# Lipolysis of LDL with phospholipase $A_2$ alters the expression of selected apoB-100 epitopes and the interaction of LDL with cells

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**Abstract** To assess the effects of perturbing the surface of low density lipoprotein (LDL) on the conformation of apoB-100, LDL (d 1.030–1.050 g/ml) isolated from normal subjects were treated with phospholipase $A_2$ (PL-$A_2$) for 0.5 to 15 min. The resulting P-LDL and concurrent control LDL (C-LDL) incubated without PL-$A_2$ were isolated by gel permeation chromatography. Approximately 50% of LDL-phosphatidylcholine was hydrolyzed in 2 min and ~85% in 5 min. Lysophosphatidylcholine compounds (LPC) and free fatty acids (FFA) accumulated during lipolysis but most of the LPC and all of FFA could be removed by adding FFA-free albumin to the lipolysis mixtures. Immunoreactivities of P-LDL and C-LDL were evaluated in competitive radioimmunoassays, using a library of anti-human LDL monoclonal antibodies directed against the major regions of apoB-100 (the T4, T3, and T2 thrombin fragments). One epitope defined by monoclonal antibody 465B6C3 and localized near the carboxyl end of the apoB-100 molecule became less immunoreactive (ED 50s increased); three other epitopes on the T2 fragment near the LDL receptor recognition site and four epitopes localized towards the middle (T3) and amino terminal (T4) regions did not change. Altered immunoreactivities were not related to LPC and FFA contents. Thus, the conformation of apoB-100 was selectively altered by phospholipolysis. The interactions of P-LDL with cultured fibroblasts were grossly altered: P-LDL were bound nonspecifically to fibroblasts of both normal and homozygous familial hypercholesterolemic subjects and P-LDL were not degraded. LPC and FFA retained in LDL did not explain these alterations, nor did changes of epitope expression near the LDL receptor recognition site. It is likely that the apoB-100 aberrant cell interaction is due to loss of surface phospholipids and "uncovering" of core lipids that react nonspecifically with cell surface components. —**Kleinman, Y., E. S. Krul, M. Burnes, W. Aronson, B. Pfleger, and G. Schonfeld.** Lipolysis of LDL with phospholipase $A_2$ alters the expression of selected apoB-100 epitopes and the interaction of LDL with cells. *J. Lipid Res.* 1988. **29:** 729–743.

**Supplementary key words** cultured fibroblasts • phospholipids • lysophospholipids • epitopes

The expression of epitopes of apoB in VLDL and in LDL is modulated by perturbations of lipoprotein structure performed either in vivo or in vitro (1). For example, when large VLDL human particles ($S_f$ 100–400) are incubated with lipoprotein lipase (LPL) in vitro, they become progressively depleted of triglycerides, and, as they do, they interact more avidly with human cellular LDL receptors and with monoclonal anti-apoB antibodies that inhibit the binding of apoB-100-containing particles to cellular LDL receptors (2–4). Analogous results are obtained with various-sized VLDL subfractions isolated from plasma by zonal ultracentrifugation. When patients with hypertriglyceridemia are treated with bezafibrate for several weeks, the post-treatment LDL particles in their plasmas are also depleted of triglycerides and they too manifest enhanced reactivities with the LDL receptors of cultured fibroblasts and with the same monoclonal anti-apoB antibodies (5, 6). These results have been interpreted as suggesting that the regions of VLDL- and LDL-apoB at or near the LDL-receptor recognition domain had been altered by perturbations of lipoprotein structures that appeared to involve major degrees of depletion of a core lipid. However, the LPL-induced changes in VLDL-apoB epitope expression could not be related directly to a single perturbation, such as loss of core triglycerides, because LPL may catalyze the hydrolysis of both surface (e.g., PC) and core lipids and this may result in complicated rearrangements of VLDL structures, including losses of apoCs and apoE (7). Results of hypolipidemic

Abbreviations: PL-$A_2$, phospholipase $A_2$; LDL, low density lipoproteins; VLDL, very low density lipoproteins; LPDS, lipoprotein-deficient serum; FCS, fetal calf serum; MEM, Eagle's minimum essential medium; PBS, phosphate-buffered saline; FFA, free fatty acid; BSA, bovine serum albumin; TLC, thin-layer chromatography; BHT, butylated hydroxyl toluene; PMSF, phenylmethylsulfonylfluoride; DTPA, diethylenetriaminepentaacetic acid; PPACK, D-phenyl-L-propyl-L-arginine chloromethyl ketone; TCA, trichloroacetic acid; Mab, monoclonal antibodies; DTT, dithiothreitol; LPL, lipoprotein lipase; PC, phosphatidylcholine; SBTI, soybean trypsin inhibitor; RIA, radioimmunoassay.

therapy on LDL also need to be interpreted with caution because, although LDL (d 1.030–1.050 g/ml) are simpler particles than VLDL (approproteins other than apoB are absent or present only in trace amounts), hypolipidemic therapy may have exerted multiple effects in the remodeling of LDL particles, inasmuch as therapy may affect several metabolic processes, including lipoprotein secretion, intravascular metabolism, and clearance from plasma (8, 9). Furthermore, after several weeks of therapy, the pre- and post-treatment LDL populations tested probably do not consist of the same particles.

For this present study, we sought to perturb primarily surface components and we limited ourselves to LDL. We also sought a more discrete method that, at least initially, would affect only a single site of a single class of surface lipids in LDL. Phospholipase $A_2$ (PL-$A_2$) specifically attacks fatty acids at the *sn*-2 positions of diacylglycerol phospholipids (10). Since it was previously shown that treatment with PL-$A_2$ yields structurally intact LDL particles (10) selectively depleted only in diacylglycerol phospholipids, we chose to study the effects of phospholipolysis with PL-$A_2$ on LDL epitope expression and LDL interaction with cultured fibroblasts.

## MATERIALS AND METHODS

### Materials

$Na^{125}I$ was purchased from Amersham Corporation, Arlington Heights, IL. Culture flasks and dishes were purchased from Falcon Labware. Media, FCS, penicillin, streptomycin, and gentamicin were obtained from Gibco Laboratories, Grand Island, NY. Microtiter plates were purchased from Dynatech Laboratories, Inc., Alexandria, VA. Phospholipase $A_2$ was from Sigma, St. Louis, MO. FFA-free BSA was from Miles Laboratories, Inc., Naperville, IL, and Iodogen was from Pierce, Rockford, IL. BHT, PMSF, DTPA, and PPACK were from Boehringer Mannheim, Germany. All chemicals and reagents were of analytical grade.

### LDL isolation

Blood from six healthy normal subjects who had fasted for ~15 hr was drawn into tubes containing 1 mM BHT, 1 mM PMSF, 1 mM benzamidine, 0.04 mM DTPA, 1% gentamicin, 0.1% chloramphenicol, 2.8 TIU/ml aprotinin, 0.05% soybean trypsin inhibitor (SBTI), 0.002% PPACK, and 0.01% sodium azide (11). LDL was isolated by sequential ultracentrifugation between the densities 1.030 and 1.050 g/ml, using KBr for density adjustment (12, 13). The lipoproteins were dialyzed exhaustively at 4°C against saline–1 mM EDTA.

After incubation with PL-$A_2$, the LDL were reisolated by gel permeation chromatography on a Superose 6 HR 10/30 (25 ml) column using the FPLC system (Pharmacia, Fine Chemical AB, Sweden) (14). Columns were equilibrated and eluted at ambient temperature with buffer consisting of 1 mM EDTA and 0.15 M NaCl, pH 8.0. LDL was applied to the column using a 1.5-ml sample loop and monitored by measurement of absorbance at 280 nm. The major LDL fractions were pooled and sterilized by passage through 0.45-$\mu$m filters. Electron microscopy, after negative staining of the particles with phosphotungstate (15), was provided by the Pathobiology Core Facility of the Diabetes Research and Training Center (NIH grant #P60AM20579).

### Phospholipolysis

Phospholipase $A_2$ from bee venom was used (Sigma-Aldrich, St. Louis, MO, sp act 1510 U/mg of protein). A solution of LDL in 0.15 M NaCl–1 mM EDTA was placed in the reaction tube. An equal volume of the reaction buffer (200 mM Tris, 1 mM $CaCl_2$, 0.15 M NaCl, pH 8.5) containing fatty acid-free BSA (0.8% or as indicated) was added. The LDL solution was preincubated for 15 min at 37°C. Then PL-$A_2$ was added for 15 min or as indicated. The ratio between PL-$A_2$ and LDL was 0.42 $\mu$g of enzyme/mg of LDL-phospholipid. The reaction was stopped either by addition of EDTA to a 10 mM final concentration or by the application of the incubation mixture immediately to the FPLC gel permeation column for chromatography. The PL-$A_2$-treated LDL are referred to as P-LDL. For each experiment a control LDL (C-LDL), consisting of the same preparation as the given P-LDL, was prepared. The C-LDL underwent all the incubation and reisolation steps as the paired P-LDL but without PL-$A_2$.

To assess whether PL-$A_2$ had any proteolytic activity, $^{125}I$-labeled LDL was incubated in a series of tubes at 37°C (as above) for 0.5 to 15 min with and without PL-$A_2$. The following proteolysis inhibitors were included in some incubations: 1 mM PMSF, 1 mM benzamidine, 2.8 TIU/ml aprotinin, 6.05% SBTI, and 0.002% PPACK. At the end of incubation, TCA (20%) was added in equal volumes to aliquots of the incubation mixtures for 30 min at 4°C. Following centrifugation, total radioactivities were determined in the tubes and, after withdrawal of the supernatants, TCA-precipitable counts were determined on the pellets. $^{125}I$-labeled P-LDL and $^{125}I$-labeled C-LDL and unlabeled LDL preparations also were analyzed by SDS-polyacrylamide gel electrophoresis.

### Chemical analysis

Triglyceride, cholesteryl ester, free cholesterol, and free fatty acids were determined by commercially available enzymatic colorometric methods (Wako Pure Chemical Industries, Ltd., Osaka, Japan). LDL protein was measured by the method of Lowry et al. (16) using BSA as a stan-

dard. For phospholipid analysis, lipid extracts of LDL were prepared using chloroform–methanol 2:1 (v/v). Phospholipid subclasses were separated by two-dimensional TLC on silica gel (Fisherbrand 06-600A, Fisher Scientific, USA). The first solvent system was chloroform–methanol–ammonium hydroxide 15:5:1 (v/v); the second, chloroform–acetone–methanol–acetic acid–water 6:8:2:2:1 (v/v) (17). Iodine vapors were used to visualize the lipid spots on the TLC plates and lipids were identified by use of standards run on paired TLC plates. The phosphorus contents of LDL-lipids and of lipid spots were determined by the method of Bartlett (18). Phospholipid mass was calculated from phosphorus analysis by multiplying phospholipid and lysophospholipid masses by 25 and 16.8, respectively.

### Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of LDL was performed in a gradient gel of 3 to 6% (gradient former Ga0991, BRL, Gaithersburg, MD) (19). Before electrophoresis, LDLs were delipidated in chloroform–methanol 2:1 (v/v) and lipid-free LDL proteins were dissolved in loading buffer (19). Protein bands were identified by Coomassie Blue staining. In certain experiments, $^{125}$I-labeled LDLs were delipidated prior to electrophoresis simply by incubation in 5× loading buffer overnight at 23°C and heating at 100°C for 5 min. Labeled bands were visualized by autoradiography.

LDL agarose gel electrophoresis was performed using 1% agarose plates (Universal Electrophoresis Film, Corning, Palo Alto, CA) (20). Electrophoresis was carried out in barbital buffer 0.045 mM, pH 8.6, at room temperature at a constant current 15 mA using an LKB 2117 Multiphor apparatus. Detection of lipoproteins was accomplished by staining the lipids with Sudan Red 7B. Migration of $^{125}$I-labeled LDLs was visualized by autoradiography.

### Iodination of LDL

LDL isolated by ultracentrifugation were labeled with $^{125}$I using Iodogen (Pierce Chemical Co., Rockford, IL) (21). The iodinated preparations were dialyzed extensively against 0.15 M NaCl, 1 mM EDTA, pH 7.4. Final specific radioactivities varied between 150 and 600 cpm/ng of protein. In all preparations >90% of the radioactivity was TCA-precipitable and <5% was extractable with chloroform–methanol. $^{125}$I-labeled LDL preparations used in immunoassays were kept in tightly capped vials, in the dark, under nitrogen at 4°C, for no longer than 4 wk.

### Acetylation of LDL

Acetyl-LDL was prepared by reaction of LDL with acetic anhydride (22).

### Monoclonal anti-human LDL antibodies

Anti-human LDL Mabs were produced in mice by using intact VLDL or LDL as immunogens and have been characterized elsewhere (2, 23).

Mabs 454B1B3, 454B1B6, and 465B6C3 define epitopes on the COOH terminal (T2/K4) region of apoB-100, Mab1888D7.2 defines an epitope on the middle portion of the molecule (T3/K3 fragment), and antibodies 1888C1.1, 1888C1.4, and 457C4D1 define epitopes on the amino terminus of the molecule (T4/K2 region) (24 and also see Fig. 13 for epitope map). Mab MB47 was kindly provided by Dr. Linda Curtiss.

### Solid phase competitive binding immunoassay

C-LDL and P-LDL from the same LDL preparations were assayed in triplicate wells in competitive displacement assays on microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) (23). Microtiter wells were coated with 0.15 ml of purified Mab solution (10 μg/ml PBS) overnight, then wells were blocked with 3% BSA-PBS. Serial dilutions of the C-LDL or P-LDL in 1% BSA-PBS were added, followed by a constant amount of $^{125}$I-labeled intact LDL also in 3% BSA-PBS. After incubation overnight at 23°C, the wells were washed and counted in a Packard gamma counter. $B/B_o$ versus LDL concentrations were plotted and slopes and mid-range (ED50) isotherm values were calculated for the various curves with the "Program to calculate parameters for the four parametric logistic function, using 2 + 2 linear regression approach" as developed by P. Munson, D. Rodbard, and M. L. Jaffe, NIH, Bethesda, MD, USA 20892, IBM PC version 1985. Coefficients of variation of replicate wells averaged 8%.

### Cell cultures

Cells were obtained from the American Tissue Type Culture collection. Normal human skin fibroblasts (GM 0203) were grown and maintained in MEM–10% FCS containing penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator (5% $CO_2$) at 37°C. Fibroblasts from sixth to fifteenth generation were used. LDL receptor-negative cells (GM 2000) were grown and maintained in MEM containing 15% FCS. LDL receptors were up-regulated by growing the cells for 48 hr in media containing 10% human lipoprotein-deficient serum (LPDS).

### Assays of $^{125}$I-labeled LDL cell association and degradation

On the days of experiments, culture media were removed, fibroblast cell monolayers were washed with PBS, and the cells were incubated with $^{125}$I-labeled LDL in MEM–10% LPDS with or without the indicated competitor lipoproteins, at the indicated concentrations. To

obtain nonspecific values of $^{125}$I-labeled LDL cell association or degradation, 100–200× excess of the respective unlabeled lipoproteins were added to some plates. At the end of incubation, the culture media were removed and assayed for $^{125}$I-labeled lipoprotein degradation as previously published (25, 26). Cells were washed three times with 50 mM Tris-Cl, 0.15 M NaCl, 0.2% BSA, pH 7.4, followed by three washes with Tris-saline alone, and dissolved in 0.1 N NaOH overnight. The radioactivity counted in the dissolved cells represented the cell-associated $^{125}$I-labeled lipoproteins. Specific cell associations and degradations were calculated by subtraction of the nonspecific values (assayed in the presence of excess unlabeled lipoprotein) from the total value (assayed with radioactive lipoprotein alone). Assays were performed in triplicate. Coefficients of variation were ~11%.

The metabolic fate of $^{125}$I-labeled LDL associated with cells was traced in more detail essentially as described by Aulinskas et al. (27) and by Greenspan and St. Clair (28). LDL receptor up-regulated cells were incubated with $^{125}$I-labeled LDL (10 μg/ml) in MEM-10% LPDS for 4 hr at 37°C. This is referred to as the "pulse period." The cells were washed with Tris-saline-BSA followed by Tris-saline (three times for each wash). The cells were then incubated with MEM-10% LPDS at 4°C for 30 min (LPDS wash). Cells were washed again three times. To confirm that washes were complete, the last LPDS wash was collected and protein was precipitated with 20% TCA in the presence of 0.1% BSA as a carrier. Cells were then warmed to 37°C and incubated with MEM-10% LPDS at 37°C for various times as stated. This is referred to as the "chase period." The cell-associated LDL at the beginning of the chase period was determined and used as the 100% value. At the specified times, the medium was spun down to remove any detached cells and 0.9 ml of the supernatant media was precipitated with ice-cold 20% TCA in the presence of 0.1% BSA as a carrier. NaOH (0.1 N) was used to dissolve the cells and to determine the remaining cell-associated LDL and cellular protein concentrations. TCA-soluble non-iodide (chloroform-insoluble, water-soluble) $^{125}$I-labeled material in the medium (cellular degradation) was determined. The results were calculated initially as ng of LDL/mg of cell protein and expressed finally as percents of the initial LDL cell associated at the beginning of the chase period (0 time).

### Determination of cholesterol esterification in cells

Cholesteryl ester deposition in cells was determined (25, 29) in monolayers of fibroblasts with up-regulated LDL receptors. Cells were incubated for 5 hr at 37°C in medium containing 10% LPDS in the presence or absence of added lipoproteins and with 0.1 mM [$^3$H]oleate complexed to 0.02 mM BSA. The specific radioactivity of oleate incubated with cells was approximately 24 mCi/mol. After incubation the medium was removed, monolayers were washed, and their lipids were extracted with hexane-isopropanol 3:2 (v/v). Then the cells were dissolved in 0.1 N NaOH for protein determination. The lipid extracts were evaporated under nitrogen and resuspended in chloroform-methanol 2:1 (v/v), spotted on TLC plates and developed (25) in petroleum ether-diethyl ether-glacial acetic 84:15:1 (v/v/v) and visualized with iodine. Cholesteryl ester spots were scraped and radioactivities were determined in a Beckman LS-2800 liquid scintillation spectrometer. Data are presented as nmol of cholesterol esterified/mg of cell protein · 5 hr$^{-1}$. Coefficients of variation of triplicate dishes were ~12%.

### Proteolytic degradation of $^{125}$I-labeled LDL by cell-free fibroblast extracts

Cell-free extracts were prepared from confluent 150-mm dishes of fibroblasts as follows (30). Cells were freed with trypsin-EDTA and transferred to a 15-ml tube. Dishes were washed with MEM and the cells were pelleted by centrifugation. The cell pellet was resuspended in 1.5 ml of water and sonicated on ice with a microprobe (Branson Sonic Power Company model W185, Danbury, CT) at approximately 30 watts for 15 sec three times. The sonicated suspension was centrifuged (2500 *g* for 20 min at 4°C) and the resulting supernatant was adjusted to contain the following: 0.1 M sodium acetate, pH 4.0, 5 mM DTT, 1 mM EDTA, and approximately $10^6$ cpm/ml of $^{125}$I-labeled LDL. After incubation at 37°C for 10 min, 0.1-ml aliquots of the reaction were removed, and 0.5 ml of ice-cold 1% BSA and 0.36 ml of 50% TCA were added. The TCA precipitates were removed by centrifugation and aliquots of the supernatant solution were extracted with chloroform in the presence of KI and hydrogen peroxide. The aqueous phases were counted to determine the amounts of $^{125}$I-labeled acid-soluble material formed. Results are presented as TCA-soluble materials formed as a percentage of the initial TCA-precipitable $^{125}$I-labeled LDL.

### Statistical analysis

Significant differences between control and modified LDL were evaluated by paired *t* test. Significance levels were obtained from statistical tables (31, 32).

## RESULTS

### Phospholipolysis of LDL

The progress of phospholipolysis over 15 min was easily followed by the increasing anodal migration of P-LDL (**Fig. 1**). Changes were noted as early as 0.5 min of enzyme incubation. As the PC contents of LDL progres-

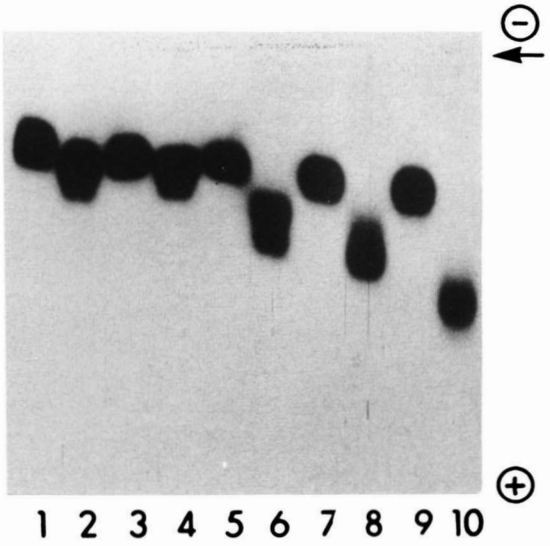


**Fig. 1.** Agarose gel electrophoresis of PL-$A_2$-modified LDL: time course experiment. $^{125}$I-Labeled LDL was incubated with and without PL-$A_2$ for 0.5–15 min. Incubation mixtures were subjected to agarose gel electrophoresis. Gels were dried and developed by autoradiography for 3.5 hr. Lanes 1, 3, 5, 7, and 9 are $^{125}$I-labeled C-LDL (incubated without PL-$A_2$), and lanes 2, 4, 6, 8, and 10 are $^{125}$I-labeled P-LDL (incubated with PL-$A_2$) for 0.5, 1, 2, 5, and 15 min at 37°C. Each lane contained 25 μg of LDL protein. The arrow indicates the origin of migration of the LDLs; +, −, electrodes; O, origin.

sively fell, LPC contents rose and sphingomyelin remained relatively stable (**Fig. 2**). Approximately 50% of the PC was hydrolyzed by 1 min, 85% by 5 min, and all by 10 min. Over 15 min the FFA contents of LDL rose from 1 to 39 μeq/mg LDL-protein. After 15 min of incubation, relative proportions of PL and protein fell and proportions of cholesterol and triglycerides rose (**Table 1**). The drop in percentage of protein suggested that protease activity could have been present in the phospholipolysis mixtures. To evaluate this possibility, LDL was iodinated and $^{125}$I-labeled LDL and PL-$A_2$ were incubated together as described in Methods and under the same conditions used in Fig. 1. Concurrent control incubations contained no PL-$A_2$. At the end of 0.5–15 min of incubation, $^{125}$I-labeled LDL was precipitated with 20% TCA at 4°C and precipitable $^{125}$I counts were determined. TCA precipitabilities in the PL-$A_2$ and control tubes (in parentheses) were as follows: (in percent of total TCA precipitable cpm) 98.2 (98.7), 102 (95.6), 97.6 (95.6), 95.6 (94.6), 88.6 (96.6), and 81.5 (92.5) at 0.5, 1, 2, 5, 10, and 15 min, respectively. Coefficients of variation in triplicate tubes were 2–4%. Aliquots of these incubations also were analyzed on 3–6% gradient SDS gels and bands were visualized by autoradiography (not shown). No differences were noted between control and PL-$A_2$ incubations until 15 min when the PL-$A_2$-containing sample appeared to contain larger amounts of degraded apoB-100. In other experiments, lipolyzed and control LDL were isolated on FPLC, delipidated, subjected to 3–6% SDS gradient gel electrophoresis, and stained with Coomassie Blue (**Fig. 3**). No differences between controls and PL-$A_2$-treated LDL were detected visually or after scanning of the gels. Thus, it appears that TCA-precipitability may be the most sensitive of the three methods for detecting proteolysis. Even with this most sensitive technique, no proteolysis was detectable, at least for the first 5 min of incubation, by which time ~85% of the PC had been hydrolyzed. This suggests that the apoB-100 of LDL was not readily accessible to any protease(s) that may have been present in the incubation mixture until the surface lipids had been lipolyzed. Similar incubations were carried out over 15 min in the presence of protease inhibitors at the same concentrations used for LDL isolation with identical results, and the degree of proteolysis also was independent of the amounts of BSA in the incubation mixtures. These data could be interpreted as suggesting that the protease(s) may have been associated with LDL.

On negative staining transmission electron microscopy, P-LDL remained as distinct, spherical, nonaggregating particles (not shown), with reduced diameters compared to C-LDL (Table 1). Elution times of P-LDL from FPLC Superose 6 columns were also retarded relative to C-LDL by ~0.15 min (i.e., 11.96 min for C-LDL, 12.11 min for P-LDL) at a flow rate of 1 ml/min.

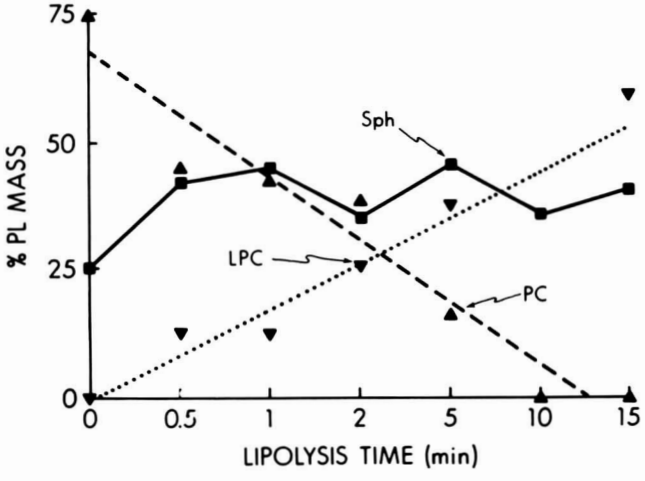


**Fig. 2.** Time course of the PL-$A_2$ hydrolysis of LDL. Phospholipolysis was carried out in the presence of 0.4% BSA for the indicated times. The reaction was stopped by loading of P-LDL samples onto the gel permeation chromatographic (FPLC) columns. Phosphorus contents of the phospholipid classes were measured after two-dimensional TLC as described in the Methods. The different phospholipid constituents were calculated as a weight percentage of total phospholipids and plotted versus time.

TABLE 1. Chemical compositions and diameters of C-LDL and P-LDL

| LDL | Protein | CE | TG | FC | PL | Diameter |
|---|---|---|---|---|---|---|
| | | | % mass | | | Å |
| Control (C-LDL) | 29.2 ± 1.52 | 28.3 ± 1.81 | 7.42 ± 1.72 | 9.96 ± 0.95 | 25.1 ± 1.30 | 228 ± 3.36 |
| Phospholipase-treated (P-LDL) | 26.9 ± 2.32 | 31.7 ± 2.54 | 10.5 ± 3.80 | 11.3 ± 0.96 | 19.8 ± 2.13 | 210 ± 2.91 |
| *P* Value | <0.05 | <0.05 | n.s. | <0.005 | <0.05 | <0.05 |

Data are means ± SEM of six experiments using LDL from six normal human volunteers. The PL-$A_2$ lipolysis was carried out for 15 min in the presence of 0.4% BSA in the reaction mixture. A control LDL (C-LDL) was prepared for each enzyme-modified LDL (P-LDL) and the paired samples were compared by paired *t*-test; n.s., not significant; CE, cholesterol; TG, triglyceride; FC, free cholesterol; PL, phospholipids.

### Immunoreactivity of LDL following phospholipolysis

In competition assays originally carried out with P-LDL produced by a 15-min incubation with PL-$A_2$, the most pronounced change in immunoreactivity was noted for antibody 465B6C3. The mean ED50 value was increased four- to sixfold. Lesser statistically nonsignificant increases were seen with antibodies 464B1B3 and 1888D7.2 and in a single experiment with MB47 (33, 34), while with antibodies 464B1B6 and 457C4D1, the mean ED50 showed nonsignificant decreases (**Table 2**). Because proteolysis may have confounded the results, the time course of modulation of the epitope defined by antibody 465B6C3 was followed during PL-$A_2$ hydrolysis of LDL (**Table 3**). Slopes appeared to increase initially and then to decrease. It took about 2 min to achieve a 50% change in the ED50 value (about the same amount of time needed to achieve a 50% drop in PC contents). Thus the time-related change in immunoreactivity at this epitope more closely mirrored the kinetics of phospholipolysis than proteolysis of LDL components.

To assess whether the retained products of lipolysis affected P-LDL immunoreactivity, increasing amounts of BSA were added to the PL-$A_2$ incubation mixtures. Total phospholipids fell from 25 to 19.8% of LDL mass when 0.4% BSA was present in the incubation mixture (**Fig. 4A**). Decreases in the total phospholipid mass became greater when increasing amounts of BSA were added to the lipolysis mixture, i.e., the relative amount of phospholipids gradually fell to 14.9% of LDL mass as the BSA content of the incubation buffer was increased to 6% (Fig. 4A). The relatively small drop in LDL phospholipids in the 0.4% BSA incubations was due to the retention of PL-$A_2$-generated lysophospholipids by the LDL particles (Fig. 4B). Addition of more BSA to incubation mixtures resulted in decreases in LDL-associated lysophospholipids (from 10.3% of LDL mass in the presence of 0.4% BSA to 4.11% with 8% BSA) (Fig. 4A). As a result of the selective removal of lysophospholipids by BSA, the amount of sphingolipids relative to the total phospholipids in LDL increased as the amounts of BSA in the incubation media rose (Fig. 4B). Free fatty acids also accumulated in LDL during phospholipolysis, but the amounts of free fatty acids associated with LDL particles diminished when phospholipolysis was carried out in the presence of increasing amounts of BSA (**Fig. 5**). As noted, P-LDL had increased anodal electrophoretic mobilities as compared with C-LDL (Figs. 1, 6). Mobility was markedly diminished by increasing the amounts of BSA in the hydrolysis reaction (**Fig. 6**). Thus, when 8% BSA was present there was virtually no difference in the electrophoretic mobilities of C-LDL and P-LDL. These data and that of others (10) suggest that negative electrostatic charges accumulated during lipolysis, probably due to

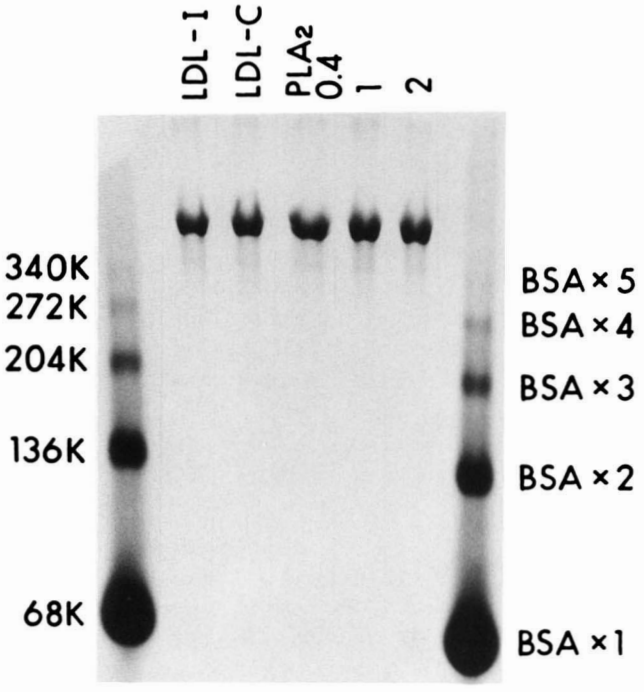


**Fig. 3.** SDS polyacrylamide gel electrophoresis of LDL prior to incubation (LDL-I), control LDL (LDL-C) incubated in 0.4% BSA buffer alone, and phospholipase-treated LDL (PL-$A_2$) incubated with enzyme in 0.4%, 1%, and 2% BSA buffers, respectively. Intact LDL (LDL-I) and LDL incubation mixtures were delipidated with ethanol–ether 3:2, and 20 μg of protein were electrophoresed in 3–6% gradient polyacrylamide gels containing 0.1% SDS. Gels were stained with Coomassie Blue R250. Bovine serum albumin oligomers cross-linked with glutaraldehyde were used as molecular weight markers.

TABLE 2. Slopes and midpoint isotherm (ED50) values of competition curves produced by C-LDL and P-LDL in radioimmunoassays

| Mabs | C-LDL | P-LDL | *P* Value |
|---|---|---|---|
| 1888C1.1 (n = 5) | | | |
| Slope | 2.12 ± 0.73 | 1.86 ± 0.38 | 0.47 |
| ED50 | 0.97 ± 0.34 | 0.99 ± 0.35 | 0.83 |
| 1888C1.4 (n = 5) | | | |
| Slope | 1.71 ± 0.11 | 1.62 ± 0.12 | 0.58 |
| ED50 | 0.97 ± 0.33 | 1.22 ± 0.53 | 0.33 |
| 457C4D1 (n = 6) | | | |
| Slope | 1.22 ± 0.09 | 1.15 ± 0.13 | 0.70 |
| ED50 | 5.23 ± 1.31 | 2.67 ± 0.45 | 0.11 |
| 1888D7.2 (n = 4) | | | |
| Slope | 1.57 ± 0.18 | 1.46 ± 0.13 | 0.14 |
| ED50 | 1.80 ± 0.43 | 2.02 ± 0.36 | 0.13 |
| 464B1B3 (n = 8) | | | |
| Slope | 1.28 ± 0.08 | 1.12 ± 0.06 | 0.17 |
| ED50 | 2.36 ± 0.28 | 3.35 ± 0.49 | 0.10 |
| 464B1B6 (n = 3) | | | |
| Slope | 1.06 ± 0.06 | 1.21 ± 0.05 | 0.21 |
| ED50 | 2.26 ± 0.11 | 1.78 ± 0.26 | 0.09 |
| MB47 (n = 1) | | | |
| Slope | 2.17 | 2.20 | |
| ED50 | 2.47 | 3.43 | |
| 465B6C3 (n = 7) | | | |
| Slope | 1.33 ± 0.15 | 1.74 ± 0.24 | 0.15 |
| ED50 | 3.16 ± 0.98 | 13.98 ± 1.69 | 0.001 |

P-LDL were incubated with PL-$A_2$ for 15 min in 0.4% BSA-containing buffer. C-LDL were incubated under identical conditions in the absence of PL-$A_2$. RIA buffer (PBS) contained 3% BSA. The slope and midpoint isotherm (ED50) values (in μg of LDL protein) were computed as described in Materials and Methods and represent the means ± SEM of the number of experiments indicated in parentheses. The significance of differences is calculated by paired *t* test; n.s., $P \geq 0.05$.

free fatty acids, and that these negative electrostatic charges disappeared along with the fatty acids when sufficient amounts of FFA-acceptor BSA were added to the reaction.

Depletion of the products of lipolysis during the incubations themselves did not affect the slopes or ED50 values produced by P-LDL in assays containing antibody 465B6C3 (**Table 4**) or any of the other antibodies tested in Table 2 (not shown), perhaps because the RIAs were carried out in buffer containing 1% BSA, i.e., P-LDL produced in lipolysis buffers containing < 1% BSA could have been depleted of LPC and FFA in the RIA buffers. In any case, the data suggest that the PL-$A_2$-induced alteration of immunoreactivity of epitope 465B6C3 was not due to the accumulation of lysophospholipids or FFA in P-LDL.

### Interaction of phospholipolyzed LDL with cells

When $^{125}$I-labeled C-LDL and $^{125}$I-labeled P-LDL were incubated with normal cultured human fibroblasts (**Fig. 7**), $^{125}$I-labeled C-LDL exhibited saturation kinetics for both cell association and degradation and 80 to 90% of the cell-associated and degraded counts were mediated by the LDL receptor. By contrast $^{125}$I-labeled P-LDL cell association and degradation curves were linear with dose (i.e., they displayed no saturation kinetics) and C-LDL was unable to compete effectively with $^{125}$I-labeled P-LDL for cell association or degradation (i.e., the vast majority of cell assocation and degradation was nonspecific). The time course of development of the altered association of P-LDL with cells was explored in an experiment where LDL was incubated with PL-$A_2$ for 0–15 min (**Fig. 8,** see also Fig. 2). Phospholipolysis was stopped at the indicated intervals of time and the resulting P-LDLs, purified on

TABLE 3. The slopes and ED50s of competition curves during timed phospholipolysis of LDL

| PL-$A_2$ Incubation Time | Slope | *ED50* |
|---|---|---|
| *min* | | *μg of LDL protein* |
| 0 | 1.43 | 2.05 |
| 0.5 | 1.74 | 5.18 |
| 1 | 1.92 | 7.82 |
| 2 | 2.06 | 10.77 |
| 5 | 1.07 | 21.80 |
| 10 | 0.95 | 14.03 |
| 15 | 0.91 | 23.39 |

LDL was incubated for the indicated times in buffer containing 0.4% BSA. Aliquots were purified by FPLC and used in competitive RIAs using Mab 465B6C3 in PBS–3% BSA. Slopes and ED50s were calculated as indicated in the Methods section. Individual points are means of triplicates.

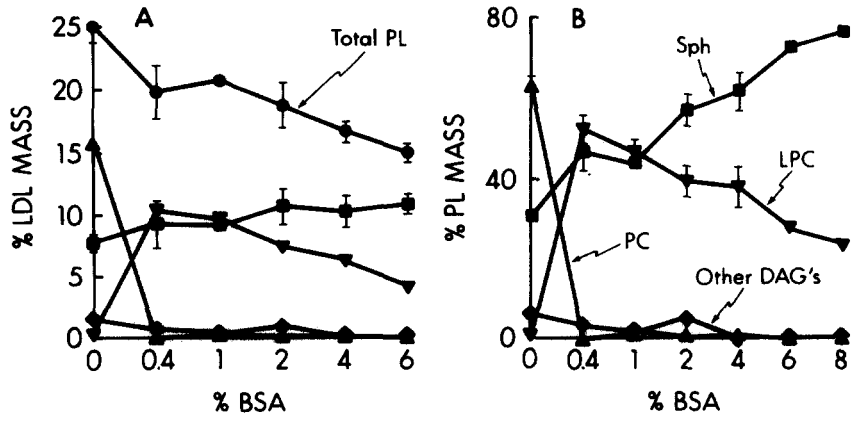


**Fig. 4.** Effect of BSA in the PL-$A_2$ lipolysis media on the chemical composition of LDL. PL-$A_2$ lipolysis was carried out for 15 min at 37°C in 100 mM Tris, 0.15 M NaCl, and 0.5 mM $CaCl_2$, pH 8.5, in a total volume of 1 ml. The indicated concentrations of BSA were present in the reaction mixtures. LDL were isolated from the incubation mixtures by gel permeation chromatography on FPLC. Concentrations of the major lipid and protein fractions were determined. Data are presented as weight percents of LDL mass (panel A). Compositions of the phospholipid classes are given as weight percent of total phospholipid mass (panel B). Each point represents three experiments ± SEM. LPC includes lysolecithin and lysophosphatidylethanolamine. Lecithin comprised more than 90% of all of the diacylglycerol phospholipids; phosphatidylserine, -ethanolamine, and -inositol comprised <10%. DAG, diacylglycerol phospholipids.

FPLC, were labeled with $^{125}I$ and incubated with normal fibroblasts. With increasing duration of LDL phospholipolysis, specific cell association (Fig. 8A) and degradation (Fig. 8B) of $^{125}I$-labeled P-LDL declined, while nonspecific association increased (Fig. 8A). The time course of these events also closely paralleled loss of LDL-PC and preceded the loss of LDL-protein.

Since C-LDL did not compete with $^{125}I$-labeled P-LDL for binding to cells, we tested whether, conversely, P-LDL was able to compete effectively with $^{125}I$-labeled C-LDL for cell interaction (**Fig. 9**). As expected, C-LDL competed very effectively with $^{125}I$-labeled C-LDL, but P-LDL did not compete. These results suggested that P-LDL indeed was not associating with the cells via the LDL receptor. Four additional experiments were performed to further verify this hypothesis. In the first experiment we determined whether the binding of $^{125}I$-labeled C-LDL or $^{125}I$-labeled P-LDL to cells was inhibited by means of a monoclonal antibody known to inhibit the binding of intact LDL to the LDL receptor (**Fig. 10**). As expected, the binding of C-LDL was completely inhibited whereas the binding of $^{125}I$-labeled P-LDL was not. In the second experiment we assessed the ability of acetylated P-LDL to interact with cultured fibroblasts (**Table 5**). It is known that acetylated LDL does not bind to LDL receptors (35). As expected, $^{125}I$-labeled C-LDL associated with normal cultured fibroblasts was specifically degraded by them, and C-LDL was able to stimulate the incorporation of $^{3}H$-labeled fatty acids into cholesteryl esters. Acetylation of C-LDL destroyed its capacity to be recognized and processed by the cells. $^{125}I$-Labeled P-LDL bound to cells, with the majority of the association being nonspecific. $^{125}I$-Labeled P-LDL was not degraded, and P-LDL did not stimulate the intracellular esterification of cholesterol. Acetylated $^{125}I$-labeled P-LDL also associated with cells (to about half the extent of $^{125}I$-labeled P-LDL and sixfold more than acetylated $^{125}I$-labeled C-LDL), but it was not degraded and did not stimulate cellular cholesterol esterification.

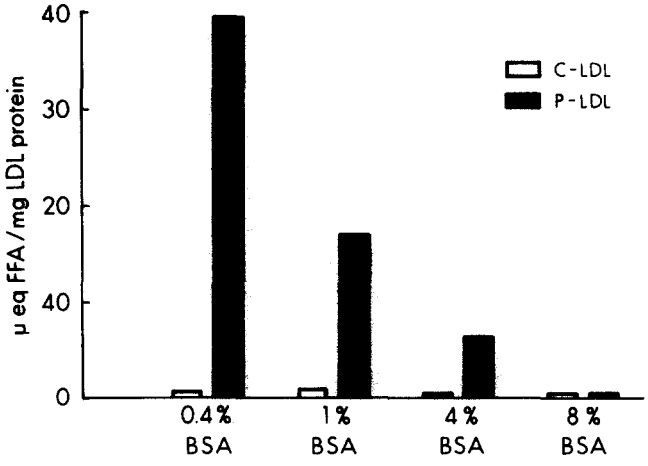


**Fig. 5.** The effect of BSA on the FFA contents of LDL. P-LDL and C-LDL were prepared as in Fig. 1, in the presence of the indicated concentrations of BSA. LDL preparations were reisolated by gel permeation chromatography and their FFA contents were determined using oleic acid as the standard. Results are expressed as μeq of FFA/mg of LDL protein. C-LDL, open bar, and P-LDL, closed bar.

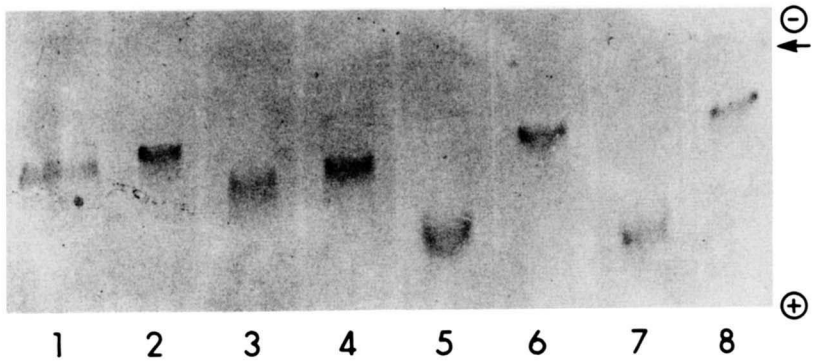


**Fig. 6.** The influence of BSA on the electrophoretic mobility of P-LDL. P-LDL and C-LDL were electrophoresed on 1% agarose gel. Lanes 2, 4, 6, and 8 are C-LDL and lanes 1, 3, 5, and 7 are P-LDL incubated in the presence of 8, 4, 1, and 0.4% BSA in the reaction mixture. Two μl of LDL solution (1–2 μg of LDL protein) was applied. Lipids were stained with Sudan Red 7B. The arrow indicates original migration of the LDLs. Anode and cathode are + and –, respectively.

In the third experiment, the binding of P-LDL to LDL receptor-deficient GM2000 fibroblasts was assessed (**Table 6**). $^{125}$I-Labeled C-LDL, as expected, bound very poorly but, by contrast, $^{125}$I-labeled P-LDL manifested an appreciable amount of binding. However, neither $^{125}$I-labeled C-LDL nor $^{125}$I-labeled P-LDL was degraded to any extent by the LDL receptor-deficient cells.

In the fourth experiment, we tested whether $^{125}$I-labeled P-LDL was removed from the surfaces of normal cultured fibroblasts by heparin. $^{125}$I-Labeled P-LDL and $^{125}$I-labeled C-LDL (each at 10 μg/ml) were incubated with cells for 4 hr at 37°C and then with heparin (10 mg/ml) for 1 hr at 4°C. Heparin-releasable material accounted for 90 and 241 ng of lipoprotein protein/mg of cell protein of $^{125}$I-labeled P-LDL and $^{125}$I-labeled C-LDL, respectively. These four experiments were intrepreted as showing that P-LDL did not interact with fibroblasts via their LDL receptors.

Next, we sought to ascertain the fate of the P-LDL that was associated with cells (**Fig. 11**). Cells were pre-loaded with $^{125}$I-labeled C-LDL or $^{125}$I-labeled P-LDL, thoroughly washed to remove any LDL particles nonspecifically adhering to cell exteriors, and then incubated for a "chase" period. After 2 hr of "chase," the association of $^{125}$I-labeled C-LDL with cells declined to approximately 60% of 0-chase time values. A large proportion of the $^{125}$I-labeled C-LDL label that disappeared from the cells appeared in the medium, mostly as TCA-soluble degradation products. Very little (<5%) of the $^{125}$I-labeled C-LDL was recovered in the medium as TCA-precipitable material, indicating that very little of the $^{125}$I-labeled C-LDL had dissociated from the cell surface back into the medium as intact particles. During the same 2-hr chase period, $^{125}$I-labeled P-LDL also was lost from the cells. However, by contrast, most of the lost material was recovered in the medium in TCA-precipitable form, presumably representing $^{125}$I-labeled P-LDL that had either entered the cells and been retroendocytosed into the medium, or had not entered the cell at all and been simply dissociated from the cell surface during the chase period. Very little (<5%) of the $^{125}$I-labeled P-LDL was degraded by the cells to TCA-soluble products.

To assure ourselves that the lack of degradation of $^{125}$I-labeled P-LDL by cells was not due to the inability of lysosomal enzymes to degrade P-LDL, a homogenate of fibroblasts was prepared and $^{125}$I-labeled C-LDL and $^{125}$I-labeled P-LDL were incubated with the homogenates in acid pH. The generation of TCA-soluble counts was used as an index of the degradation of LDL protein by lysosomal acid proteases. Thirty eight percent of $^{125}$I-labeled C-LDL and 37.4% of $^{125}$I-labeled P-LDL counts became TCA-soluble over 10 min of incubation.

Finally, we wished to assess to what extent the products of phospholipolysis (lysophospholipids and/or free fatty acids) remaining with LDL particles contributed to the altered interaction of P-LDL with fibroblasts (**Fig. 12**). Incubations of LDL with PL-$A_2$ were carried out in the presence of 0.4% BSA. The resulting P-LDL were isolated

TABLE 4. The lack of effect of BSA in the LDL lipolysis buffer on the immunoreactivity of Mab 465B6C3

| | C-LDL 0.4% BSA | P-LDL[a] 0.4% | 1% | 2% | 4% | 6% |
|---|---|---|---|---|---|---|
| Slope | 1.06 | 1.63 | 2.8 | 1.38 | 1.37 | 1.64 |
| ED50 | 2.70 | 15.97 | 14.87 | 15.87 | 13.12 | 18.78 |

The slopes and midpoint isotherm values of the competition curve (ED50 in μg of LDL protein) were computed as described in Materials and Methods.

[a] P-LDL was lipolyzed with PL-$A_2$ in the presence of the indicated concentrations of FFA-free BSA in the lipolysis buffer, at 37°C for 15 min.

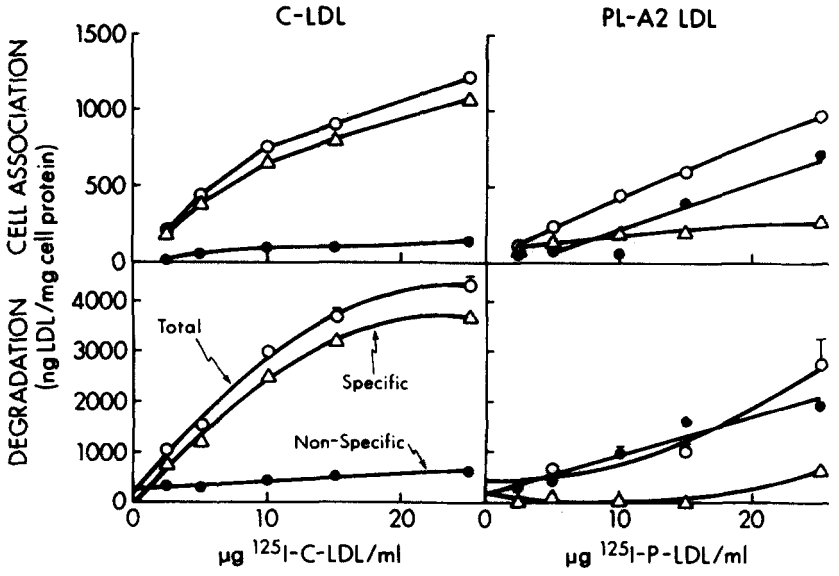


**Fig. 7.** Cell association and proteolytic degradation of $^{125}$I-labeled C-LDL and $^{125}$I-labeled P-LDL by cultured fibroblasts. Cellular LDL receptors were up-regulated by incubation for 48 hr in medium containing 10% LPDS. Cells then were incubated for 5 hr at 37°C with medium containing 10% LPDS and the indicated amounts of $^{125}$I-labeled C-LDL or $^{125}$I-labeled P-LDL in triplicate dishes. Nonspecific association and degradation were determined in dishes containing the indicated concentrations of $^{125}$I-labeled LDL with 500 μg/ml of C-LDL. High affinity (specific) cell association and degradation were obtained by subtraction of nonspecific from total cell-associated and total degraded radioactive counts, respectively.

by FPLC, labeled with $^{125}$I, and incubated with cultured fibroblasts in MEM-10% LPDS to which were added increasing amounts of BSA. Parallel control cell incubations were performed with $^{125}$I-labeled C-LDL also prepared in 0.4% BSA. The greater the amount of BSA in the media, the smaller was the association of $^{125}$I-labeled P-LDL with cells. However, at every BSA concentration, the majority of the cell association was nonspecific and almost none of the $^{125}$I-labeled P-LDL was degraded. By contrast, the interaction of $^{125}$I-labeled C-LDL with cells was not affected by BSA in the culture medium. These data suggest that the interaction of P-LDL with cells was conditioned by the presence of some of the products of phospholipolysis in the P-LDL particles, but that this did not completely account for the altered character of the association with and degradation of $^{125}$I-labeled P-LDL by cells.

## DISCUSSION

PL-$A_2$ selectively, rapidly, and completely hydrolyzed the diacylglycerol phospholipids of LDL causing the accumulation of lysophospholipid compounds and FFA in the LDL particles (Figs. 2 and 5). The retained products of phospholipolysis altered the electrophoretic mobilities of the LDL particles (Fig. 1), but most of the lyso compounds and virtually all of the FFA could be removed by including more FFA-free BSA in the phospholipolysis mixtures (Figs. 4 and 5). On electron microscopy, P-LDL remained as discrete, unfused spherical particles that appeared to be slightly smaller in diameter than C-LDL (Table 1). The retention times of P-LDL on gel permeation chromatography were slightly prolonged relative to C-LDL, also suggesting that the sizes of the particles may have been reduced.

The RIA competition curves produced by P-LDL and C-LDL indicated that the expression of only one epitope was consistently altered by phospholipolysis. When slopes of RIA curves calculated by computer analysis are close to 1, the LDL-antibody reaction probably involves the binding of a single ligand (in this case an epitope on apoB-100) to a homogeneous population of binding sites (on monoclonal antibodies), and the reaction is compatible with the law of simple mass action (36). Under those conditions, ED50 values represent equilibrium constants. Departures of slopes from 1.0 indicate some heterogeneity of LDL preparations with respect to their affinities and binding constants for the homogeneous monoclonal antibodies. Even when slopes depart from 1.0, ED50s represent useful indexes of differences between two ligands tested under identical conditions. LDL heterogeneity has

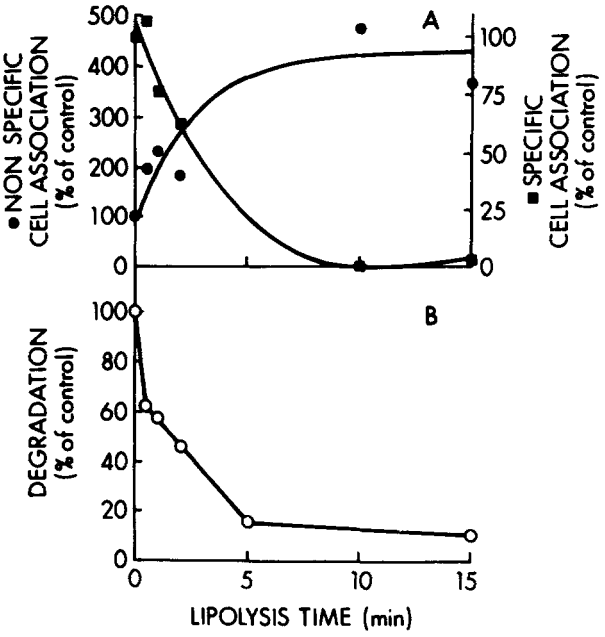


**Fig. 8.** Evolution of the interaction of $^{125}$I-labeled P-LDL with cultured fibroblasts with increased phospholipolysis. Cellular LDL receptors were up-regulated as described in Materials and Methods. P-LDLs phospholipolyzed to varying extents were generated by incubation of LDL with PL-$A_2$ (0.42 μg PL-$A_2$/mg of LDL phospholipids) in 0.1 M Tris, 0.15 M NaCl, 0.5 mM $CaCl_2$, 0.4% BSA, pH 8.5, at 37°C for 0.5 min to 15 min as indicated. P-LDL were immediately applied to a Superose 6 FPLC column and isolated by gel permeation chromatography. P-LDLs and C-LDLs were iodinated by the Iodogen method (150–600 cpm/ng of LDL protein) and incubated with the fibroblasts. Specific and nonspecific cell association values of the enzyme-treated LDL preparations are given in panel A, expressed as a percentage of the specific or nonspecific cell association of $^{125}$I-labeled C-LDL. Data on cellular degradation of $^{125}$I-labeled P-LDL expressed as a percentage of the degradation of $^{125}$I-labeled C-LDL are given in panel B. Each data point represents a mean of triplicate dishes. One hundred percent (or 0-time of enzyme incubation) values for nonspecific and specific cell associations of $^{125}$I-labeled C-LDL were 156 ± 10 and 370 ± 4 ng of LDL/mg of cell protein (panel A). Degradation values of $^{125}$I-labeled C-LDL (or 0-time of enzyme incubation) were 250 ± 12.5 ng of LDL/mg of cell protein/5 hr (panel B).

been documented by us using competition RIAs (6, 37), and by others using several physical techniques, particularly in patients with hypertriglyceridemia (38). Based on the slopes obtained, the normal subjects used in these studies manifested considerably less immunologic heterogeneity at all epitopes than did the previously studied hypertriglyceridemic patients (6). In the present experiments, phospholipolysis altered the immunologic heterogeneity of the particles (slopes) to a lesser extent than it did the affinities (ED50s) of selected epitopes of apoB-100. The greatest and most consistent alterations were noted in the epitope closest to the COOH terminal (465B6C3) (see epitope map in **Fig. 13**). The epitopes near the cell receptor recognition sites (454B1B3, 454B1B6, and MB47) were less consistently affected and affected to a much lesser

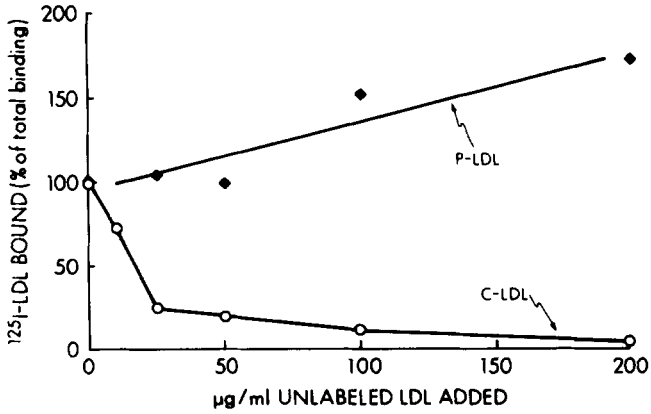


**Fig. 9.** Inhibition of cellular binding of $^{125}$I-labeled C-LDL by P-LDL. Fibroblast LDL receptors were up-regulated as in Fig. 7. The dishes were chilled and each dish then received 1 ml of ice-cold medium containing 10% LPDS, 5 μg/ml of $^{125}$I-labeled C-LDL (sp act 196 cpm/ng) and either C-LDL or P-LDL at the indicated concentrations. After incubation for 2 hr at 4°C, the monolayers were washed extensively and the amount of $^{125}$I-labeled C-LDL bound to cells was determined in triplicate dishes. Results represent % of $^{125}$I-labeled LDL bound = ($^{125}$I-LDL bound in the presence of P-LDL or C-LDL/ $^{125}$I-LDL bound in the absence of P-LDL or C-LDL) × 100; 100% binding = 387 ± 40.0 ng/mg of cell protein.

extent. The expression of epitopes in the rest of the molecule appeared not to be affected. The extent of changes in ED50 at epitope 465B6C3 was closely related to the extent of phospholipolysis (Fig. 2 and Table 2), but retention of products of lipolysis did not account for the altered immunoreactivity of P-LDL (Tables 1 and 4).

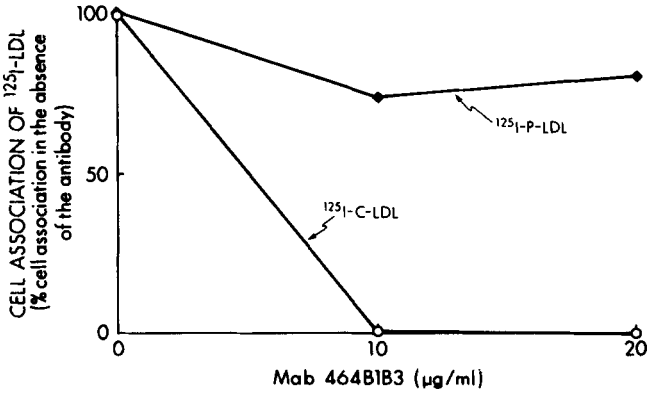


**Fig. 10.** Inhibition of the cell association of $^{125}$I-labeled C-LDL and $^{125}$I-labeled P-LDL by anti-LDL monoclonal antibody 464B1B3. Monolayers of fibroblasts were prepared as in Figs. 7 and 8. Each dish then received 1 ml of medium containing 10% LPDS, 10 μg/ml of either $^{125}$I-labeled C-LDL or $^{125}$I-labeled P-LDL and the indicated amounts of Mab 464B1B3. After incubation for 5 hr at 37°C, the medium was removed, the cells were washed extensively and dissolved in 0.1 N NaOH. Aliquots were used to determine $^{125}$I-labeled LDL cell association and cellular protein concentrations. Specific cell association was calculated by subtracting the nonspecific values obtained from dishes containing excess unlabeled C-LDL (200 μg/ml). Data are expressed as the percents of cell-associated $^{125}$I-labeled C-LDL or $^{125}$I-labeled P-LDL; 100% (0 antibody) values for $^{125}$I-labeled C-LDL and $^{125}$I-labeled P-LDL are 806 ± 8 and 438 ± 40 ng/mg cell protein, respectively.

TABLE 5. Comparison of cell association, cellular degradation, and lipoprotein-induced cholesterol esterification by acetylated and nonacetylated C-LDL and P-LDL in normal (GM203) cultured fibroblasts

| LDL | Cell Association | Cellular Degradation | Cholesterol Esterification |
|---|---|---|---|
| | *ng LDL* | *ng LDL per mg of cell protein* | *μmol cholesterol* |
| C-LDL | | | |
| Total | 196 ± 2.33 | 156 ± 22.0 | 1.78 ± 0.23 |
| Nonspecific | 61.2 | 26.6 | |
| Ac-C-LDL | | | |
| Total | 19.1 ± 0.5 | 0 | 0 |
| Nonspecific | 21.1 | 0 | |
| P-LDL | | | |
| Total | 250 ± 16.2 | 0 | 0.4 |
| Nonspecific | 170 | 0 | |
| Ac-P-LDL | | | |
| Total | 120 ± 4.58 | 0.35 ± 0.1 | 0 |
| Nonspecific | 103 | 4.2 | |

Monolayers of fibroblasts were grown in MEM, 10% FCS. Forty eight hr before the experiment, medium was replaced with MEM, 10% LPDS to up-regulate LDL receptors. P-LDL were lipolyzed by PL-$A_2$ for 15 min in buffer containing 0.4% BSA and were reisolated by gel permeation chromatography. LDLs were radiolabeled by the Iodogen method to specific radioactivities of 150–600 cpm/ng of protein. Cells were incubated in 1 ml of medium containing 10% LPDS and 2.5 μg/ml of the indicated radiolabeled lipoprotein in the presence or absence of excess C-LDL, 500 μg/ml, for 5 hr at 37°C. Total and nonspecific cell association and degradation were calculated as described. To determine nonspecific cell association and degradation, excess C-LDL was used with $^{125}$I-labeled C-LDL or $^{125}$I-labeled P-LDL and excess Ac-C-LDL was used with $^{125}$I-labeled Ac-C-LDL or $^{125}$I-labeled Ac-P-LDL. In a parallel set of culture dishes, fibroblasts were incubated with 1 ml of MEM, 10% LPDS, 25 μg/ml of unlabeled LDL or acetyl-LDL, and 0.1 mM [$^3$H]oleate (sp act 24 mCi/mol) complexed to BSA (0.02 mM). After 5 hr incubation at 37°C, the medium was removed and the cells were extracted with hexane–isopropanol; cholesteryl esters were separated by TLC and the radioactivity of the cholesteryl ester spots was determined. Four dishes without lipoproteins were used to assess baseline esterification of cholesterol. The results are means ± SEM of triplicate dishes.

The interaction of P-LDL with cells was grossly different from that of C-LDL (Fig. 7). Whereas C-LDL interacted specifically with the LDL receptor and were internalized and degraded, P-LDL appeared to adhere nonspecifically (Figs. 9 and 10; Tables 5 and 6), and to enter the cells poorly if at all. $^{125}$I-Labeled P-LDL were not degraded; instead they "leaked" back into the culture media (Fig. 11). As in the case of immunoreactivity (Table 2), the evolution of altered cell interaction over time occurred rapidly and was related to the extent of phospholipolysis rather than to proteolysis of apoB (Fig. 8). But whereas immunoreactivity at the epitopes tested was not affected by retained products of phospholipolysis (Table 4), the interactions of P-LDL with cell surfaces were greatly affected by these products. Nevertheless, when most of the retained products were removed by albumin, cell association and degradation still remained abnormal (Fig. 12). It seems likely that PL-$A_2$ catalyzed the removal of surface lipids and the exposure of underlying core lipids may have significantly altered the interaction of LDL particles with cells.

It seems unlikely that proteolysis of LDL apoB-100 greatly contributed to the altered immunoreactivity or cell reactivity of P-LDL. First, no evidence of degradation of $^{125}$I-labeled LDL was seen after PL-$A_2$ treatment, at least for the first 5 min of incubation with PL-$A_2$ (Fig. 3), by which time immunoreactivity and cell reactivity were substantially altered (Table 2). Second, as we previously reported, proteolysis of LDL both by trypsin and staphy-

TABLE 6. Cell association and degradation of $^{125}$I-labeled C-LDL and $^{125}$I-labeled P-LDL by normal and LDL receptor-deficient fibroblasts

| | Fibroblasts | |
|---|---|---|
| | Normal GM203 | FH Homozygotes GM2000 |
| | *ng of LDL/mg of cell protein* | |
| $^{125}$I-Labeled C-LDL | | |
| Cell association | 456 ± 4.48 | 27.9 ± 1.01 |
| Degradation | 689 ± 13.6 | 33.2 ± 6.54 |
| $^{125}$I-labeled P-LDL | | |
| Cell association | 418 ± 9.29 | 196 ± 3.18 |
| Degradation | 0 | 0 |

GM203 fibroblasts were grown in MEM with 10% FCS. GM2000 fibroblasts (FH, receptor-negative cells) were grown in MEM with 15% FCS. The LDL receptors of fibroblasts were up-regulated by incubation for 48 hr with 10% LPDS prior to experiment. Then each of the monolayers received 1 ml of medium with 10% LPDS and 2.5 μg/ml of either $^{125}$I-labeled C-LDL or $^{125}$I-labeled P-LDL in triplicate dishes. After incubation for 5 hr at 37°C, cellular degradation (total) and cell association (total) were determined. The results are means ± SEM of triplicate dishes.

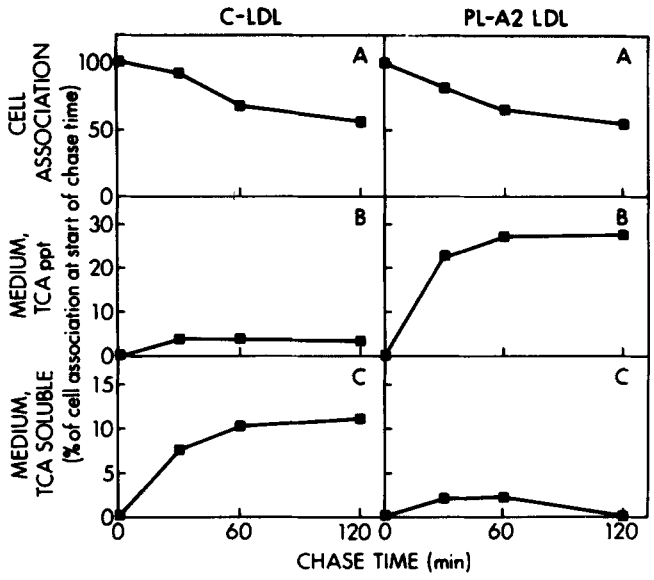


**Fig. 11.** The "pulse-chase" experiment. Up-regulated fibroblasts were incubated with MEM, 10% LPDS, and 10 μg/ml of either $^{125}$I-labeled C-LDL or $^{125}$I-labeled P-LDL for 4 hr at 37°C ("pulse" period). The monolayers were then washed extensively with ice-cold Tris–saline–BSA followed by Tris–saline alone and incubated further with LPDS at 4°C for 30 min. After this period, the medium was changed and the cells were washed again and incubated in MEM, 10% LPDS at 37°C for the indicated times ("chase" period). At the indicated times, media were collected, centrifuged to pellet any cells, and 0.9 ml of the supernatant media was precpitated with ice-cold 20% TCA in the presence of 0.1% BSA. Total cell-associated radioactivity (panels A), TCA-precipitable $^{125}$I-labeled protein in the medium during the chase period (panels B), and TCA-soluble non-iodide (chloroform-insoluble, water-soluble) $^{125}$I-labeled material in the medium (panels C) were determined. Each point represents the mean of triplicate dishes. The data are expressed as percents of the total cell-associated radioactive counts at the beginning of the chase period (0 time); these were 1207 ± 81 and 1171 ± 53 ng of LDL/mg of cell protein for $^{125}$I-labeled C-LDL and $^{125}$I-labeled P-LDL, respectively.

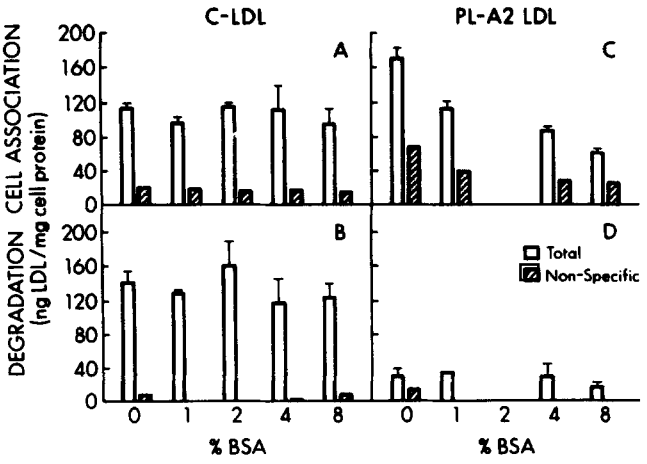


**Fig. 12.** Effects of depletion of phospholipolysis products of the association and degradation of $^{125}$I-labeled P-LDL by cultured fibroblasts. P-LDL were prepared by co-incubating LDL and PL-$A_2$ in buffer containing 0.4% of BSA. P-LDL and C-LDL then were radioiodinated. Cell association and degradation assays were carried out in media containing the indicated amounts of BSA as described in Methods.

lococcal V-8 protease *increased* the expression of the epitope defined by Mab 464B1B3, while expression of epitope 457C4D1 did not change or *decreased.* Phospholipolysis, if it produced any changes at all, *decreased* the expression of epitope 464B1B3 and tended to *increase* that of 457C4D1 (41). Third, P-LDL and C-LDL achieved the same extent of saturable binding under conditions of lipoprotein excess to solid phase monoclonal antibodies (data not shown). These data suggest that the kinetics of binding of the epitopes were altered (competitive binding assays) but the chemical structure of the epitopes was not destroyed since binding of the P-LDL to antibodies did occur, albeit with much lower affinity. Fourth, in previous experiments reported by us (41) and by others (42, 43), proteolysis with trypsin either slightly *decreased* specific binding of tryptic core LDL to fibroblasts or *increased* binding to porcine adrenal cell membranes. Staphylococcal protease had *little* effect on LDL–fibroblast interactions. By contrast, phospholipolysis produced *profound decreases* in specific binding to fibroblasts (Figs. 7 and 10).

Finally, these experiments confirm and extend our previous observations that altering the lipid compositions of apoB-100-containing lipoproteins affects the expression of epitopes of apoB-100 and the interactions of VLDL and LDL with cultured fibroblasts. Treatment of VLDL with lipoprotein lipase yields *smaller,* triglyceride-depleted particles that manifest *enhanced* expression of epitopes near the LDL-receptor recognition region of apoB-100 and *increased* interactions with cells. Treatment of hypertri-


**Fig. 13.** Linear map of apoB-100 indicating the location of epitopes recognized by the monoclonal antibodies used in this study. The major sites of cleavage of apoB-100 by thrombin are indicated by the arrows. Epitope assignments of the anti-apoB-100 monoclonal antibodies (identified by abbreviated numbers) are indicated by boxes. Details are provided in ref. 39. The assignment of the epitope recognized by Mab MB47 was adapted from Knott et al. (40).

glyceridemic patients with bezafibrate results in the accumulation in plasma of *LDL* particles that, too, are triglyceride-depleted but somewhat *larger* than pretreatment LDL. Post-treatment LDL also interact *more readily* with fibroblasts. Thus, changes of VLDL and LDL *sizes* in *opposite* directions altered immunoreactivities and cell reactivities of these particles in the *same* direction, suggesting that there is an optimal size for the apoB-100-containing particles at which epitopes near the LDL receptor recognition domain are maximally expressed. PL-$A_2$ treatment yields an altogether different result. Primarily a single epitope became altered in expression and specific interaction with cells was virtually abolished. Clearly, selective depletion of surface phospholipids produces very different effects on the conformations of apoB-100 than do the more physiologic perturbations of core lipoprotein lipids produced by LPL and by bezafibrate therapy.

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