore, recognition of PC was used as a positive control for individual experiments, either covalently bound to KLH (PC-KLH) as a carrier protein or as a free salt. Fig. 3 presents the competition curves of Ox-LDL, POVPC-BSA, and PC-KLH for their ability to inhibit the binding of EO6 to OxLDL. In this figure, we present the data as the moles of protein added as competitor, although it should be appreciated that each modified protein has a large but different number of reactive epitopes. EO6 recognized epitopes on all modified proteins but did not react with the unmodified proteins (data not shown). Note that LDL modified with numerous malondialdehyde groups is not reactive.

We then studied a variety of individual lipids for their ability to compete with EO6 for binding to OxLDL to define the structures responsible for their antigenicity. The phosphorylcholine head group was essential for EO6 reactivity. Compounds analogous to phosphorylcholine such as phosphorylethanolamine and phosphorylserine did not interact with EO6 (Fig. 4). Glycerophosphorylcholine, the compound formed by the addition of a glycerol backbone to phosphorylcholine, bound nearly as well to EO6 as PC. The addition of two short fatty acids to glycerophosphorylcholine, yielding either 1,2-dipropionyl-*sn*-glycero-3-phosphorylcholine (diC_3PC) or 1,2-divaleroyl-*sn*-glycero-3-phosphorylcholine (diC_5PC), also gave compounds with good antigenicity (Fig. 4). Note that choline alone reacted only at very high concentrations, suggesting that the phosphate moiety is an important component of the antigen.

To test how the degree of phospholipid hydrophilicity affects the antigenicity of the compounds, we compared 1-caproyl-2-hydroxy-*sn*-glycero-3-phosphorylcholine ($lysoC_6PC$) with 1,2-dicaproyl-*sn*-glycero-3-phosphorylcholine (diC_6PC) and C_6OVPC (Fig. 5). C_6OVPC was the most potent antigen, whereas diC_6PC was the least potent among the three caproyl chain-containing phosphatidylcholines in a competition study against EO6, a finding that correlates with the relative hydrophilicity of these lipids. The level of hydrophilicity of a lipid is inversely correlated with its tendency to aggregate (note that just because a lipid is below its classical critical micellar concentration, there can still be some degree of aggregation, so one may not have true monomers). Among the molecules listed above, the hydrophilicity varies, although only diC_6PC forms micelles at high concentration. Thus, in the case of monomeric phospholipids, there is a notable correlation of antibody recognition to hydro-

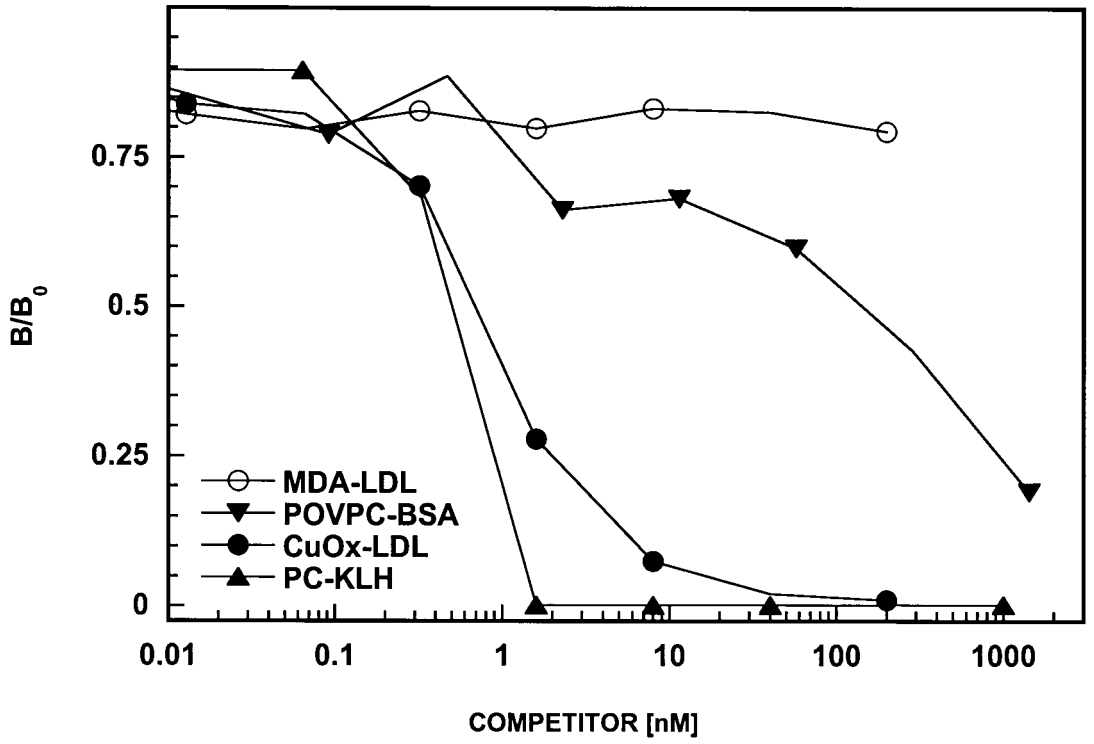

FIG. 3. **Competition immunoassay showing binding of a fixed and limiting amount of EO6 to CuOx-LDL in the absence or presence of increasing amounts of malondialdehyde (*MDA*)-LDL (malondialdehyde-modified LDL, a negative control for the antibody; ○), POVPC bound to BSA (*POVPC-BSA*; ▼), CuOx-LDL (●), and phosphorylcholine bound to KLH (PC-KLH; ▲).** The protein modifications were approximately as follows. The malondialdehyde-LDL has 75% of apoB lysines modified (apoB-100 has about 356 mol/mol of apoB). The POVPC-BSA has about 45% of lysines modified. The modification of OxLDL was not determined, although we know on average about 75 mol of lysines are modified with intact phospholipids. The PC-KLH is a commercial product and contains 11 mol of PC/mol of KLH. The amount of antibody bound was then measured with alkaline phosphatase-labeled goat anti-mouse IgM antibody using chemiluminescent techniques, as described under "Experimental Procedures." Values shown are the means of triplicate determinations, measured in relative light units for 100 ms and expressed as the ratio of B/B_0, where B represents the binding of EO6 to OxLDL in the presence, and B_0, in the absence of competitor. The error in these experiments was smaller then the size of the symbols. The experiment shown is representative of at least five experiments.

philicity of the lipid; an increase in aggregation levels appears to decrease the EO6 recognition. The IC_{50} values, the concentration of competitor yielding 50% inhibition in the EO6 binding assay to OxLDL, for the short chain phospholipids are shown in Table I.

Long Fatty Acid Chain Phospholipids—POVPC, P(hydroxy)VPC, P(carboxy)VPC, PONPC, PAPC, lysoPC (1-palmitoyl-*sn*-glycero-3-phosphorylcholine), platelet-activating factor (PAF), lysoPAF (1-*O*-hexadecyl-*sn*-glycero-3-phosphorylcholine) were tested for antibody binding to EO6. None of these long chain phospholipids were antigenic (Table II). The failure of POVPC to bind (inset Fig. 5*B*) was surprising, since in an earlier study we had observed it to be antigenic (31). This is addressed below.

We have attempted to synthesize the phosphatidylethanolamine and phosphatidylserine analogs of POVPC by ozone-based oxidation of 1-palmitoyl-2-arachidonoyl phosphatidylethanolamine (PAPE) and 1-palmitoyl-2-linoleoyl phosphatidylserine (PLPS), respectively. However, upon formation, the aldehyde immediately reacted in either an intermolecular or intramolecular fashion with the head group amine, yielding various Schiff base products. These "polymerized" products of PAPE and PLPS oxidations (oxPAPE and oxPLPS, respectively) did not bind to the antibody (Table II).

Schiff Base Products—The non-antigenic aldehyde containing long chain phospholipids (POVPC and PONPC) were incubated with free amine-containing compounds of peptide and phospholipid origin to determine whether Schiff base formation would lead to increased antibody recognition (Table II). Lysine, the two lysine-containing peptides, and albumin were incubated with POVPC, yielding an imine, a Schiff base formed by reacting the aldehyde with the ϵ-amine. Both peptides contained a single lysine, yielding 1 POVPC-lysine moiety/mol. In the case of albumin, containing ~66 lysines/mol of protein, the number of POVPC-lysine adducts per mole was ~30 mol/mol of albumin, based on picrylsulfonic acid (TNBS) assay (37) (Fig. 3). The POVPC-lysine adducts were tested either as imines or as secondary amines upon reduction with $NaCNBH_3$, and both showed reactivity with EO6 (Table II). The peptide and albumin products were all reduced to secondary amines. The analogous complex of PONPC with albumin was also prepared. All of these adducts exhibited high antigenicity toward EO6 (Table II). The pure lysine and lysine-containing peptides as well as albumin failed to bind to the antibody (data not shown). Forming a Schiff base between POVPC and PE through the ethanolamine head group also yielded a non-antigenic product both in the imine as well as the secondary amine form (data not shown).

Aldol Condensation Products—As noted above, the failure to observe binding to freshly prepared POVPC was surprising. However, we noted that when the POVPC was allowed to "age" a few weeks after preparation, it exhibited an aging-dependent recognition. Because the starting POVPC was 99.5% pure, we postulated that it underwent intermolecular reactions and, in particular, aldol condensation. To test this hypothesis P(POVPC)VPC was synthesized as described under "Experimental Procedures." To verify the structure, NMR, IR, and mass spectroscopy analysis was performed. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.86 (6H t $J_{H\text{-}H}$ = 6.8 Hz CH_3), 1.28 (48H m CH_2), 1.47 (3.9H m H at C-3 of sn-1), 1.6 (3H m O-CH-(CH_2)-CH), 1.95 (5H

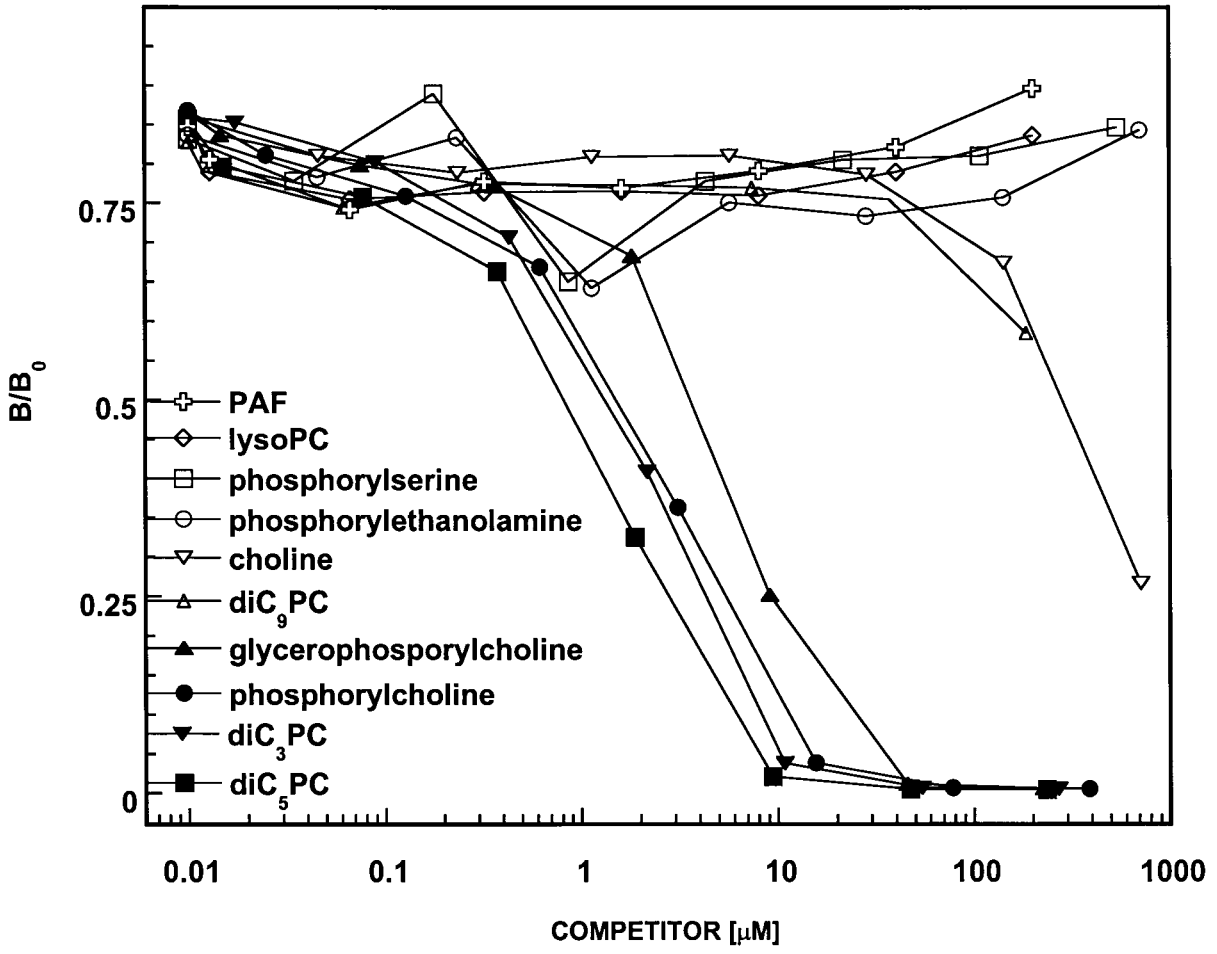

FIG. 4. **Competition immunoassay showing binding of a fixed and limiting amount of EO6 to CuOx-LDL in the absence or presence of increasing amounts of the following competitors: ✣, PAF; ◇, lysoPC; □, phosphorylserine; ○, phosphorylethanolamine; ▽, choline; △, diC$_9$PC; ▲, glycerophosphorylcholine; ●, phosphorylcholine; ▼, diC$_3$PC; and ■, diC$_5$PC.** The techniques are the same as described in legend for Fig. 3.

dd $J_{H\text{-}H}$ = 6 Hz, 7.3 Hz H at C-3 of sn-2), 2.31 (4.1H m H at C-2 of sn-1), 2.41 (4.1H m H at C-2 of sn-2), 2.6 (4.1H m H at C-4 of sn-2), 3.36 (18H s $(CH_3)_3$-N), 3.99 (4H m CH_2-OC), 4.18 (8H m $POCH_2CH_2N$), 4.24 (1H m OH) 4.62 (4H m sn-3 CH_2-OP), 5.25 (2.1 H m sn-2 H), 9.8 (1H s aldehyde); Fourier transform IR ($CDCl_3$) 3021, 2927, 2855, 2362, 1740, 1735, 1601, 1467, 1430, 1380, 1244, 1168, 1092, 1066, 970 cm^{-1} and *m/z* = 1188 are consistent with aldol condensation product shown as P(POVPC)VPC in Fig. 1. Indeed, P(POVPC)VPC, the aldol condensation product of POVPC, was found to be antigenic in contrast to its starting material, POVPC (Fig. 6). ^{1}H NMR analysis of aged and freshly prepared POVPC showed a distinct difference. The integration ratio between the aldehydic hydrogen and the choline head group in the freshly prepared POVPC sample was 1:9, as expected for the monomer. However, after about 1.5 months the ratio was 1:24, even though the sample was stored in deuterated chloroform at −20 °C. This would explain the previously reported antigenic (31) reaction of the so-called POVPC, which we believe was actually modified via intermolecular reaction. The reduced P(POVPC)VPC yielding a hydroxyl moiety instead of the aldehyde (Fig. 2) was found to bind to EO6 as well as the actual aldol condensate. We also synthesized another POVPC aldol condensation analog, the di-lysoPC-C9, a dimer of lysoPC (Fig. 2), which is the P(POVPC)VPC analog without the aldehydic residue but containing instead a C9 alkyl chain between the two sn-2 hydroxyl residues of the lysophosphatidylcholine molecules. This compound appeared to be equally antigenic, perhaps indicating the importance of the distance between the phosphocholine head groups.

From the oxidation of 1,2-diarachidonoyl-*sn*-glycero-3-phosphorylcholine, three very potent antigens of EO6 were isolated. ^{1}H NMR analysis indicated that the antigens did not include the theoretically predicted 1,2-di(5′-oxo)-valeroyl-*sn*-glycero-3-phosphorylcholine (Fig. 2), as the integration ratios of the aldehydic hydrogen *versus* the choline residue hydrogen atoms were different from the expected 2:9. The reaction products were purified and isolated as described under "Experimental Procedures," and the NMR analysis indicated compounds resulting from either intermolecular or intramolecular reaction yielding "polyphospholipids." This result was further confirmed by mass spectrometry indicating formation of a dimer (*m/z* = 882), a trimer (*m/z* = 1326), and a tetramer (*m/z* = 1793). According to the ^{1}H NMR spectra of the isolated products, the ratio of the aldehydic hydrogen to the trimethylamine hydrogens of the choline head group was 1:6 for the dimer as expected. For the trimer and tetramer, the measured ratios were 1:6.9 and 1:7.1, which are within 2% of the theoretical ratio of 4:27 and 5:36, respectively. The immunoassay testing of these products in a competition study against CuOx-LDL indicated high reactivity of these compounds (Fig. 7), whereas freshly prepared POVPC did not bind.

Reaction with Cardiolipin—We previously reported that EO6 reacted with cardiolipin that had undergone oxidation but not un-oxidized cardiolipin (25). However, the demonstration that EO6 requires the presence of a PC head group for reactiv-

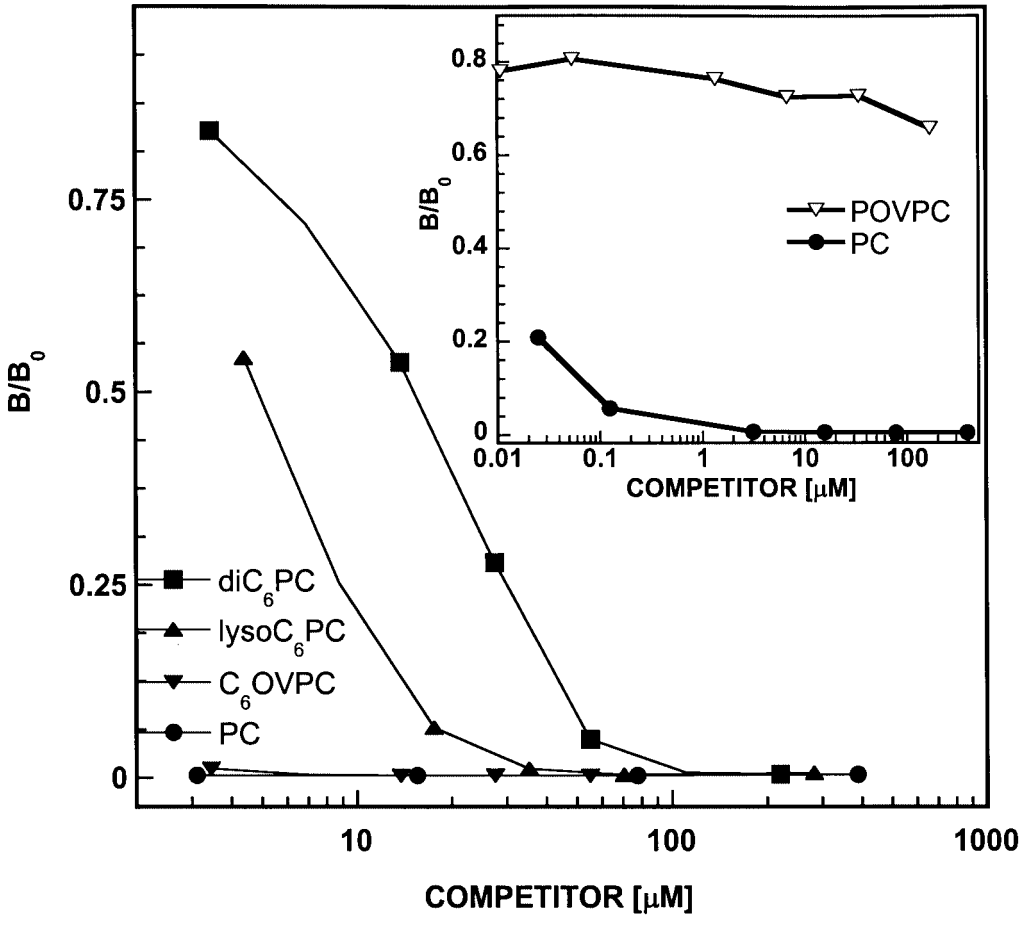

FIG. 5. **Competition immunoassay showing binding of a fixed and limiting amount of EO6 to CuOx-LDL in the absence and presence of increasing amounts of various caproyl-containing phospholipids: ■, diC_6PC; ▲, $lysoC_6PC$; ▼, C_6OVPC; ●, PC.** The techniques are the same as given in the legend for Fig. 3. The *inset* shows a separate experiment measuring the binding of EO6 to CuOx-LDL, which shows that there was no competitive binding of POVPC (▽) to EO6, whereas PC (●) effectively competed even at nanomolar concentrations.

TABLE I
IC_{50} for competition with CuOx-LDL binding to EO6

Competitor	IC_{50} (μM)
CuOx-LDL	0.000044
Phosphorylcholine-KLH	0.000044
Phosphorylcholine	0.095
Glycerophosphorylcholine	0.23
diC_3PC	0.095
diC_5PC	0.025
diC_9PC	9
Choline	11.8
Phosphorylethanolamine	Not detected
Phosphorylserine	Not detected

TABLE II
Antigenicity of various phospholipids and Schiff base adduct

Phospholipid	Antigenicity
PAPC (130 μM)	No
LysoPC (200 μM)	No
POVPC (170 μM)	No
P(hydroxy)VPC (170 μM)	No
P(carboxy)VPC (160 μM)	No
PONPC (160 μM)	No
PAF (190 μM)	No
LysoPAF (210 μM)	No
OxPAPE (polymerized)	No
OxPLPS (polymerized)	No
POVPC-phosphatidylethanolamine (10 μM)	No
POVPC-Lys (imine)	Yes
POVPC-Lys (reduced to an amine)	Yes
POVPC-AcAAKAY	Yes
POVPC-LDL-peptide	Yes
POVPC-albumin	Yes
PONPC-albumin	Yes

ity is in conflict with this observation since cardiolipin does not contain a PC group. We have now repeatedly tested the ability of EO6 to bind to a number of different pure cardiolipin preparations subjected to varying degrees of oxidation. However, no binding was observed (data not shown). Because the commercially obtained cardiolipin is purified from extracts of beef heart, we postulated that the earlier findings were due to contamination of the cardiolipin with small amounts of phosphatidylcholines containing polyunsaturated fatty acids. To test this hypothesis, we added small amounts of PAPC to fresh pure cardiolipin preparations and subjected them to oxidation as described previously (25). In these experiments, cardiolipin was spiked with increasing amounts of PAPC, as a percent of the total phospholipid, and various oxidation times were used with each mixture. In these experiments we never saw binding of EO6 to preparations in which only cardiolipin was present irrespective of the time of oxidation. In mixtures containing PAPC, EO6 binding was seen when the PAPC constituted 5–10% of the total phospholipid present and the oxidation had taken place for 3.5–4.5 h. With a higher percent of PAPC present, for example, 10–25%, increased binding was seen after only 30 min of air oxidation. The data reported here confirm that even minor contamination of commercial preparations of cardiolipin with phosphorylcholine-containing phospholipids can give misleading results when testing by sensitive immunoassays.

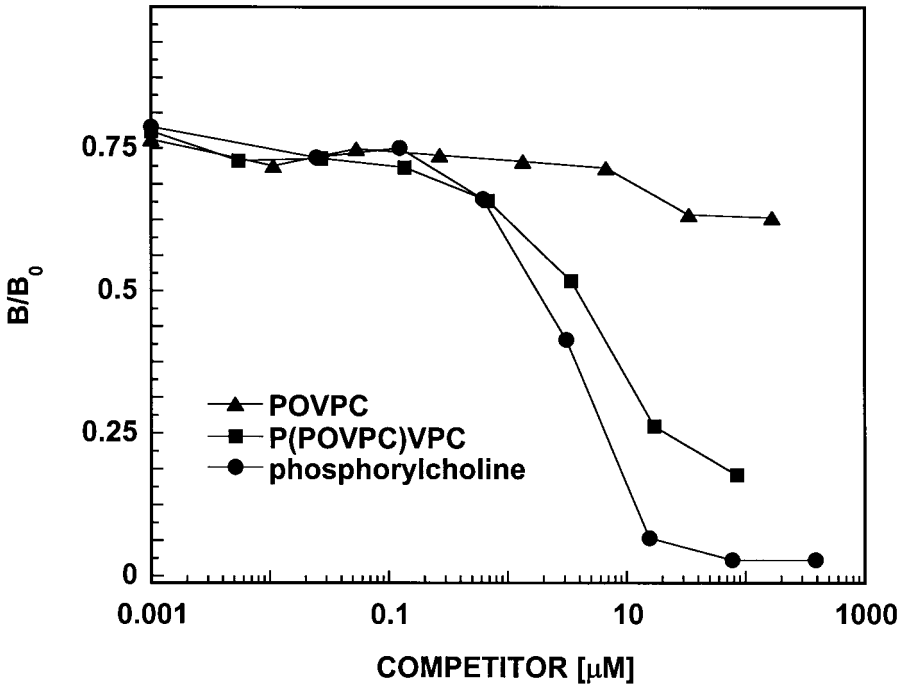

FIG. 6. **Competition immunoassay showing binding of a fixed and limiting amount of EO6 to CuOx-LDL in the absence or presence of increasing amounts of POVPC (▲), P(POVPC)VPC, the aldol condensate of POVPC (■), and phosphorylcholine (●).** The techniques are the same as in the legend of Fig. 3. In this experiment a different preparation of CuOx-LDL was used than in Fig. 5.

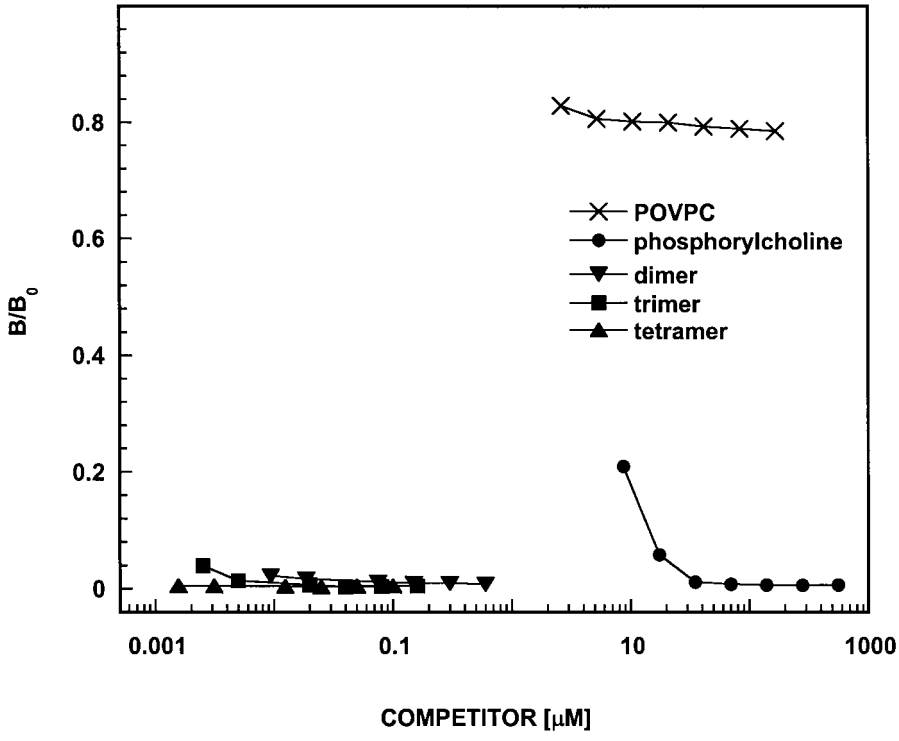

FIG. 7. **Competition immunoassay showing binding of a fixed and limiting amount of EO6 to CuOx-LDL in the absence and presence of increasing amounts of POVPC (×), phosphorylcholine (●), and the oxidation products of 1,2-diarachidonoyl phosphatidylcholine, dimer (▼), trimer (■), and tetramer (▲).** The techniques are the same as given in the legend to Fig. 3.

DISCUSSION

We have found several correlations between phospholipid structure and its antigenicity toward EO6. One is based on fatty acid chain length, whereas another is related to structural modification of the fatty acid chain due to oxidation-induced changes. The head group variation experiments showed the requirement for the phosphorylcholine head group, as it seems that the phosphorylcholine motif is vital for the antibody recognition, consistent with previous reports (33). Phosphorylcholine and glycerophosphorylcholine, as expected, showed recognition by the antibody. Indeed, phosphorylcholine was a highly effective competitor of EO6 binding to OxLDL, even in the nanomolar range (Figs. 5–7). Elimination or substitution of the choline from the head group abolished this recognition, whereas choline by itself was antigenic only at exceedingly high concentrations (*e.g.* >50 μM), again indicating the necessity of the phosphate moiety.

Decreasing the fatty acid chain length, thus decreasing hydrophobicity and the tendency toward aggregation, increased antibody recognition. Short fatty acid chain-containing phosphatidylcholine molecules that are monomeric and have a lower tendency to aggregate due to higher solubility were found to be antigenic. Short chain lysophospholipids also exhibited antigenicity. To further evaluate the effect of the fatty acid composition while testing short chain phospholipids, we compared C_6OVPC with diC_6PC and lysoC_6PC. C_6OVPC was

clearly the best antigen, a fact that can perhaps be related in part to the much higher hydrophilic properties of the molecule. For similar reasons the lysoC$_6$PC was a better competitor than diC$_6$PC for EO6.

Aggregation of phospholipids depends on hydrophobicity. In turn, increased levels of aggregation lead to formation of more organized aggregates, micelles. Apparently short chain monomeric phosphorylcholine-containing lipids are completely soluble and are able to present their PC head group readily for EO6 binding. In contrast, phosphatidylcholine molecules containing long chain fatty acids are more likely to aggregate, decreasing PC head group availability for recognition by EO6. Long chain fatty acid-containing phosphatidylcholines as well as lysophosphatidylcholines, PAF, and lysoPAF did not bind to EO6.

Originally, samples of POVPC were supplied by various laboratories as isolated lipids, and we found them to be antigenic for EO6 (31). In retrospect, we believe that although pure when synthesized, the POVPC underwent some kind of modification to become antigenic during storage or conceivably even during the plating when tested in direct binding assays. We have observed repeatedly that freshly synthesized samples prepared as described herein, which were thoroughly characterized and shown to be monomeric, were not antigenic when immediately tested for EO6 recognition. In contrast, if the samples were allowed to age, even when stored at −20 °C before testing, then the samples became progressively more reactive. We have now demonstrated clearly that the "aged" POVPC forms aldol condensation adducts and possibly other polymerized products, and these are clearly antigenic. Thus we conclude that the pure POVPC is not antigenic *per se*.

Our results confirm that the key epitope is phosphorylcholine. Among various breakdown products, the oxidation of the phospholipids yields reactive, aldehyde-containing phospholipids that can react with proteins via Schiff base formation, altering the presentation of the phosphorylcholine head group and allowing its recognition and subsequent binding to the antibody. We have now discovered an additional route to the productive presentation of the epitope, namely through aldol condensation and polymerization of the oxidized aldehyde-containing phospholipids. Such compounds appear to be as antigenic as the protein adducts. It appears that the association of more than one epitope on the chemical backbone and/or that its precise structure or conformation is an important element for antibody recognition and antigenicity.

The aldehyde-containing phospholipids with a long chain fatty acid on the sn-1 position had non-detectable binding to EO6. Interestingly, the recognition was found to be very high upon reaction of the phospholipid aldehyde with an $\epsilon-$amine of free lysine or peptides containing lysine. Apparently covalent adduct formation with lysines causes exposure of the PC head group, thus making it available for recognition by the EO6 antibody. This supports the hypothesis that EO6 recognizes the oxidized phospholipid as a hapten in this setting. Our data do not support a role for the aldehydic residue as part of the epitope recognized by EO6. However, it is interesting to speculate that such haptens might be part of the actual antigens leading to the expansion of EO6 clones *in vivo*. PC-KLH and CuOxLDL, as mentioned earlier, were found to be the preferred ligands, but the number of epitopes they carry as well as their presentation is not well defined. We postulate that their levels of antigenicity are correlated with the number of available epitopes.

EO6 binds avidly to OxLDL, and this binding is inhibited by Schiff base adducts and aldol condensation products (Figs. 3, 6, and 7). It is intriguing to speculate that similar Schiff base adducts occur on apoB when LDL undergoes oxidation, and indeed we have provided evidence in support of this previously (21). In brief, when OxLDL was subjected to SDS Western-blotting technique and the membrane was probed with EO6, there was staining of the fragments of oxidized apoB, presumably reflecting the covalent adduct formation between the oxidized phospholipid and apoB. To demonstrate that there was the direct incorporation of intact PC-containing phospholipid into apoB when LDL undergoes oxidation, we demonstrated an equal incorporation into the OxLDL of phosphate, choline, and saturated fatty acid (actually about 75 mol of each) and that this was all lost when the OxLDL was subjected to saponification, which released the phospholipid backbone containing the PC head group and sn-1 fatty acid. When the saponified oxidized apoB was immunostained with EO6 on Western blot, the immunoreactivity was completely lost. Furthermore, if the lysines of native LDL were blocked by reductive methylation before oxidation, there was an 80% decrease in the incorporation into LDL of phospholipid, clearly supporting the role of phospholipid-lysine adducts of apoB. It is unclear exactly why such stable adducts are formed in the OxLDL, but we speculate that the highly reactive nature of the aldehyde-containing oxidized phospholipids and the hydrophobic core of LDL contribute to stabilization of the Schiff bases formed. Because EO6 also binds to the isolated lipids of OxLDL, we would also hypothesize that aldol condensation products of oxidized phospholipids are similarly formed.

Both the EO6 antibody and POVPC adducts with lysine or BSA block the binding and uptake of OxLDL by macrophages (38). In addition, EO6 and POVPC-BSA block the uptake of the isolated oxidized lipids and protein moieties of OxLDL (38). Furthermore, EO6 and POVPC-BSA block the uptake of OxLDL by cells transfected with CD36 (39) or SR-B1 (40). These data suggest that some structurally related motifs of POVPC adducts are also directly recognized by CD36, SR-B1, and possibly other macrophage scavenger receptors. In this context it would be of great interest to know if aldol condensation products represent ligands either as free lipids or even possibly as adducts with apoB. By determining the minimal structural requirements of the epitope for EO6 and testing the ability of such compounds to inhibit OxLDL uptake by macrophages, we may be able to develop small molecules that could block OxLDL uptake. Because uptake of OxLDL by scavenger receptors such as CD36 appears to be proatherogenic (41), the availability of such molecules could prove to be of therapeutic value.

Acknowledgments—We thank to Professor Yitzhak Tor, Raymond Deems, and David Six from the Department of Chemistry and Biochemistry, University of California San Diego, for fruitful discussions and useful critiques.

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Correlation of Antiphospholipid Antibody Recognition with the Structure of Synthetic Oxidized Phospholipids: IMPORTANCE OF SCHIFF BASE FORMATION AND ALDOL CONDENSATION

Peter Friedman, Sohvi Hörkkö, Daniel Steinberg, Joseph L. Witztum and Edward A. Dennis

J. Biol. Chem. 2002, 277:7010-7020.
doi: 10.1074/jbc.M108860200 originally published online December 14, 2001
