Limited proteolysis selectively destroys epitopes on apolipoprotein B in low density lipoproteins

Kyung-Soo Hahm, Matti J. Tikkanen,[1] Ratna Dargar, Thomas G. Cole, Joseph M. Davie, and Gustav Schonfeld

Lipid Research Center, Departments of Preventive Medicine and Medicine, Washington University School of Medicine, St. Louis, MO 63110

Abstract Apolipoprotein B (apoB), the major apoprotein in human low density lipoprotein (LDL), is a large protein and complex immunogen, against which seven monoclonal IgG antibodies have been produced in our laboratory. These antibodies were used to define at least five individual epitopes on holo-LDL. The aim of the present experiments was to ascertain whether limited proteolysis of LDL would selectively affect the expression of the epitopes. LDL was digested with staphylococcal protease (SP), trypsin (T) or pronase; ~20% of LDL protein was lost by treatment with SP and T, and ~60% by treatment with pronase. Structurally stable SP- and T-LDL cores and soluble peptides were separated by gel permeation chromatography and zonal ultracentrifugation. The cores resembled holo-LDL in composition and flotation rates but apoB was fragmented into peptides <100,000 in molecular weight. On analysis by competitive radioimmunoassays some epitopes of the LDL cores were destroyed partially, some completely, and some were spared. The enzymes each produced individual patterns of epitope modulation. SP- and T-LDL cores retained most of their abilities to be bound and degraded by normal human cultured fibroblasts. Some soluble peptides also manifested both antigenicity and cell reactivity. The retention of activities by the cores while active fragments are lost is compatible with, but does not constitute unequivocal evidence for, the hypothesis that apoB may consist of repeating structures.—**Hahm, K-S., M. J. Tikkanen, R. Dargar, T. G. Cole, J. M. Davie, and G. Schonfeld.** Limited proteolysis selectively destroys epitopes on apolipoprotein B in low density lipoproteins. *J. Lipid Res.* 1983. **24:** 877–885.

Supplementary key words zonal ultracentrifugation • Western blots

Lipoproteins are complex particles consisting of several types of lipids of varying hydrophobicity and of apoproteins. The amphipathic phospholipids and apoproteins form the surface regions of the particles, whereas the hydrophobic cholesteryl esters and triglycerides fill the inner core regions. The association between apoproteins and lipids is thought to occur at the hydrophobic surfaces of amphipathic helical regions of apoproteins, whereas the hydrophilic faces of the helices probably interact with polar regions of phospholipids, other apoproteins, or water (1). The amino acid sequences of several apoproteins including apoA-I, A-II, and the C apoproteins are known and their lipid binding regions have been identified. This was possible because these apoproteins are of relatively small molecular weight and are water-soluble. Less is known about the interactions of apoB with lipids and with the surface regions of lipoproteins. Two major (B-100 and B-48) and two minor (B-74 and B-26) forms of apoB have been described (2). All are large proteins that aggregate in aqueous solutions. Their amino acid sequences are unknown, but it has been suggested that they may be composed of repeating subunits of smaller molecular weight (3).

Monoclonal antibodies, because they are directed against discrete epitopes, are powerful probes of the structures of proteins, even of proteins whose chemical structure is not defined. In previous work we have produced seven monoclonal anti-apoB antibodies (4). In experiments where ^{125}I-labeled and unlabeled antibodies were made to compete against each other for binding to LDL, at least five non-overlapping epitopes of apoB were defined in holo-LDL. Two antibodies defining epitope 2 [or two closely related epitopes: 2a (antibody 464B1B3) and 2b (antibody 464B1B6)] inhibited the binding of ^{125}I-LDL to cultured normal human fibroblasts, indicating that the epitopes interacting with these antibodies also were involved in the binding of LDL to its cellular receptor.

Previous studies have shown that limited proteolysis of LDL results in the production of relatively stable lipoprotein 'core' particles that retain many physical, chemical, and immunological properties of the native lipoprotein (5–7). In the experiments to be described we sought to ascertain whether perturbations of the structure of apoB by limited proteolysis had selective effects on the expression of various epitopes in holo-

Abbreviations: ^{125}I-LDL, ^{125}I-labeled LDL, LDL, low density lipoprotein; P-LDL, pseudodigested LDL; SP, staphylococcal protease; T, trypsin.

[1] Fogarty International Fellow in Preventive Medicine. Supported also by the Paavo Nurmi Foundation, Helsinki, Finland.

LDL. In an attempt further to distinguish individual epitopes from each other, we also sought to ascertain whether the various epitopes could be localized to different proteolytic fragments of apoB. Limited proteolysis also provided water-soluble cleavage peptides that could be examined for immunoreactivity. Finally, in order to link structure with function, the abilities of digested particles and cleavage peptides to interact with the LDL receptors of cultured human fibroblasts was examined.

METHODS

Enzymatic proteolysis of low density lipoprotein

Low density lipoprotein (LDL) was isolated between densities 1.025–1.050 g/ml (8) with two ultracentrifugations carried out at each density. Freshly isolated LDL was used for enzymatic digestions at a concentration of about 20 mg/ml in 1 mM EDTA–0.16 M NaCl, pH 8.2. The digestions were performed at 37°C under a nitrogen atmosphere at an enzyme to substrate ratio of 1/40 (w/w) using the following enzymes: *a*) trypsin-TPCK (Millipore Corp., Freehold, NJ) dissolved in 1.0 mM acetic acid–0.1 mM calcium chloride (9), *b*) *Staphylococcus aureus* V8 protease (Miles Laboratories, Inc., Elkhart, IN) dissolved in 50 mM ammonium bicarbonate buffer, pH 8.4 (10), and *c*) pronase (protease from *Streptomyces griseus*) (Sigma Chemical Co., St. Louis, MO) dissolved in 0.1 M Tris, pH 7.4 (11). The enzyme solutions were prepared fresh before digestion. Incubations were for periods of time indicated below. "Pseudodigestions" of LDL (P-LDL) were performed as described above but without including enzymes. Digestions were stopped by addition to 5 ml of digestion mixtures of 10 μl of 1% PMSF (phenylmethyl sulfonyl fluoride) in isopropyl alcohol.

Isolation of proteolysis cores

The enzyme digestion mixtures were cooled to 10°C and applied to a 2.5 × 95 cm column of Sephadex G-50 superfine previously equilibrated with 1 mM EDTA–0.16 M NaCl, pH 7.4. Proteolysis cores eluted at the void volume of the column and were separated from cleaved peptides of molecular weight smaller than 10,000. In preliminary tests some effluent fractions containing cleavage peptides exhibited binding to cultured fibroblasts or monoclonal antibodies. Effluent fractions containing binding activity were pooled and subjected to further analysis. The pooled void volume fractions were concentrated to a final concentration of ~10 mg/ml in an Amicon ultrafiltration cell equipped with a PM-10 Diaflo membrane, filtered through 0.45 μm filters (Millipore), and stored at 4°C.

In some instances, LDL cores were characterized further. Aliquots of ^{125}I-LDL (10^6 cpm, 10 ng of protein) were added to each of the proteolysis cores, which were then subjected to rate zonal ultracentrifugation through a linear density gradient of 1.00 to 1.30 g/ml NaBr (12). The rotor effluent as collected in 10-ml fractions and the positions of lipoproteins were established by the ultraviolet absorption, protein concentration, and radioactivity of each fraction.

Gel electrophoresis and immunoblotting

The peptides of proteolysis cores were separated in polyacrylamide slab gels containing 0.1% SDS, 3.5–15% linear gradient acrylamide, and 0.4% bisacrylamide cross-linked with ammonium persulfate (13). The electrode buffer was 0.025 M Tris, 0.195 M glycine in 0.1% SDS, pH 8.3. Sample solutions containing 20% sucrose, 2.5% SDS, 5% β-mercaptoethanol, 0.0075% bromophenol blue, and 10–50 μg of protein per 40 μl were heated for 2 min at 100°C before applying to the gels. Electrophoresis was performed at room temperature in the model 220 vertical slab electrophoresis cell (Bio-Rad Laboratories, Richmond, CA) at a constant voltage of 32 V for 17–18 hr. Gels were stained with Coomassie blue or were subjected to electrophoretic transfer of peptides (Western blotting) (14). Aminophenylthioether (APT) paper was prepared from Whatman #50 paper by reacting the paper with 1,4-butanediol diglycidyl ether and 2-aminothiophenol (15). APT paper was diazotized to diazophenylthioether (DPT) paper using $NaNO_2$/HCl (15). Before transfer, polyacrylamide gels were cut longitudinally, one section was stained as above and the rest was mounted in a Trans-Blot cell (Bio-Rad Laboratories). Electrophoretic transfer was performed at 1.6 Å for 2 hr at 5°C. Coomassie blue-stainable material was quantitatively transferred by the procedure. After transfer, the DPT paper was washed by gentle shaking in TEG buffer (0.4 M Tris-(hydroxymethyl)aminomethane, pH 8.8, 40% (v/v) ethanolamine, 1% (w/v) gelatin) for 2 hr at room temperature, rinsed in deionized water, blotted on paper, and placed into heat-sealable freezer bags. ^{125}I-labeled monoclonal antibodies were diluted (0.5 ml per lane containing 0.7 × 10^6 cpm) in TENG-T buffer (50 mM Tris(hydroxymethyl)aminomethane, pH 7.4, 4 mM Na_2 EDTA, 150 mM NaCl, 0.25% (w/v) gelatin, 0.05% (v/v) Triton X-100) containing also nonimmune ascites fluid (10%, v/v), and added to the freezer bags containing the transferred proteins. In control incubations an ^{125}I-labeled nonrelated monoclonal antibody (antihuman C3 complement antibody) was used. The DPT papers were thoroughly wetted with antibody solution and excess solution was squeezed out. Bags were heat-sealed and incubated at room temperature for 18 hr. DPT papers

were rinsed and washed with TENG-T buffer for 2 hr. The papers were then rinsed in deionized water, blotted, air-dried, and radioautographed at −70°C with Dupont Cronex Lightning-Plus XL intensifying screens using Kodak X-Omat AR X-ray film.

Immunoassays

Monoclonal IgG class antibodies directed against intact LDL were produced in a mouse spleen-myeloma system and purified by affinity chromatography on insolubilized LDL as described (4). The seven antibodies under study defined at least five epitopes on LDL-apoB (4). They are identified in this text as follows: 1a = 457C4D1; 1b = 457C4D6; 2a = 464B1B3; 2b = 464B1B6; 3 = 465B6C3; 4 = 465C3D1; 5 = 465D3D5.

Competition assays were carried out in 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) that were precoated overnight at 23°C with affinity purified antibody solution (5 μg/ml) using 150 μl per well. After incubating the wells with 3% BSA-PBS (300 μl per well) for 2–3 hr and emptying the wells, increasing amounts of sample protein (pseudodigested LDL, proteolytic LDL cores, or proteolytic peptides) in 130 μl of BSA-PBS were added together with 100,000 cpm of ^{125}I-LDL (in 20 μl of BSA-PBS). After incubation at 4°C overnight, wells were emptied, washed three times with PBS, sliced, and counted in a Packard Autogamma Spectrometer. The results of these competitive displacement assays were expressed as apparent apoB contents in the samples (as % of P-LDL).

Cell studies

The cell reactivity of proteolytic LDL cores was assessed in competitive binding assays at 37°C using cultured normal human fibroblasts (16, 17). Cells were grown in Eagle's minimal essential medium (15% newborn calf serum) for 5 days and in 10% lipoprotein-deficient serum for 48 hr. At the beginning of the experiments the dishes received the indicated amounts of LDL or proteolytic LDL cores as well as 5 μg/ml ^{125}I-LDL (sp act ~100 cpm/ng). Incubations in triplicate were carried out for 4 hr, after which cell-associated counts and ^{125}I-LDL degradation were determined.

The capacity of proteolytic LDL peptide fragments to bind to cells was tested in competitive binding assays at 4°C using ^{125}I-LDL and the same cell culture system as above. Before the experiment cells and media were cooled (30 min, 4°C). The indicated amounts of peptides or acetylated peptides were added to the culture media and 2 hr later 100,000 cpm of ^{125}I-LDL (5 μg/ml) was added. After incubation in triplicate (2 hr, 4°C), media were removed and plates were washed three times with cold Tris-BSA and twice with cold Tris. Cells then were incubated (1 hr, 4°C) with dextran sulfate (4 mg/ml) in saline, and aliquots were removed for determination of dextran sulfate-releasable counts. Following washing with cold Tris, cells were suspended in 1 ml of 0.1 M NaOH and aliquots were taken for measurement of residual bound counts and for protein determination.

RESULTS

Characterization of lipoprotein cores after proteolytic digestion of LDL

Proteolysis with trypsin and staphylococcal protease resulted in the loss of ~20% of LDL protein from the lipoprotein particles during 24 hr of treatment. Each enzyme also produced characteristic elution profiles of water-soluble cleavage peptides (**Fig. 1**). In addition, SDS polyacrylamide gel electrophoresis (4–15% gradient) of the isolated T and SP cores indicated that each protease also produced a characteristic pattern of cleavage products (**Fig. 2**). No additional cleavage peptides were found in the Sephadex G-50 column effluent when the incubation was carried out for more than 24 hr, and no change occurred in the pattern of cleavage products on SDS polyacrylamide gels. In order to find out whether digestion of apoB subspecies was complete, different amounts (5–50 μg per lane) of SP cores were electrophoresed together with pseudodigested LDL on SDS polyacrylamide (4–15% gradient). The results showed that all SP core chains detectable by Coomassie blue were located below the apparent molecular weight range of intact apoB subspecies (**Fig. 3A**). In 3% SDS polyacrylamide gels, comparison with appropriate molecular weight markers indicated that the SP core chains were of <100,000 molecular weight (not shown). To explore the possibility that minute amounts of protein, not detectable by Coomassie blue staining, would be present in the apparent molecular weight range of intact apoB, a Western blot of an identical gel to that shown in Fig. 3A was prepared and incubated with labeled antibody 2a; this antibody has been shown to bind to intact apoB-100 and B-74 on Western blots (18). On the resulting radioautogram (Fig. 3B), no binding of the label in the apparent molecular weight range of non-digested apoB subspecies was noted. Similar results were obtained when the experiments were carried out with T cores. Despite the major perturbations of their apoproteins, solutions of T cores and SP cores were optically clear, suggesting that they had retained properties of intact lipoproteins. Also, the percent chemical compositions of the T cores and SP cores remained similar to that of pseudodigested LDL (**Table 1**). Pronase treat-

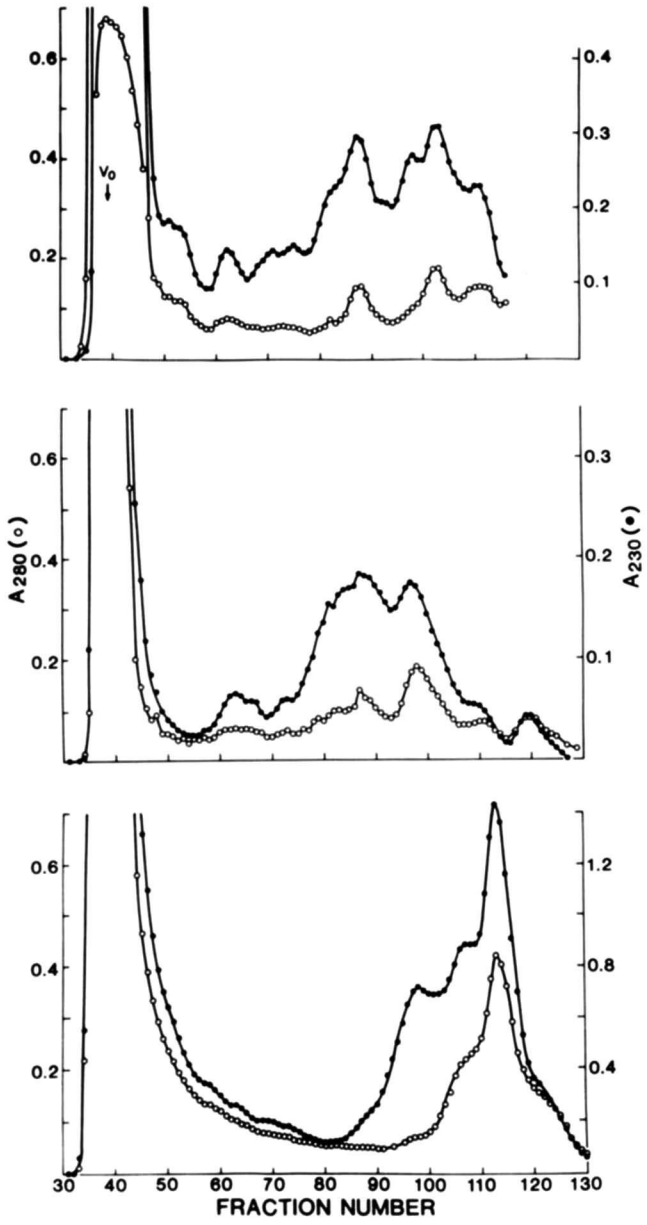

Fig. 1. Elution profiles of enzymatic proteolysis mixtures. Top, trypsin; middle, staphylococcal protease; bottom, pronase. After 24 hr of incubation, mixtures were gel-filtered on Sephadex G-50 (2.5 × 95 cm) at 10°C in 1 mM EDTA–0.16 M NaCl and 2.3-ml fractions were collected. Fractions 35–40 containing the proteolysis cores were pooled and characterized further. Fractions containing small cleavage peptides were pooled for further analysis as follows. Staphylococcal protease-treated peptides: SP-IV, fractions 91–99; SP-V, fractions 101–113. Trypsin-treated peptides: T-IV, fractions 73–77; T-V, fractions 78–83; T-VII, fractions 95–99; T-VIII, fractions 100–107.

ment over 24 hr caused ~60% losses of protein from LDL and on gel electrophoresis most of the core peptides were small (<44,000). Cleavage peptides also were small, eluting near the salt volume of the column (Fig. 1). Solutions of pronase cores were turbid, indicative of a disruption of the structure of the lipoprotein.

The isolated T cores and SP cores were characterized further by zonal ultracentrifugation (**Fig. 4**). Some heterogeneity of the cores was observed, but the flotation properties of the major peaks (peaks III in Fig. 4) differed only to a minor degree from those of pseudodigested LDL, and remained unchanged relative to the ^{125}I-labeled LDL used as internal standard. About 65% of the protein of T- and SP-LDL was recovered in peaks III; recovery for pseudo-LDL was 98%. The percentage chemical composition of the zonal peaks III is given in **Table 2.** Peptide profiles of zonal LDL cores on SDS gel electrophoresis were indistinguishable from those shown in Fig. 2 for the cores isolated by gel permeation chromatography (not shown).

Immunoreactivity of proteolysis cores and cleavage peptides with monoclonal anti-LDL antibodies

Five monoclonal antibodies designated as antibodies 1–5 that are directed against at least five nonoverlap-

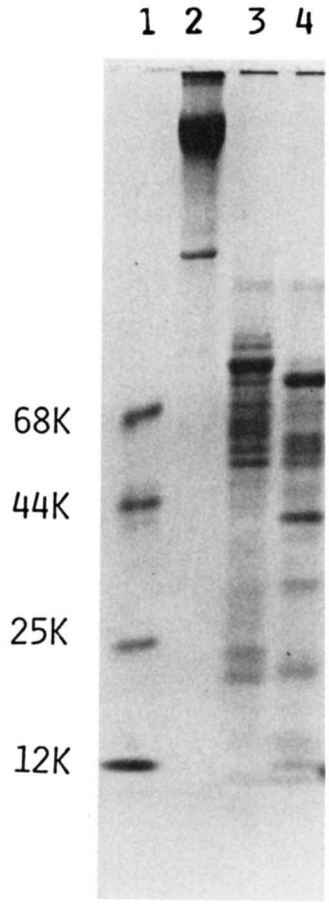

Fig. 2. Twenty-four hour enzymatic digests of LDL. Enzymatic proteolysis and 4–15% gradient polyacrylamide gel electrophoresis of the isolated proteolysis cores followed by Coomassie blue staining were carried out as described under Methods. 1, mol wt markers; 2, P-LDL; 3, SP-LDL; 4, T-LDL. Mol wt markers: 68 K, bovine serum albumin; 44 K, ovalbumin; 25 K, α-chymotrypsinogen; 12 K, cytochrome C.

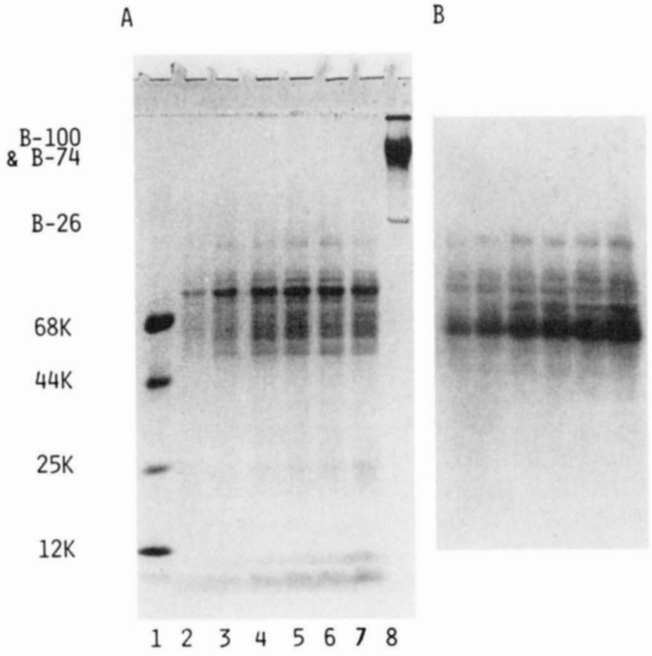

Fig. 3. Gradient polyacrylamide gel electrophoresis of varying quantities of SP-LDL protein. A, Coomassie blue staining of SP core peptides following 4–15% gradient polyacrylamide gel electrophoresis. 1, mol wt markers (see legend to Fig. 2); lanes 2 through 7 received increasing amounts of SP-LDL as follows: 2, 5 μg; 3, 10 μg; 4, 15 μg; 5, 20 μg; 6, 30 μg; 7, 50 μg. Lane 8, P-LDL. B, Radioautogram of ^{125}I-labeled antibody 2a bound to electrophoretically transferred SP core peptides. Lanes correspond to lanes 2–7 in A. Gel electrophoresis was carried out as in A. After transfer of the core protein to diazophenylthioether paper, the paper was incubated with ^{125}I-labeled antibody 2a, and sites of antibody binding were localized by radioautography.

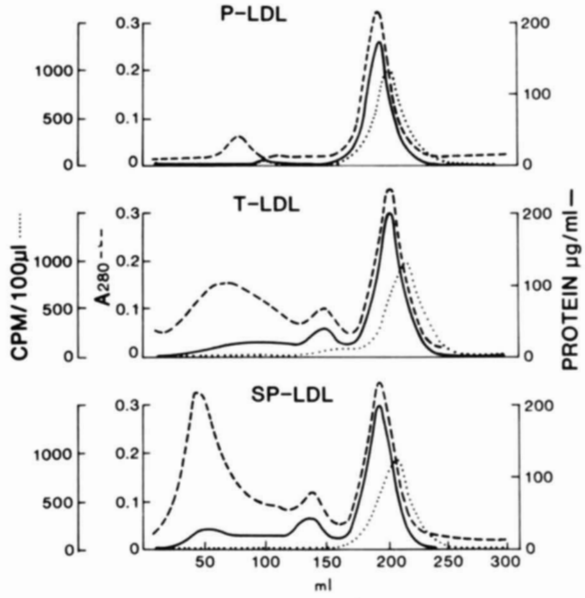

Fig. 4. Zonal ultracentrifugation of proteolysis cores. After enzymatic proteolysis and gel filtration, fractions 30–45 (Fig. 1) were pooled, concentrated, mixed with 10^6 cpm of ^{125}I-LDL, and subjected to rate zonal ultracentrifugation through a linear density gradient (1.00–1.30 g/ml NaBr). Rotor effluent was collected in 10-ml fractions. The effluent peaks (with increasing density from left to right) were designated peaks I, II, and III. The peak III's (effluent fractions 170–220 ml) were pooled and used for further analyses. Peaks I and II were not analyzed further. T-LDL, trypsin; SP-LDL, staphylococcal protease; P-LDL, pseudodigested LDL (incubation in the absence of enzyme with subsequent gel filtration on Sephadex).

ping antigenic determinants on LDL-apoB (4) were used in the immunoblotting analysis. All five antibodies have been shown to react with apoB-100 when Western blots of nondigested LDL were incubated with labeled antibodies (18). Three antibodies (1a, 2a, and 3) bound to at least one core peptide on Western blots (**Fig. 5**). Antibodies 4 and 5 did not bind to core peptides. The overall patterns of binding revealed that the epitopes detectable by immunoblotting could be separated from the others, confirming that the epitopes were distinct.

The effects of 24 hr of limited proteolysis on the expression on epitopes in holo-LDL were determined in competitive radioimmunoassays on microtiter plates. Representative displacement curves for 24 hr-digested T- and SP-LDL cores with antibodies 1 and 4 are shown in **Fig. 6.** Digestion produced differential effects on the immunoreactivity of LDL with each of the antibodies, destroying some epitopes, leaving some decreased and others unaffected or even increased (**Table 3**). With

TABLE 1. Chemical compositions of LDL proteolysis cores islolated by gel filtration

	P	PL	TG	UC	CE
			Weight %		
T-LDL	14.6	21.9	4.2	15.0	44.3
SP-LDL	15.8	22.4	4.6	13.9	43.3
P-LDL	20.1	20.2	4.7	13.6	41.3

After 24 hr of proteolysis, the incubation mixtures were filtered on a 2.5 × 95 cm Sephadex G-50 column (see Fig. 2) and fractions 30–45 were pooled for analysis. Abbreviations: T-LDL, SP-LDL, and P-LDL indicate trypsin, staphylococcal protease, and pseudodigested LDL, respectively. P, protein; PL, phospholipid; TG, triglyceride; UC, unesterified cholesterol; CE, cholesteryl ester.

TABLE 2. Chemical compositions of LDL proteolysis cores fractionated by zonal ultracentrifugation

	P	PL	TG	UC	CE
			Weight %		
T-LDL	18.4	25.1	1.1	14.3	41.2
SP-LDL	20.1	26.4	2.2	14.0	37.4
P-LDL	18.6	22.5	1.5	11.1	46.2

After proteolysis and gel filtration, fractions 30–45 (Fig. 2) were pooled, concentrated, and subjected to zonal ultracentrifugation (Fig. 4). Effluent fractions 170–220 ml (Peaks III) were pooled and analyzed. Abbreviations: See Table 1.

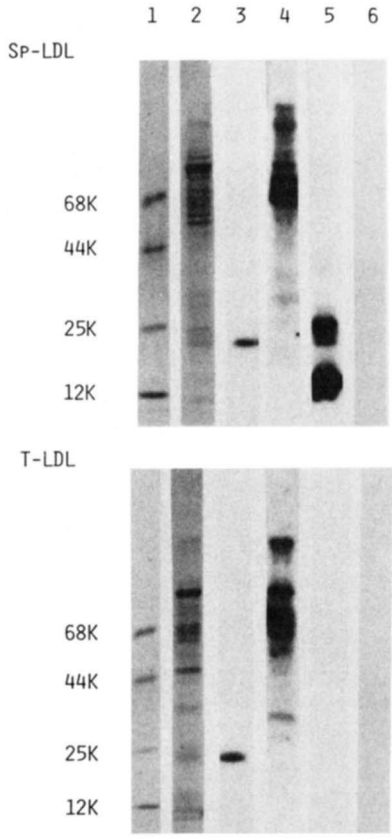

Fig. 5. Radioautogram of ^{125}I-labeled monoclonal antibodies bound to electrophoretically transferred T core and SP core peptides. Following 3.5–15% gradient polyacrylamide gel electrophoresis of enzyme-treated LDL cores isolated by Sephadex column chromatography and transfer of the core protein to diazophenylthioether paper, the paper as incubated with ^{125}I-labeled monoclonal anti-LDL antibodies, and sites of antibody localization were visualized by radioautography. Lane 1, mol wt markers: 68 K, bovine serum albumin; 44 K, ovalbumin; 25 K, α-chymotrypsinogen; 12 K, cytochrome C; Coomassie stain. Lane 2 top: SP-LDL core (Coomassie stain); Lane 2 bottom: T-LDL core (Coomassie stain). Lane 3: ^{125}I-antibody 1a; Lane 4: ^{125}I-antibody 2a; Lane 5: ^{125}I-antibody 3; Lane 6: ^{125}I-antihuman C3 complement antibody (control). Antibody 3 bound to SP-LDL but not to T-LDL. Antibodies 4 and 5 (not shown) were not well visualized on several attempts.

some exceptions, the apparent apoB contents of the zonal peaks III resembled those of the cores obtained from the Sephadex G-50 column effluents (Table 3). Pronase treatment caused the most marked decreases in the apparent apoB content of LDL, including a nearly total loss of antigenicity towards five of the seven antibodies tested. Virtually all of the immunoreactivity of the T and SP cores was retained vis a vis antibody 2, which is known to inhibit the binding of ^{125}I-LDL to fibroblasts (4). By contrast, immunoreactivity of LDL with antibody 3 was almost totally lost after trypsin digestion and with antibody 4 after treatment with staphylococcal protease. The progressive destruction of one epitope (antibody 4) during digestion was monitored in a time-course experiment in which an LDL preparation was treated with staphylococcal protease for increasing periods of time and the ability of chromatographically isolated core LDL to compete with ^{125}I-LDL for binding to antibody 4 was tested. Pseudodigestions of LDL were carried out in parallel. After incubations of 3 and 6 hr, the apparent apoB contents per mass core LDL protein decreased to 82 and 45% of pseudodigested LDL, respectively. After incubations of 24 or 30 hr, the apparent apoB contents were reduced to zero.

The immunoreactivities of various water-soluble peptide fractions also were determined in competitive microtiter plate radioimmunoassays (examples of displacement curves are shown in Fig. 6 and results for those cleaved peptides that showed detectable competitive activity are given in **Table 4**). Immunoreactivity was shown particularly in assays containing antibodies 1a, 2a, 2b, and 3. The maximum apparent apoB content was 1% of the standard LDL-apoB. Note that, in spite of the removal of peptides with immunoreactivity vs antibody 2 from holo-LDL, the immunoreactivity of the LDL cores with antibody 2 was retained or increased (Table 3).

Interaction of proteolysis cores and peptides with cultured human fibroblasts

The competitive ability of the 24-hr SP cores vs ^{125}I-LDL for binding to cells was identical with that of pseudodigested LDL, whereas the competitiveness of T cores was significantly diminished (**Fig. 7**). The water-soluble cleavage peptide fractions obtained from the gel filtration column effluents also were tested in cell assays (**Table 5**). The material in fractions T-IV and SP-IV caused inhibition of binding of ^{125}I-LDL to cells. Acetylation, which has been shown to eliminate the ability of LDL to compete with ^{125}I-LDL for binding to fibroblasts (19), also abolished the inhibiting capacity of active peptides. Similar inhibiting activity was present in T-V and SP-V peptides; other peptides had no activity (not shown).

DISCUSSION

The aim of these studies was to ascertain whether epitopes on holo-LDL could be selectively perturbed by limited proteolysis, and to find out whether this process could provide information on the structure and organization of apoB in LDL. Digestion of LDL by staphylococcal protease and trypsin yielded LDL core particles which, on separation by gel permeation chromatography and zonal ultracentrifugation (Figs. 1 and 4), had protein and lipid compositions that greatly resembled control "pseudodigested" LDL (Tables 1 and 2).

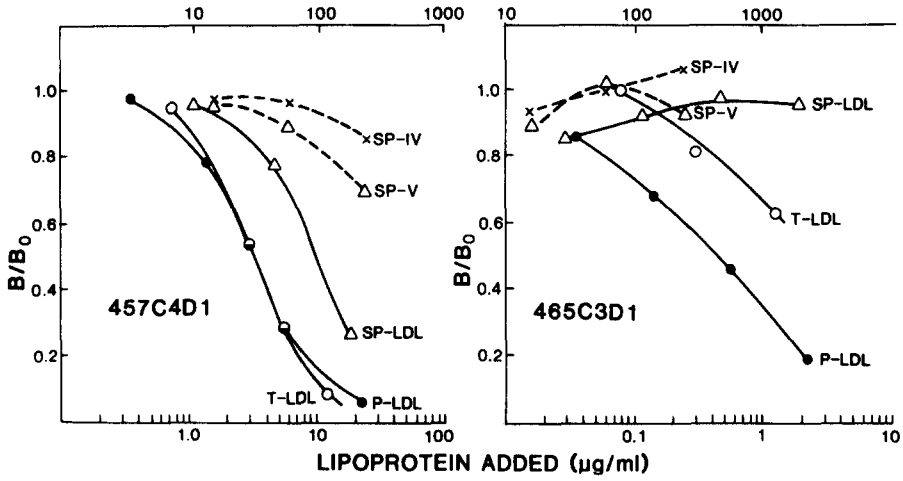

Fig. 6. Competitive displacement of ^{125}I-labeled LDL by trypsin and staphylococcal protease-treated cores or peptides. Microtiter wells were coated with 5 μl/ml of monoclonal antibody 1 or 4 overnight at 23°C, rinsed with PBS, and blocked with PBS-BSA (3 hr, 23°C). Wells then received increasing amounts of unlabeled enzyme-treated LDL (T-LDL or SP-LDL), or pseudodigested LDL (P-LDL) isolated by gel permeation chromatography, or cleavage peptides (for definition of SP-IV and SP-V fractions, see legend to Fig. 1) as well as a constant amount (~100,000 cpm) of ^{125}I-labeled LDL. After incubation (16 hr, 23°C), wells were rinsed with PBS, sliced, and counted in a gamma counter. Note: Dashed line curves relate to abscissas *on the top;* continuous curves relate to abscissas *on the bottom* of the figure.

This suggested that the SP and T cores were quite stable in structure, in spite of digestion of LDL proteins (Fig. 2). By contrast, pronase digestion caused more extensive digestion of apoB and disruption of particle integrity.

In the previous communication we suggested that the anti-LDL monoclonal antibodies produced in our laboratory defined at least five distinguishable epitopes on holo-LDL (4). Monitoring of digestions over time revealed that by 24 hr of digestion proteolysis had reached its maximum extent. The absence of LDL protein in the apparent molecular weight range of nondigested apoB (>100,000) suggests that all particles had been digested. Thus, retention of any immunoreactive or biologic activity by cores probably was not due to retention of "native" apoB chains by some LDL particles in the digestion mixtures.

All of the epitopes were altered by proteolysis, and trypsin and staphylococcal protease yielded different patterns of perturbation in the expression of the epitopes (Fig. 6 and Table 3). Pronase, a nonspecific pro-

TABLE 3. Effects of limited proteolysis on apparent apoB contents of LDL "cores"

	Apparent ApoB Contents				
	T-LDL		SP-LDL		Pro-LDL
Antibody[a]	Column	Zonal	Column	Zonal	Column
1a	107	98	68	75	0
1b	95	61	62	46	0
2a	140	126	115	154	32
2b	114	92	103	85	34
3	0	0	42	33	10
4	18	9	0	0	0
5	28	42	30	61	8

The results are means of two assays. Two LDL samples were separately digested and LDL cores were isolated on Sephadex and subjected to zonal ultracentrifugation. The apparent apoB contents were determined by competition assays on microtiter plates. For each LDL preparation, duplicate determinations at 3–5 doses were carried out with coefficients to variation of ~8%. Data are expressed as percent of protein relative to the pseudodigested LDL protein standard. Column fractions represent LDL cores recovered after fractionation on Sephadex G-50 (see Fig. 2). Zonal fractions represent peaks III recovered after zonal ultracentrifugation of LDL cores (see Fig. 4).

[a] Identification of antibodies; see Immunoassays under Methods.

TABLE 4. Apparent apoB contents of LDL proteolytic cleavage peptide fractions separated from "cores" by gel filtration

Antibody[a]	T V	T VII	T VIII	SP IV	SP V
1a	0	0	0	0.4	0.7
1b	0	0	0	0	<0.1
2a	0	0	0	0.5	0.9
2b	<0.1	0	<0.1	0.5	1.0
3	0	0	0	0.3	0.9
4	0	0	0	0	0
5	0	0	0	<0.1	0

Results are expressed as percent of protein relative to LDL protein standard. Duplicate determinations of 2–3 doses were carried out with coefficients of variation of ~8%. Peptide fractions are identified in the legend to Fig. 2. See also Fig. 6.

[a] Identification of antibodies, see Immunoassays under Methods.

tease, produced a more generalized destruction of LDL immunoreactivity. This suggests that epitopes indeed were distinctively expressed in holo-LDL and epitopes were sufficiently close to the surface of the LDL particle to be accessible to enzymes. The immunoblotting experiments (Fig. 5) demonstrated that it was possible physically to separate some of the epitopes from each other. The results of immunoblotting confirm our previous findings (4) of the presence of distinct epitopes on apoB and lend strength to the conclusions that epitopes on holo-LDL are differentially affected by proteolytic perturbation of LDL structure.

We have shown that Fab fragments of antibody 2 inhibited the binding of ^{125}I-LDL to cultured normal human fibroblasts, whereas the other monoclonal antibodies had no inhibitory effect (4). These results were taken to indicate that whereas epitopes 1, 3, 4, and 5 were not involved in the interaction of apoB with its cellular receptor, epitope 2 might overlap or be located near the cellular binding cite on apoB (4). The present results seem to confirm the dissociation of some epitopes from the cellular binding site. Thus, epitope 4 was de-

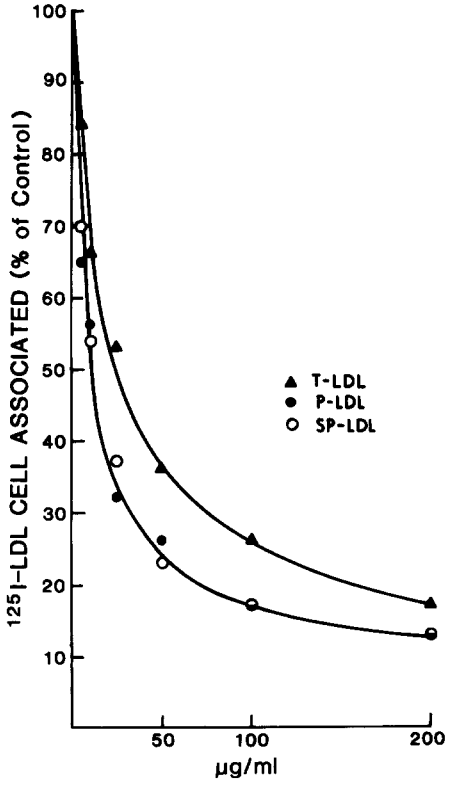

Fig. 7. Binding of ^{125}I-labeled LDL to cultured normal human fibroblasts in the presence of T-LDL or SP-LDL. Cells were grown in Eagle's minimal essential medium (15% fetal calf serum) for 5 days and in 10% lipoprotein-deficient serum for 48 hr. At the start of the experiment, 5 µg/ml of ^{125}I-LDL (~100 cpm/ng) and increasing amounts of T-LDL, SP-LDL, or P-LDL were added to the culture media. At the end of incubations (4 hr at 37°C), cell-associated LDL counts were determined. All incubations were carried out in triplicate. The displacement curve produced by T-LDL differed significantly (two-tailed t test; $P < 0.05$) from those produced by P-LDL and SP-LDL. The assay was repeated once using T-LDL and SP-LDL and once using T and SP cores reisolated by zonal ultracentrifugation (peak III's, see Fig. 4). The results were the same in both instances. Similar results were obtained for ^{125}I-LDL degradation (not shown).

TABLE 5. Inhibition of ^{125}I-LDL binding to cultured human fibroblasts by proteolytic LDL peptides

	^{125}I-LDL Bound in the Presence and Absence of Inhibitors			
Inhibitor Dose	T IV	SP IV	Ac T IV	Ac SP IV
µg/ml	*% of control*			
50	56	49		
100	44	40		
200	56	33	102	98
none	100	100	100	100

Cultured fibroblasts grown in 10% LPDS for 48 hr before the experiment were exposed to the indicated peptide inhibitors at 4°C for 2 hr at the indicated concentrations. Five µg of ^{125}I-LDL was added and incubation was continued for another 2 hr at 4°C. Dextran sulfate-releasable ^{125}I-LDL was then determined. Results are expressed as % of control with no inhibitors present; 100% binding was 45 ± 3 ng/mg cell protein. T, tryptic; SP, staphylococcal protease; Ac T, acetylated T peptides; Ac Sp, acetylated SP peptides. For identification of fractions T-IV and SP-IV in the gel filtration column effluent, see legend to Fig. 2. Determinations for T and SP peptides were carried out in duplicate and those for Ac peptides, in triplicate.

stroyed by treatment with staphylococcal protease (Table 3) but cell reactivity did not change at all (Fig. 7). Also, trypsinization destroyed epitope 3 but cell reactivity, although diminished, was still retained. On the other hand, treatment of LDL with staphylococcal protease did not diminish its immunoreactivity towards antibody 2, nor its cellular reactivity. Also trypsinized LDL retained its immunoreactivity with antibody 2 but cell reactivity was decreased (Fig. 7). The fact that epitope 2 and cell reactivity were spared to a different degree during trypsinization suggests that epitope 2 and the cellular binding site are not identical.

Although SP-LDL and T-LDL retained (or seemingly somewhat increased) their immunoreactivity with antibody 2, partial removal of this epitope must have occurred since some cleavage peptide fractions reacted with antibody 2 (Table 4). Similarly, despite complete retention of cell reactivity by SP cores, some SP peptides reacted with fibroblasts (Table 5) indicating loss of cellular binding sites from LDL during digestion. These findings of retained immunoreactivity and cell reactivity in the face of losses of active peptides are consistent with the possibility that epitopes and cellular binding sites may occur than once in LDL particles. Provided that enzymatic digestion of all particles is uniform and completely homogeneous, they constitute evidence for the hypothesis that repeating structures occur in apoB. It is, however, also possible that cleavage sites are diversely exposed in different LDL particles resulting in heterogeneous digestion. If so, active peptides could be released from some particles but retained in others. Accordingly, the present results provide supportive but not unequivocal evidence for the repeating structure hypothesis of apolipoprotein B.

Manuscript received 18 October 1982 and in revised form 25 January 1983.

REFERENCES

1. Gotto, A. M., Jr., R. L. Jackson, J. D. Morrisett, H. J. Pownall, and J. T. Sparrow. 1976. Molecular association of lipids and proteins in plasma lipoproteins: a review. *In* Lipoprotein Metabolism. Heiner Greten, editor. Springer Verlag, Berlin. 152–157.
2. Kane, J. P., D. A. Hardman, and H. E. Paulus. 1980. Heterogeneity of apolipoprotein B: Isolation of a new species from human chylomicrons. *Proc. Natl. Acad. Sci. USA.* **77:** 2465–2469.
3. Deutsch, D. G., R. L. Heinrikson, J. Foreman, and A. M. Scanu. 1978. Studies of the cyanogen bromide fragments of the apoprotein of human serum low density lipoproteins. *Biochim. Biophys. Acta.* **529:** 342–350.
4. Tikkanen, M. J., R. Dargar, B. Pfleger, B. Gonen, J. M. Davie, and G. Schonfeld. 1982. Antigenic mapping of human low density lipoprotein with monoclonal antibodies. *J. Lipid Res.* **23:** 1032–1038.
5. Triplett, R. B., and W. R. Fisher. 1978. Proteolytic digestion in the elucidation of the structure of low density lipoprotein. *J. Lipid Res.* **19:** 478–488.
6. Chapman, M. J., S. Goldstein, and G. L. Mills. 1978. Limited tryptic digestion of human serum low-density lipoprotein: isolation and characterization of the protein-deficient particle and of its apoprotein. *Eur. J. Biochem.* **87:** 475–488.
7. Goldstein, S., and M. J. Chapman. 1979. Radioimmunological study of the surface protein of the human serum low-density lipoprotein: comparison of the native particle and the products obtained by tryptic treatment. *Biochem. Biophys. Res. Commun.* **87:** 121–127.
8. Schonfeld, G., R. S. Lees, P. K. George, and B. Pfleger. 1974. Assay of total plasma apolipoprotein B concentration in human subjects. *J. Clin. Invest.* **53:** 1458–1467.
9. Hahm, K-S. On the primary structure of pig heart aconitase. Ph.D. Dissertation. 1979. Duquesne University.
10. Drapeau, G. R. 1977. Cleavage of glutamic acid with staphylococcal protease. *Methods Enzymol.* **47:** 189–191.
11. Levine M. J., M. C. Herzberg, M. S. Levine, S. A. Ellison, M. W. Stinson, H. C. Li, and T. Van Dyke. 1978. Specificity of salivary-bacterial interactions: role of terminal sialic acid residues in the interaction of salivary glycoproteins with *Streptococcus sanguis* and *Streptococcus mutans*. *Infect. Immun.* **19:** 107–115.
12. Patsch, J. R., W. Patsch, S. Sailer, and H. Braunsteiner. 1976. Isolation and partial characterization of two abnormal human plasma lipoproteins: LP-X_1 and LP-X_2. *Biochim. Biophys. Acta.* **434:** 419–427.
13. Swaney, J. B., and K. S. Kuehl. 1976. Separation of apolipoproteins by an acylamide-gradient sodium dodecyl sulfate gel electrophoresis system. *Biochim. Biophys. Acta.* **446:** 561–565.
14. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **76:** 4350–4354.
15. Kelley, P. M., and M. J. Schlesinger. 1982. Antibodies to two major chicken heat shock proteins cross-react with similar proteins in widely divergent species. *Mol. Cell Biol.* **2:** 267–274.
16. Goldstein, J. L., S. K. Basu, G. Y. Brunschede, and M. S. Brown. 1976. Release of low density lipoprotein from its cell surface receptor by sulfated glycosaminoglycans. *Cell.* **7:** 85–95.
17. Ostlund, R. E., Jr., B. Pfleger, and G. Schonfeld. 1979. Role of microtubules in low density lipoprotein processing by cultured cells. *J. Clin. Invest.* **63:** 75–84.
18. Tikkanen, M. J., T. G. Cole, K-S. Hahm, E. S. Krul, and G. Schonfeld. 1983. Differential reactivity of apolipoprotein B in human very low density lipoprotein subfractions with monoclonal anti-LDL antibodies. Submitted for publication.
19. Basu, S. K., J. L. Goldstein, R. G. W. Anderson, and M. S. Brown. 1976. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc. Natl. Acad. Sci. USA.* **73:** 3178–3182.