Regional specificities of monoclonal anti-human apolipoprotein B antibodies

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Abstract The usefulness of monoclonal antibodies as probes of protein structure is directly related to knowledge of the structures and locations of the epitopes with which they interact. In this report we provide a detailed map of 13 epitopes on apoB-100 defined by our anti-apoB monoclonal antibodies based on current information on the amino acid sequence of apoB-100. To localize antibody specificities to smaller regions along the linear sequence of the apoB-100 molecule we used *a*) thrombin- and kallikrein-generated fragments of apoB-100; *b*) β-galactosidase– apoB fusion proteins; *c*) heparin; and *d*) antibody versus antibody competition experiments. Most of the monoclonal antibodies elicited by immunization with LDL were directed towards epitopes within the first 1279 amino terminal (T_4/K_2 fragments) or last 1292 carboxyl terminal amino acid residues (T_2/K_4 fragments) of apoB-100. One epitope localized to the mid-portion of apoB-100 was elicited by immunization with VLDL (D7.2). Saturating amounts of heparin bound to LDL did not inhibit the binding of any of the monoclonal antibodies to their respective epitopes on apoB-100, indicating that none of the antibody determinants is situated close to any of the reported heparin binding sites on LDL apoB. We examined the expression of apoB epitopes on VLDL subfractions and LDL isolated from a normolipidemic donor. The apparent affinities with which the antibodies interacted with their respective epitopes on the VLDL subfractions and LDL uniformly increased as follows: LDL > $VLDL_3$ > $VLDL_2$ > $VLDL_1$, suggesting that each of the major regions of apoB-100 is progressively more exposed as normal VLDL particles become smaller in size and epitopes are most exposed in LDL. Previous experiments utilizing hypertriglyceridemic VLDL subfractions yielded similar results, but the rank order of VLDL subfractions and LDL was not the same for all antibodies tested. Thus, differences in apoB epitope expression on VLDL particles of differing sizes is a general phenomenon, but the expression of apoB epitopes in hypertriglyceridemic VLDL appears to be more heterogeneous than is the case for VLDL from normolipidemic donors. Only those of our antibodies directed against the T_2/K_4 fragments (last 1292 carboxyl terminal amino acid residues) inhibited the binding of LDL to fibroblast apoB,E-receptors, suggesting that the recognition site for the apoB,E receptor may be confined to that region of apoB-100.—**Krul, E., Y. Kleinman, M. Kinoshita, B. Pfleger, K. Oida, A. Law, J. Scott, R. Pease, and G. Schonfeld.** Regional specificities of monoclonal anti-human apolipoprotein B antibodies. *J. Lipid Res.* 1988. **29:** 937–947.

Supplementary key words apoB,E receptor • VLDL • apoB-100

In recent years several libraries of monoclonal antibodies directed against human apoB have become available (1–6). These highly specific reagents have been useful in studies of apolipoprotein (apo)B structure and in the development of assays for quantitation of apoB concentrations in biological fluids (7). For example, monoclonal antibodies were used to demonstrate that the expressions of given apoB epitopes on VLDL density subfractions and on LDL differ from each other (8–11) and that various perturbations of the structures of the lipoproteins affect apoB epitope expression (i.e., apoB conformation) in a region-specific manner (12–14). Some of the antibodies also identify genetic variants of apoB (15–18) and others inhibit the binding of LDL cellular apoB,E receptors (6, 19, 20). Because the amino acid sequence of apoB was unknown until recently, it was impossible to relate any of the epitopes defined by the monoclonal antibodies to specific structural regions of apoB.

Recently, the amino acid sequence of apoB-100 was deduced from the nucleotide sequence of its mRNA (21–23) leading us to attempt to map the epitopes defined by our antibodies in relation to known sequences. We used immunoblots of kallikrein- and thrombin-generated fragments of apoB (24, 25) and antibody versus antibody competition assays (6) for initial assignment of epitopes along the linear sequence of apoB-100. The cDNA probes used for mRNA sequencing, when inserted in β-galactosidase (lac z) gene-containing vectors and transformed into suitable bacterial hosts, express β-galactosidase-apoB fusion proteins containing relatively short linear segments of apoB-100 with

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoprotein; $VLDL_1$, S_f 120–400; $VLDL_2$, S_f 60–120; $VLDL_3$, S_f 20–60; LDL, low density lipoprotein; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PBS, 0.01 M phosphate-buffered saline; HRH, high-reactive heparin; FPLC, fast protein liquid chromatography; ITPG, isopropyl β-D-thiogalactopyrososide; TMU, tetramethylurea.

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amino acid sequences that can be deduced from the nucleotide sequences of the cDNA inserts (21). Immunoblots using these fusion proteins and our monoclonal antibodies have enabled us to map the epitope specificities of some of our monoclonal anti-apoB antibodies to the shorter segments of apoB-100. Several heparin binding regions on apoB-100 have been identified very recently (26–28); we also assessed whether any of our antibodies are directed against heparin binding regions. In addition to the antibodies we have reported on previously (6), we have recently produced several new monoclonal anti-apoB antibodies. In this study we report on the regional specificities of our previous and also our new anti-apoB antibodies. We assessed the expression of epitopes defined by our new antibodies on VLDL subfractions and LDL, and also examined the abilities of the newer antibodies to inhibit the binding of LDL to cellular receptors of cultured fibroblasts.

METHODS

Materials

D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) was obtained from Calbiochem-Behring Co. (San Diego, CA); polybrene, benzamidine, aprotinin, phenylmethylsulfonyl fluoride (PMSF), lima bean and soybean trypsin inhibitors, human plasma kallikrein (16 units/mg), human plasma thrombin (≈2000 NIH units/mg), fluoresceinamine and bovine serum albumin were obtained from Sigma (St. Louis, MO); sodium [^{125}]iodide was obtained from Amersham Corp. (Arlington Heights, IL); electrophoresis grade sodium dodecyl sulfate (SDS) was obtained from Bio-Rad (Richmond, CA).

Isolation of LDL

Blood from normolipidemic fasting donors (triglycerides < 100 mg/dl and cholesterol < 180 mg/dl) was collected by venipuncture into 50-ml plastic centrifuge tubes containing EDTA (0.5% w/v), polybrene (25 μg/ml), benzamidine (2 mM), aprotinin (100 kallikrein-inhibitory units/ml), PPACK (1 μM), lima bean and soybean trypsin inhibitors (20 μg/ml), NaN_3 (0.01%), and PMSF (0.5 mM) according to the protocol of Cardin et al. (24). Plasma was obtained by centrifugation. VLDL were separated by ultracentrifugation at a density of 1.006 g/ml. The density of the infranatant was adjusted to 1.050 g/ml and was re-spun to isolate LDL. LDL was washed by a second spin at 1.050 g/ml and pelleted by a third spin at 1.030 g/ml. The LDL was then dialyzed against EDTA–saline, pH 8.2, and used promptly for thrombin and kallikrein digestions. For some experiments, VLDL subfractions and LDL were isolated by zonal ultracentrifugation from a normal donor (29).

Digestion of LDL B-100 with thrombin and kallikrein

Enzymic digests were performed at 37°C with 150 μg of LDL B-100 protein in 10 mM Tris-HCl, pH 8.0, at an enzyme–apoB-100 protein ratio of 1:30 in a total volume of 150–500 μl. Digests were incubated for 8–18 hr. Digestions were terminated by the addition of electrophoresis sample buffer consisting of 5% (w/v) SDS, 40% (w/v) sucrose, 10% (v/v) β-mercaptoethanol, and 0.01% (w/v) bromophenol blue.

Fusion proteins

Lysates of transformed *E. coli* containing cDNA inserts of apoB-100 gene fragments were generous gifts from Dr. S. O. Olofsson (Gothenberg, Sweden, here called group I) and Dr. J. Scott (London, England, here called group II). Characterizations of these and other clones have been described elsewhere (21, 30). Lysates were prepared as follows. *E. coli* were grown in Luria-Bertani (LB) broth containing 100 μg/ml ampicillin and 250 μg/ml ITPG at 37°C overnight. Bacteria were pelleted by centrifugation and lysed by sonication for 60 sec at 30 watts in 5% (w/v) SDS. Lysates were transferred to microfuge tubes, centrifuged at 12,000 rpm at 23°C for 15 min. Supernates (30 μl) plus 6 μl of 5-times concentrated loading buffer were boiled together for 2 min and applied to 7.5% polyacrylamide gels (containing 0.1% (w/v) SDS) for electrophoresis as described below.

Gel electrophoresis and immunoblotting

Separation of apoB-100 fragments after enzymic digestion was performed by electrophoresis on 3–6% polyacrylamide gels containing 0.1 M phosphate and 0.025% (w/v) SDS (31). Electrophoresis was carried out in 0.1 M phosphate buffer, 0.1% SDS, pH 7.2, at a current of 22 mA/1.5 mm × 15 cm × 15 cm slab gel for 20 hr. Ten μg of LDL protein was applied per gel lane. For *E. coli* fusion proteins, electrophoresis was carried out in 0.05 M Tris, 0.38 M glycine, and 0.1% SDS at a current of 30–40 mA for 5 to 6 hr. Ten to 20 μg of lysate protein was applied per gel lane.

For immunoblotting, proteins were transferred from gels onto nitrocellulose electrophoretically using 25 mM phosphate buffer, pH 6.5, and dried as described (13). The dried nitrocellulose transfers were incubated for 1 hr at 23°C with 3% BSA in phosphate-buffered saline (PBS), pH 7.4. The buffer was then replaced with a solution containing 10 μg/ml monoclonal antibody (purified on the FPLC Mono Q^R column (Pharmacia)) in PBS containing 3% BSA and 3% nonimmune serum. The transfers were incubated at 23°C with this solution overnight. Transfers were then washed with PBS and incubated with a solution containing 750,000 cpm/ml of ^{125}I-labeled goat anti-mouse IgG in 3% BSA-PBS with 3% nonimmune serum. These incubations were carried out for 4 hr at 23°C. Immunoblots

were then washed extensively with PBS followed by a final wash in 0.02% Tween-20 in PBS. For immunoblots of the fusion proteins, washes with 0.1%-Nonidet P40-PBS were performed after the blocking step and after antibody incubations in addition to the washes with PBS. Autoradiography was carried out by exposing the immunoblots to Kodak XAR-5 film at −70°C.

Production of antibodies, antibody versus antibody competition assay, cell cultures

Production and partial characterization of the apoB monoclonal antibodies have been described previously (6). Antibody versus antibody competition assays were performed using immobilized LDL as antigen, purified ^{125}I-labeled monoclonal antibodies as tracers, and unlabeled purified monoclonal antibodies as competitors. Interactions of ^{125}I-labeled LDL with cultured fibroblasts were carried out as described (6).

Purification of high reactive heparin (HRH), binding of HRH to LDL, inhibition by HRH of antibody binding to LDL

HRH was isolated from bovine lung heparin (Hepar Industries, Incorporated, Franklin, OH) by affinity chromatography on LDL columns as described by Cardin et al. (27) with the exception that CNBr-activated Sepharose 4B (Pharmacia) instead of Affi-Gel 10 was used for preparation of the column.

HRH was radiolabeled using Iodo-Beads (Pierce, Rockford, IL) after coupling with fluoresceinamine as described by Smith and Knauer (32). Binding of HRH to LDL was quantified using 96-well microtiter plates. LDL (150 μl containing 10 μg of LDL protein/ml) was loaded into each well and incubated overnight at 4°C. After washing the wells with PBS, various amounts of ^{125}I-labeled HRH in 10 mM Tris-Cl (pH 8.0) 10 mM CaCl were added to each well and incubated for 3 hr at room temperature. After incubation, each well was washed and the radioactivity was counted in a gamma counter.

Competitive inhibition binding assays between HRH and the apoB monoclonal antibodies also were performed in 96-well microtiter plates. LDL-coated wells (1.5 μg/well as above) were incubated with HRH (250 ng of uronic acid)/well. After incubation for 1 hr at room temperature, the unbound HRH was removed by washing the wells and anti-apoB monoclonal antibodies, 0.4-1.4 μg/well, were added to each well and incubated overnight at room temperature. The bound antibodies were detected using ^{125}I-labeled goat anti-mouse IgG antibody. ^{125}I-labeled LDL was used to assess the amount of LDL immobilized in the wells. Of the 1.5 μg of LDL protein added, 0.1 μg was bound. Direct binding assays of antibodies to partially purified CNBr peptide mixtures of apoB-100 known to contain HRH-binding activity also were performed. Sephacel DEAE-unbound and MonoS-bound and -unbound peptides prepared from CNBr digests of apoB-100 (26) (kindly sent by Dr. Alan Cardin) were immobilized on microtiter plates. Wells were washed and blocked with 3% BSA-PBS after which monoclonal antibodies were added in 1% BSA-PBS and the binding of antibodies was detected with ^{125}I-labeled goat anti-mouse IgG antibodies.

Other methods

Protein was determined by the method of Lowry et al. (33) using bovine serum albumin as standard. Uronic acid was measured according to the procedure of Bitter and Muir (34).

RESULTS

The antibody library representing 15 stable hybridomas, the immunogens used in their production, and the antibody heavy chain isotypes are given in **Table 1**. Antibodies described in previous publications (6, 8, 11) are distinguished from more recently developed antibodies by a superscript b. All antibodies were IgGs. Note that the full length identifying numbers are used only in Table 1; shorter numbers are used in the text, figures, and the rest of the tables.

In antibody versus antibody competition assays (**Table 2**), the antibodies yielded nine distinctive non-cross-competing groups, identified by Roman numerals in Table 1. To compare the specificities of some of our antibodies with those of others, additional antibody versus antibody and competition assays were performed using monoclonal anti-apoB antibodies MB24 and MB47 obtained from Dr. Linda Curtiss (19). MB24 is reported to be directed against an epitope on the T_4/K_2 fragment of apoB-100; the MB47 epitope is on the T_2/K_4 fragment and inhibits the binding of ^{125}I-labeled LDL to the apoB,E-receptor on fibroblasts. MB47 partially cross-competed against our antibodies B1B3 and B1B6, both of which also are assigned to the T_2/K_4 fragment (see below) and also inhibit ^{125}I-labeled LDL binding to fibroblasts (6). MB24 did not compete against either MB47, B1B3, or B1B6.

The amino acid and nucleotide base maps of apoB-100 are shown in **Fig. 1**. Sites of thrombin cleavage are indicated as T_4/T_3 and T_3/T_2, giving rise to T_2, T_3, and T_4 fragments. Cardin et al. (24) and Knott et al. (21) have established the following correspondence between kallikrein and thrombin fragments: $K_2 \approx T_4$, $K_3 \approx T_3$, $K_4 \approx T_2$. The T_1 and K_1 fragments referred to in **Table 3** and **Fig. 2** (not shown in Fig. 1) correspond to $T_4 + T_3$ and $K_3 + K_4$, respectively. Representative immunoblots of T fragments are provided in Fig. 2 and summarized in Table 3. Eight of our antibodies (C1.1, C1.4, C3A10, BD1.1, C3D1, CC3.4, D3D5, and C4D1) were bound to the K_2 and/or T_4 NH_2-terminal fragments. One antibody (D4B10) reacted only

TABLE 1. Panel of anti-human apoB monoclonal antibodies

Anti-apoB Antibodies	Immunogen	Isotype	Antibody versus Antibody Competition Group
1888C1.1	VLDL	IgG1	I
1888C1.4	VLDL	IgG1	I
1363C3A10	apoB[a]	IgG1	I
1363D4B10	apoB[a]	IgG1	I
1584BD1.1	LDL	IgG1	II
465C3D1[b]	LDL	IgG1	III
1583CC3.4	LDL	IgG1	IV
1888D7.1	VLDL	IgG2b	n.d.
1888D7.2	VLDL	IgG2b	V
465D3D5[b]	LDL	IgG1	VI
457C4D1[b]	LDL	IgG1	VII
457C4D6[b]	LDL	IgG1	VII
464B1B6[b]	LDL	IgG1	VIII
464B1B3[b]	LDL	IgG1	VIII
465B6C3[b]	LDL	IgG1	IX

[a]Delipidated apoB isolated by heparin-Sepharose chromatography.
[b]Antibodies previously reported (6).

with the T_1 fragment, one (D7.2) reacted with K_3 and T_3, and three (B1B6, B1B3, and B6C3) reacted with K_4 and T_2. Antibody D7.2 reacted with apoB-48 but not with the abnormal apoB-37 (35).

The restriction map and the location of the cDNA probes directing the transcription of the β-galactosidase-apoB-100 group I fusion proteins (identified as 2, 17, 10, and 3) and group II fusion proteins (PH1-N5, a and b) along the linear amino acid and mRNA base sequences are shown in Fig. 1. All lysates contained prominent fusion proteins that were larger than β-galactosidase and smaller than apoB-100 (**Fig. 3**) as determined by SDS polyacrylamide gel electrophoresis. Clones a and b expressed fusion proteins that did not contain the full β-galactosidase protein (fusion protein molecular weights are approximately 110 and 120 kDa for clones a and b, respectively). Only numbers 10 and 3 of the group I fusion proteins reacted with any of our antibodies (not shown). Representative blots with the group II fusion proteins are given in Fig. 3 and **Fig. 4**. Table 3 and Fig. 1 summarize the findings of the immunoblot experiments using the thrombin and kallikrein fragments and the fusion proteins. Note that in most cases binding to thrombin and kallikrein fragments and to one or more fusion proteins produced consistent results, mutually confirming the assignments of epitopes. Such inconsistencies as there were (e.g., B1B3 binding to fusion proteins Y, 10, a, and b but

TABLE 2. Results of antibody versus antibody competition assays

	^{125}I-Labeled Antibody											
Unlabeled Ab	C1.4	C3A10	D4B10	C3D1	CC34	D7.2	D3D5	C4D1	B1B6	B1B3	B6C3	MB47	MB24
C1.1, C1.4	+ +	+	+ +		−	−							
C3A10	−	+ +	±		−	−			−				
D4B10	±	±	+ +		−	−			−				
BD1.1	−	−	−		−	−							
C3D1	−	−	−	+ +		−	−	−	−		−		
CC34	−	−	−		+ +	−							
D7.2	−	−	−		−	+ +							
D3D5	−	−	−	−	−	−	+ +	±	−		−		
C4D1	−	−	−	−	−	−	−	+ +	−		−		
C4D6				−			−	+	−	−	−		
B1B6	−	−	−	−	−	−	−	−	+ +	+ +	−	±	−
B1B3	−	−	−	−	−	−	−	−	+ +	+ +	−	+	−
B6C3	−	−	−	−	−	−	−	−	−		+ +		
MB47									+ +	+ +		+ +	−
MB24									−	−		−	+ +

Microtiter plates were coated with LDL from a normolipidemic donor. Increasing doses of competitor antibody (indicated vertically) were added to the wells (1–100,000 ng/ml) with a constant amount of ^{125}I-labeled monoclonal antibody (indicated horizontally). Symbols are as follows: − , no competition at doses tested; ± , competition at high doses (dose to achieve 50% inhibition > 10 times dose of homologous antibody); + , competition at moderately high doses (dose to achieve 50% inhibition 2–10 times dose of homologous antibody); + + , competition at doses equivalent to those of homologous antibody.

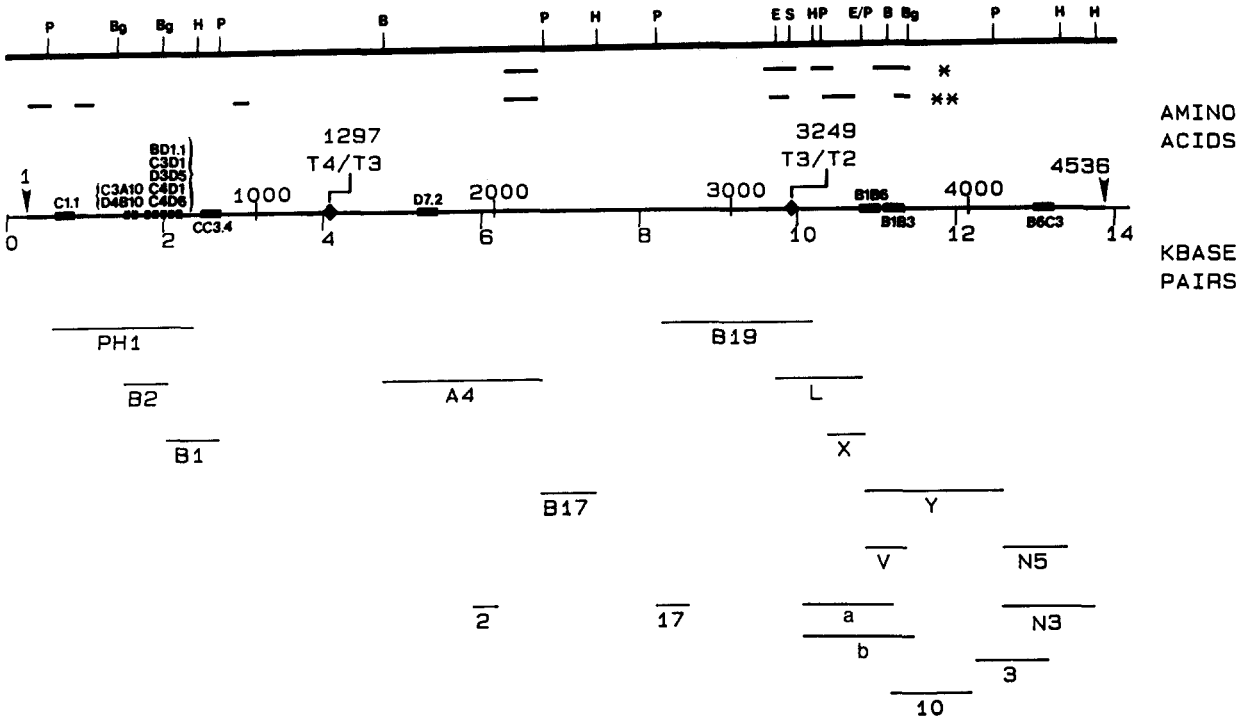

Fig. 1. Linear map of apoB-100 and assignments of epitopes defined by the designated monoclonal anti-apoB-100 antibodies. Kilobase pairs starting at the 5' terminus of apoB-100 mRNA and amino acids starting at the corresponding NH_2 terminus of the mature apoB-100 protein are indicated. The amino acid residues corresponding to the major thrombin cleavage sites on apoB-100 (T_4/T_3) and (T_3/T_2) are indicated by the diamonds. Regions of apoB-100 coded for by the fusion proteins employed in this study are indicated below the apoB-100 chain. Heparin binding sequences of apoB-100 are indicated by the bars above the apoB-100 chain. *, Heparin binding sequences reported by Hirose et al. (26); **, heparin binding sequences reported by Weisgraber et al. (28). A partial restriction map of apoB-100 cDNA (indicating those sites giving rise to the indicated fusion proteins) is shown at the extreme top of the figure. Abbreviations: P, PstI; Bg, BglI; H, HindIII; B, BamHI; E, EcoRI; S, SstI. Epitope assignments of the anti-apoB-100 monoclonal antibodies (identified by their abbreviated numbers) are indicated by boxes along the linear map of apoB-100. For those epitopes clustered together, antibodies are listed in approximate $NH_2 \rightarrow COOH$ order from top to bottom.

not to V) may be due to an inappropriate conformation of the epitope on an inert protein or the location only of part of the epitope on any inert proteins. The assignments of epitopes in Fig. 1, in addition to employing the results of immunoblotting, also took into account the results of antibody versus antibody competitive immunoassays.

To assess whether any antibodies are directed towards one or more of the heparin binding regions of apoB-100,

TABLE 3. Immunologic reactivities of monoclonal anti-apoB-100 antibodies with apoB-100 fragments and *E. coli* fusion proteins

	Fragments								Fusion Proteins																		
	Kallikrein				Thrombin																						
Antibody	K_1	K_2	K_3	K_4	T_1	T_2	T_3	T_4	PH1	B2	B1	BX148	A4	2*	B17	17*	B19	L	a b	X	V	10*	Y	3*	N5	N3
C1.1, C1.4	+	+	−	−	+	−	−	+	+	−	−	−	−	~	−	−	−	−		−	−	−	−	−	−	−
C3A10	+	+	−	±	+	±	−	+	+	+	−	−	−	~	−	−	−	−		−	−	−	−	−	−	−
D4B10	+	−	−	−	±	−	−	−	+	+	−	−	−	~	−	−	−	−		−	−	−	−	−	−	−
BD1.1	−	+	−	−	−	−	−	+	+	+	−	−	−	~	−	−	−	−		−	−	−	−	−	−	−
C3D1									+	+	−	−	−	~	−	−	−	−		−	−	−	−	−	−	−
CC34	+	+	−	−	±	−	−	+	−	−	+	−	−	~	−	−	−	−		−	−	−	−	−	−	−
D7.1					+	−	−	−																		
D7.2	+	−	+	−	+	−	+	−	−	−	−	−	+	~	−	−	−	−		−	−	−	−	−	−	−
D3D5	+	+	−	−	+	−	−	+	+	+	−	−	−	~	−	−	−	−		−	−	−	−	−	−	−
C4D1	−	+	−	−	+	−	−	+	+	+	±	−	−	~	−	−	−	−		−	−	−	−	−	−	−
C4D6	−	−	−	−	−	−	−	+																		
B1B6	−	−	−	+	−	+	−	−	−	−	−	−	−	~	−	−	−	−	+ +	−	−	−	−	−	−	−
B1B3	−	−	−	+	−	+	−	−	−	−	−	−	−	~	−	−	−	−	+ +	−	−	−	+	+	−	−
B6C3	+	−	−	+	+	+	−	−	−	−	−	−	−	~	−	−	−	−		−	−	−	−	+	+	+

ApoB-100 fragments and *E. coli* β-galactosidase-apoB-100 fusion proteins were separated on SDS-polyacrylamide gels. Proteins were electrotransferred to replicate strips of nitrocellulose which were incubated with the indicated monoclonal antibodies as described in Methods. Reactions were visualized after incubation with ^{125}I-labeled goat anti-mouse IgG, by autoradiography.

*These fusion proteins were a generous gift from Dr. Sven Oloffson of Gothenborg, Sweden.

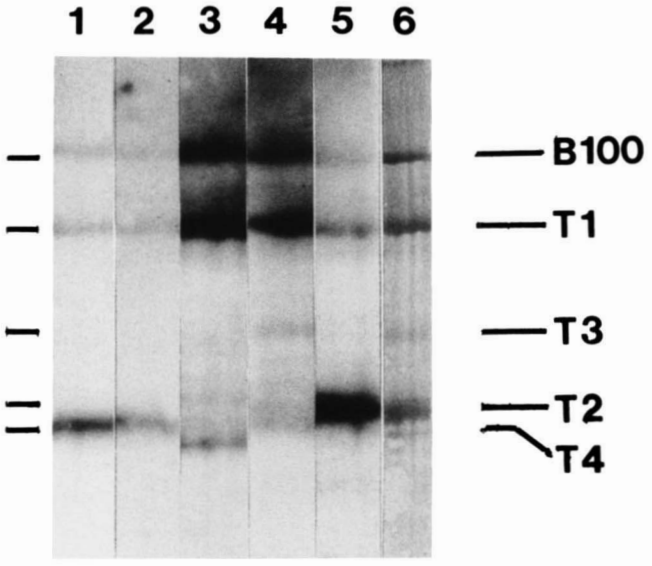

Fig. 2. Immunoblots of thrombin and kallikrein fragments of LDL-apoB-100. LDL (d 1.025–1.050 g/ml) was treated with thrombin and kallikrein as described in Methods. The resulting digest was electrophoresed on SDS-polyacrylamide (3–6% gradient) and proteins were transferred to nitrocellulose paper. Replicate nitrocellulose strips were incubated with the anti-human LDL monoclonal antibodies (1–12). Binding of antibodies was visualized, after incubation with ^{125}I-labeled goat anti-mouse IgG, by autoradiography. Lanes indicated by numerals were incubated with the following anti-apoB monoclonal antibodies: 1, C4D1; 2, D3D5; 3, D7.1; 4, D7.2; 5, B1B3; 6, Amido Black-stained nitrocellulose strip.

we investigated the inhibitory effects of HRH bound to LDL on the binding of antibodies to LDL. HRH was isolated by LDL-affinity chromatography, derivatized with fluoresceinamine and radioiodinated. Using ^{125}I-labeled HRH of known specific radioactivity (3564 cpm/ng of uronic acid) we ascertained the amount of HRH needed to saturate the binding sites of the LDL-protein coating the microtiter wells. Under these conditions, the addition of 250 ng of HRH (as uronic acid)/well resulted in the binding of 4 ng of uronic acid at maximum. Since 0.1 μg of LDL protein was bound per well (above), it was calculated that ≈9 moles of HRH (mol wt ≈13,000) were bound per mole of LDL protein (mol wt = 512,000), concordant with the results of Cardin et al. (Fig. 6 in Ref. 27). Having ascertained how much HRH was needed to saturate the LDL binding sites, we proceeded to assess whether HRH inhibited the binding of any of our antibodies to LDL. Sufficient amounts of HRH (250 ng of uronic acid) to saturate LDL heparin binding sites were added to 0.1 μg of immobilized LDL in microtiter wells as above, followed by 0.4 to 1.4 μg/well of purified monoclonal antibodies. HRH was omitted in control wells. The quantity of antibodies bound to LDL was determined with a ^{125}I-labeled goat anti-mouse IgG antibody (**Table 4**). None of the anti-apoB antibodies was inhibited from binding to LDL by HRH.

These results were confirmed in direct binding assays where CNBr fragments of apoB-100, containing HRH

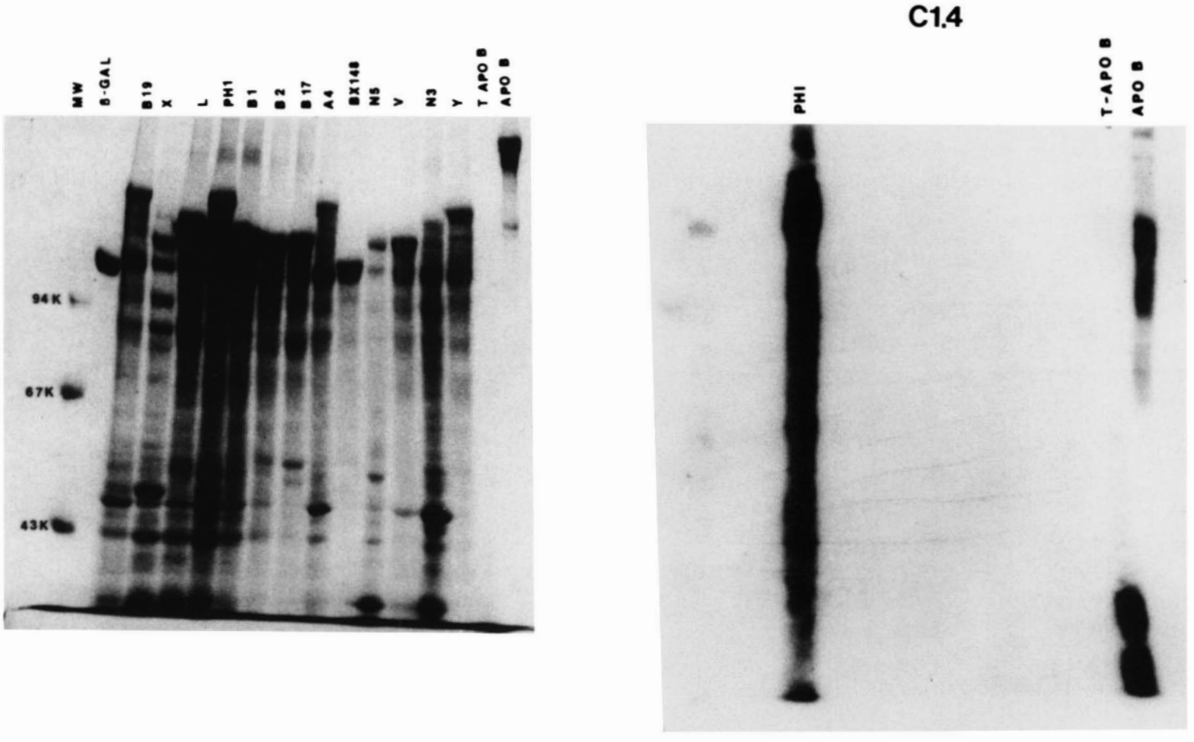

Fig. 3. Immunoreactivities of β-galactosidase group II apoB-100 fusion proteins. *E. coli* lysates were electrophoresed in 7.5% polyacrylamide SDS-gels and stained with Coomassie Blue. Transfer blots of proteins were prepared and incubated with antibody C1.4. Positive reaction is limited to fusion protein PH1, trypsin-digested LDL (T-apoB), and apoB. MW, Molecular weight markers; β-gal, purified β-galactosidase.

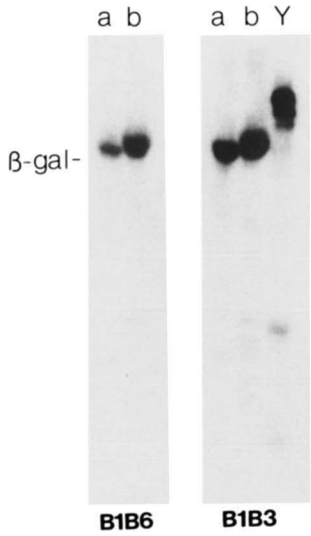

Fig. 4. Immunoreactivities of group II fusion proteins. A 7.5% polyacrylamide SDS-gel, containing *E. coli* proteins, a, b, and y, was run as indicated and proteins were transferred to nitrocellulose paper. The transfers were incubated with antibodies B1B3 and B1B6 and localization of antibodies was detected by autoradiography. Position of β-galactosidase is indicated.

binding activity were immobilized in microtiter wells. Monoclonal antibodies were incubated in the wells and the amount of binding of antibodies was quantified with ^{125}I-labeled goat anti-mouse IgG antibody (**Table 5**). Immobilized LDL was used as a positive control. Only antibody CC3.4 was significantly bound by any of the apoB-100 CNBr fragments. The DEAE-Sephacel-unbound and the Mono S-bound fractions contained HRH binding activity, while the Mono S-unbound fraction did not (26). There were no significant differences between the Mono S-bound and -unbound fractions for any of the antibodies except for antibody CC3.4, suggesting that none of the antibodies (except perhaps antibody CC3.4) was directed against heparin binding regions.

Because of an apparent discrepancy between direct binding and inhibition assay results with antibody CC3.4, the inhibition experiment with HRH and insolubilized LDL was repeated with the same and with double the mass of CC3.4 antibody. Similar results were obtained (not shown). The DEAE-Sephacel and Mono S fractions were applied to SDS polyacrylamide gels for electrophoresis, were then electrotransferred to nitrocellulose strips which were then incubated with antibody CC3.4 (not shown). Strong reactions were obtained with two peptides of 30–40 kDa and three peptides of 12–16 kDa that were minor components of the column fractions as determined by Coomassie Blue staining. No reaction was obtained with the major $\approx$20 kDa peptides that were reported to be the HRH binding peptides by Hirose et al. (26). The DEAE-Sephacel and Mono S fractions, in addition to containing HRH binding peptides, also contain several peptides that do not bind HRH (26). Based on the two HRH-antibody inhibition assays and on immunoblotting of the DEAE-Sephacel and Mono S fractions, it was concluded that antibody CC3.4 was not recognizing an epitope close to a heparin binding region. Rather, it was binding to one or more of the contaminating peptides that did not bind heparin.

We next explored which of our antibodies defined epitopes near the LDL receptor recognition region of apoB-100. None of the antibodies with epitopes located on the T_4/K_2 or T_3/K_3 fragments was able effectively to inhibit the interaction of LDL with its receptors on cultured fibroblasts (not shown). Only two antibodies, B1B3 and B1B6, both directed against the T_2 region were inhibitory. Antibody B6C3 also directed against a T_2 epitope did not inhibit.

Finally, we studied the expression of the monoclonal antibody epitopes on apoB-containing lipoproteins of various sizes isolated from a normolipidemic donor obtained by rate zonal ultracentrifugation. ApoB epitope expression of zonal VLDL and LDL subfractions was assessed in competitive assays using immobilized antibodies, ^{125}I-labeled LDL as tracer, and LDL and $VLDL_1$, $VLDL_2$, and $VLDL_3$ subfractions as competitors (**Table 6**). As in our previous reports on hypertriglyceridemic VLDL subfractions (11), epitopes of apoB on VLDL subfractions showed heter-

TABLE 4. Lack of inhibition of binding by heparin of monoclonal anti-LDL antibodies

Antibodies	Binding of Anti-LDL to LDL	
	Without HRH	With HRH
	cpm	
C1.1 (371 μg/ml)	29749	30973
C1.4 (270 μg/ml)	30892	31980
C3A10 (1262 μg/ml)	19495	18983
D4B10 (477 μg/ml)	12972	13129
BD1.1 (5900 μg/ml)*	3590	3491
C3D1 (280 μg/ml)	11039	10349
CC3.4 (900 μg/ml)	21203	23374
D7.1 (296 μg/ml)	30909	32403
D7.2 (728 μg/ml)	29578	32931
D3D5 (992 μg/ml)	14984	16248
C4D1 (742 μg/ml)	20712	21659
C4D6 (11380 μg/ml)*	16581	16044
B1B6 (928 μg/ml)	31847	53411
B1B3 (689 μg/ml)	31348	32355
B6C3 (854 μg/ml)	18424	18317
A5.6 (1900 μg/ml) (control)	1325	1310

All Mabs were purified using the FPLC Mono Q column, except for the two Mabs (*) which were used as $(NH_4)_2SO_4$ precipitates. Microtiter plates (96-well) were coated with 150 μl of LDL (10 μg/ml) overnight at 4°C. After washing each well, 100 μl of HRH (250 ng of uronic acid) was added to each well and incubated for 1 hr at room temperature. After 1 hr incubation, 50 μl of 100 times diluted Mabs was added to each well and incubated overnight at room temperature. After washing each well, ^{125}I-labeled anti-mouse IgG antibody was added to each well and incubated for 4 hr at room temperature. Bound ^{125}I-labeled goat anti-mouse IgG antibody was counted in a Micromedics gamma counter. A5.6 is an anti-apoA-I monoclonal antibody added as control.

TABLE 5. Reactivity of monoclonal antibodies with cyanogen bromide cleaved heparin binding peptides of apoB[a]

Antibodies	Cyanogen Bromide Peptides			LDL
	$A_{unbound}$[b, c] (DEAE Sephacel)	$B_{unbound}$[b] (Mono S)	C_{bound}[b, c] (Mono S)	
C1.4	102	104	196	41711
C3A10	0	0	38	37593
D4B10	0	30	0	22351
BD1.1	41	20	28	888
C3D1	0	0	65	7055
CC3.4	8894	1216	8875	21923
D7.2	774	172	153	45739
D3D5	70	101	48	9728
C4D1	44	90	76	12923
B1B6	0	0	69	21784
B1B3	0	0	100	25035
B6C3	290	0	9	9207
WU E-4[d]	726	204	360	14891

Microtiter wells were coated with apoB CNBr peptides or LDL (d 1.019–1.063 g/ml) (20 μg/ml protein) in PBS overnight. Plates were blocked with 3% BSA-PBS and then 10 μg/ml of purified monoclonal antibodies (150 μl) in 1% BSA-PBS was incubated in the wells at 4°C overnight. Plates were then rinsed and 150,000 cpm/well of ^{125}I-labeled goat anti-mouse IgG was added to each well and incubated for 4 hr at 23°C, after which the wells were washed, dried, and counted for radioactivity.

[a]The values represent the cpm per well (mean of duplicate determinations) after subtracting the nonspecific binding of monoclonal antibodies to wells coated with BSA alone (backgrounds were less than 700 cpm/well for all antibodies).

[b]Peptides $A_{unbound}$, $B_{unbound}$, and C_{bound} correspond to the CNBr peptides of apoB isolated by ion exchange chromatography as described by Hirose et al. (26).

[c]Heparin binding activity; $B_{unbound}$ does not have heparin binding activity (26).

[d]WU E-4 is an anti-apoE monoclonal antibody. The reactivity with LDL is due to the presence of trace amounts of apoE.

ogeneity of expression. In general, apoB epitopes were most expressed in LDL (lowest ED_{50} values), next in the smallest, slowest floating $VLDL_3$, and least expressed in the largest, fastest floating $VLDL_1$ subfractions. In fact, ED_{50} values for $VLDL_1$ were not obtained with some antibodies due to the flatness of the competition curves.

DISCUSSION

The aims of the present study were: *a*) to map the epitopes of our new anti-human apoB monoclonal antibodies; *b*) to assign precisely the epitopes of our previously reported antibodies; *c*) to ascertain whether any of the antibodies

TABLE 6. Relative competitive potencies of VLDL subfractions and LDL versus ^{125}I-labeled LDL for binding to monoclonal antibodies

Antibody	ED_{50} Values			
	$VLDL_1$	$VLDL_2$	$VLDL_3$	LDL
		μg/ml apoB ± SEM		
C1.4	-[a]	5.6 ± 0.6	1.9 ± 0.3	0.9 ± 0.1
D4B10	-	27.4 ± 9.3	12.8 ± 4.4	6.6 ± 2.7
C4D1	39.5 ± 24.6	37.6 ± 5.1	39.5 ± 10.7	9.8 ± 2.5
D3D5	-	31.0 ± 2.2	13.8 ± 1.3	3.8 ± 0.2
CC3.4	-	25.6 ± 3.9	18.4 ± 2.2	3.8 ± 0.2
D7.2	18.6 ± 3.8	9.3 ± 1.9	8.5 ± 1.7	3.5 ± 0.7
B1B6	63.6 ± 9.1	19.4 ± 1.6	7.6 ± 0.7	3.2 ± 0.2
B1B3	-	20.2 ± 4.7	7.6 ± 1.8	6.2 ± 1.1
B6C3	-	37.2 ± 12.2	23.3 ± 0.7	4.6 ± 0.1

VLDL subfractions and LDL were isolated from a normolipidemic donor. Increasing doses (1–100 μg/ml apoB protein determined as TMU-insoluble protein) of these lipoproteins were added to microtiter wells (coated with the indicated monoclonal antibodies) as competitors for the binding of autologous ^{125}I-labeled LDL. Microtiter plates were processed as described in Methods, and slopes and ED_{50} values of the displacement curves produced by the competitor lipoproteins were calculated by the ALLFIT program (36). Slopes were similar for all curves produced by the individual lipoprotein species with any given antibody.

[a]-, No competition (< 10%) over range of apoB concentrations studied.

recognize protein determinants close to heparin binding sites; *d*) to assess the abilities of the antibodies to inhibit the binding of LDL to its cellular receptors and to relate epitope assignment to inhibitory capacity; and *e*) to examine the expression of epitopes on normal VLDL subfractions. VLDL from a normolipidemic donor was examined because our previous studies had employed only VLDL isolated from hypertriglyceridemic subjects (11) and the apoB-100 of the latter could contain abnormal conformations. In addition, now we were able to examine a greater number of more exactly assigned epitopes.

Antibody versus antibody competition assays were useful initially in defining nonoverlapping epitopes (Table 2). Epitopes were later assigned to the major linear regions of apoB represented by the thrombin and kallikrein fragments (Table 3). It was noted by us that some antibodies reacted with two complementary fragments of apoB-100 (i.e., T_1 and T_2), or that unexpected reactions occurred (i.e., with both T_1 and K_1). Perhaps this is due to the high degree of homology of some of the repetitive sequences that span the entire length of the apoB-100 molecule (37). Some of these sequences may manifest partial cross-reactivity with some of our antibodies. It is also possible that the LDL isolated from plasma even in the presence of protease inhibitors contained trace amounts of T_1 and/or K_1 fragments. If this is true, the T_1 bands of thrombin digests on SDS gels could have contained traces of K_1 and, conversely, the K_1 bands could have contained traces of T_1.

More precise localization within the thrombin- and kallikrein-generated fragments of apoB-100 was subsequently achieved by immunoblotting the β-galactosidase-apoB fusion proteins (Table 3 and Fig. 1). Because we employed enzymatic cleavage fragments and fusion proteins that in several cases overlapped (Fig. 1), it was possible to assign epitopes with confidence. Based on this more thorough and technically more sophisticated set of experiments, we confirmed the epitope localizations of several antibodies but we also changed the assignment of the D3D5 and C4D1 epitopes (38).

The inability of heparin (HRH) to inhibit the binding of any of the antibodies to LDL and the inability of heparin-binding fragments of apoB to bind any of our antibodies were interpreted as showing that our antibodies were not directed against any heparin binding regions. Although the data support this conclusion, perhaps they are not definitive because it is possible that in the competition experiments (Table 4) heparin (HRH) was displaced from LDL particles by the antibodies, and that the apoB heparin-binding fragments did not bind antibodies (Table 5) because the epitopes in the fragments were disrupted either due to cleavage or change in conformation upon gel electrophoresis. Any possible objection to our interpretation of the absence of antibody binding to HRH binding fragments is difficult to counter, but we believe complete displacement of HRH from LDL by antibodies was unlikely. Hirose et al. (26) reported curvilinear Scatchard plots of ^{125}I-labeled HRH binding to LDL, suggesting that there are more than one HRH binding site per LDL particle, with affinity constants ranging 1–10 μM. Results obtained in the microtiter plate assays reported here are similar. The binding of monoclonal antibodies to LDL occurs with affinity constants of the same order of magnitude (10, 39). Therefore, while the more loosely bound HRH could have been displaced from LDL by antibodies, it is unlikely that all HRH could have been displaced. Thus, the more reasonable interpretation of the competition data (Table 4) is that HRH bound to LDL did not inhibit the binding of antibodies to LDL because HRH and antibodies were binding to different sites on apoB-100.

At this stage of epitope assignment, antibody versus antibody competition assays were more sensitive in identifying distinct epitopes than the fusion proteins, which themselves are quite large. For example, the B2 clone coding for amino acids 470 → 652 (or 182 amino acids) appeared to contain at least six distinguishable epitopes. The bunching of epitopes suggests that this region is particularly antigenic. The relatively high antigenicity of this region may be due to relatively high mobility, hydrophilicity, or helicity (40). Analysis of a hydrophilicity profile employing a window of six amino acids (40) (not shown) reveals at least five distinct regions of relatively high hydrophilicity in this region of the apoB-100 molecule. Furthermore, we have previously shown (13) that the immunoreactivity of epitope C3D1 is destroyed by proteases and the reactivities at epitopes C4D1 and D3D5 are cut by ≈50% providing support for the concept that this region of apoB-100 is surface accessible. Other antigenic "hot spots" may be located near the T_3/T_2 junction, since we and others (19, 20, 41) have generated several monoclonal antibodies to this region of apoB-100 as well. The rest of the epitopes defined by our monoclonal antibodies are distributed over the remainder of the apoB-100 molecule.

There has been some controversy as to whether there are one or more LDL receptor recognition sites per apoB-100 molecule based on the identification of amino acid sequence homologies in apoB-100 with the LDL receptor recognition region of apoE. Most workers have concluded that at least one recognition region is located near the T_3/T_2 junction (near amino acid 3249). Our results are compatible with the latter conclusions since only those of our antibodies that define epitopes between amino acids 3665–3780 (antibodies B1B3 and B1B6) nearest to the T_3/T_2 junction specifically inhibited the binding of LDL to its receptor on fibroblasts. Several other antibodies produced in different laboratories (4G3, 3F5, 3A10, 5E11, MB47) also inhibit LDL-fibroblast interactions (19, 20). Assignments of the epitopes defined by all of these antibodies range from amino acids 3029 → 3780, while the putative receptor-binding region is assigned to two regions linked by S–S bonds and involving amino acids 3147–3157 and 3359–3367. The ability

of antibodies as many as 300 or more amino acids away from the putative receptor region to inhibit LDL-fibroblast interactions suggests that apoB in this region may be folded, bringing these linear regions close to each other. This postulate also is supported by the abilities of MB47 and antibody B1B3 to cross-compete (Table 2) even though their epitopes are > 160 amino acids apart (ref. 21 and Fig. 1) while the six antibodies assigned to a 181 amino acid segment of T_4 (Fig. 1) do not cross-compete.

Finally, we examined the expression of all of our apoB epitopes on VLDL subfractions and on LDL isolated from a normolipidemic donor. ED_{50}s decreased (immunoreactivity increased) as VLDL particles decreased in size, while slopes of curves changed very little. In general, these results are similar to those reported before with VLDL and LDL isolated from hypertriglyceridemic subjects (11). However, with hypertriglyceridemic VLDL there were simultaneous changes in both slopes and ED_{50}s and a uniform progression of ED_{50}s from $VLDL_1$ > $VLDL_2$ > $VLDL_3$ > LDL occurred only with some but not all antibodies. This confirms the concept that apoB-containing lipoproteins of hypertriglyceridemic subjects tend to be more highly heterogeneous than those of normal subjects (42).

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