Rat monoclonal antibodies to human apolipoprotein B: advantages and applications

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Abstract Eight monoclonal antibodies (Mabs) to human serum low density lipoprotein (LDL) were derived from the fusion of spleen cells, from LOU rats immunized with human LDL, and the rat myeloma line IR983F. These Mabs were characterized in terms of isotype, specificity, and affinity. Competitive experiments indicated that the epitopes that were recognized could be grouped into three patterns depending on their apparent affinity for apoB-containing lipoprotein particles such as LDL, very low density lipoproteins (VLDL), or intermediate density lipoproteins (IDL). Six epitopes have been mapped in relation to elements of the sequence of apolipoprotein B-100 (apoB-100) and some have been assigned to the middle part of the median thrombolytic fragment T_3, a region not yet well targeted by mouse Mabs. The presence of lipids for the expression of the epitopes was studied and confirmed a lipid dependence for epitopes that are close to the T_2/T_3 cleavage site. The capacity of binding to the LDL receptor was also tested; among the Mabs we described, one inhibited the uptake and degradation of LDL to HeLa cells receptor. Finally, some antibodies were able to precipitate LDL in gel.—**Fievet, C., C. Durieux, R. Milne, T. Delaunay, G. Agnani, H. Bazin, Y. Marcel, and J. C. Fruchart.** Rat monoclonal antibodies to human apolipoprotein B: advantages and applications. *J. Lipid Res.* 1989. **30:** 1015–1024.

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Monoclonal antibodies (Mabs) against apolipoproteins have been very useful in studies of lipoprotein structure, heterogeneity, and function (1). This has been particularly true in the case of Mabs against apolipoproteins (apo) B. Anti-apoB Mabs have been used to delineate apoB functional domains (2-4), to distinguish between different immunpenotypes (5-8), to detect changes in apoB conformation (9–11), to distinguish between apoB- and apoE-mediated receptor binding (2,3,12,13), and to isolate separately, apoB-containing lipoproteins of intestinal and hepatic origin (14). Anti-apoB Mabs have also been used in many immunoassays and their unique intramolecular specificity has allowed the development of a new format of assays (15–18).

Despite the fact that apoB is readily prepared in substantial amounts from human plasma, because of its large size and solubility properties, it has been proven to be the most difficult of the apolipoproteins for biochemical characterization. The complete primary structure of apoB has recently been deduced from the sequence of its cDNA (19–23) and the secondary structure has been predicted by computer analysis. The mature hepatic form of apoB, apoB-100, is composed of 4536 amino acids and its primary structure resembles an integral membrane protein. ApoB-48, the intestinal apoB species, is composed of the amino terminal 2251 amino acids and is apparently generated by a co- or post-transcriptional modification of the apoB-100 transcript (24). This new information should increase the value of anti-apoB Mabs as structural probes.

All of the anti-apoB Mabs developed to date are mouse immunoglobulins prepared using hybridoma techniques as originally described by Kohler and Milstein (25). Here we describe the generation of eight cloned and stable rat hybridoma lines that produce antibodies specific for human plasma apoB in large quantities. These Mabs are characterized in terms of specificity and affinity. We also tested their ability to inhibit LDL binding to its receptor and their ability to precipitate LDL in gel. Finally, we speculate on the future uses of such anti-apoB antibodies in biology and medicine.

MATERIALS AND METHODS

Isolation of apoB-containing lipoproteins

From pooled fresh normal plasma that contained antibiotics, protease inhibitors, and antioxidants (26), very

Abbreviations: apoB, apolipoprotein B; Mabs, monoclonal antibodies; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; Ig, immunoglobulin.

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low, intermediary, and low density lipoproteins (VLDL ($d < 1.006$ kg/l), IDL ($1.006 < d < 1.019$ kg/l), LDL_2 ($1.019 < d < 1.063$ kg/l), and LpB ($1.040 < d < 1.053$ kg/l) were isolated by sequential preparative ultracentrifugation (27). Chylomicrons were removed from plasma of a normal subject after ingestion of a fatty meal by flotation ($120 \times 10^3 g$-min for 2 h) through a layer of saline (0.15 M NaCl, 0.01 g/l EDTA) and washed twice by recentrifugation. The protein content of these lipoprotein fractions was determined by a modified Lowry procedure (28). Their immunoreactivity with Mabs was estimated by assaying the apoB content before and after precipitating the apoB with isopropanol (29).

Production and purification of Mabs

Female LOU (LOU/C or LOU/M) rats were immunized by three intraperitoneal injections of 100–200 μg LpB at 2-week intervals as previously described (30). Six months later, 4 days before the fusion, a booster of 100–200 μg LpB in 0.15 M NaCl was given intraperitoneally. Splenocytes were then fused with nonsecreting IR 983 F myeloma cells. A classic enzyme-linked immunosorbent assay was developed to screen for antibody production using solid-phase LDL and peroxidase-conjugated mouse monoclonal antibodies anti-rat kappa light chain (Mark 1 + Mark 3).

The production and purification protocols of rat Mabs have been published earlier (31). The immunoglobulin class of each antibody was assessed by a double immunodiffusion test (32) using mouse antibodies to immunoglobulin isotypes. Their homogeneity was confirmed by electrophoresis on agarose as described (30).

Immunoblotting technique

Chylomicrons, LDL, and VLDL were delipidated by boiling for 3 min in electrophoresis sample buffer that contained 25 g/l SDS, 640 mM beta-mercaptoethanol, 10% (v/v) glycerol, 0.2 mM bromophenol blue, and 20–50 μg of protein per 10 μl. Electrophoresis was performed in 3–6 g/l acrylamide slab gel gradients containing 1 g/l sodium dodecyl sulfate (SDS) at constant voltage (150 V) for 45 min in a vertical slab gel apparatus (70 × 80 × 0.75 mm, Mini Protean II, Bio-Rad).

After migration, the separated protein bands were transferred electrophoretically (Mini Trans Blot, Bio-Rad) at a constant current (150 mA, 1 h at 4°C) to nitrocellulose paper in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3. Remaining active binding sites on the nitrocellulose were saturated by soaking for 90 min in 20 mM Tris-HCl buffer, pH 8, 150 mM NaCl, 0.1% Tween (v/v),and 15% heat-inactivated serum (v/v). The nitrocellulose paper was then cut into strips and each strip was placed individually into a solution of each Mab in the Tris-HCl buffer–5% serum (v/v) for 1 h. After washing the strips with the same buffer, we incubated them for 1 h at room temperature in a 2000-fold dilution of peroxidase-labeled MARK 1 + MARK 3. After two washings with the Tris-HCl buffer and one final wash with phosphate-buffered isotonic saline buffer (PBS), the color was developed in the strips by staining with 4-chloro-1-naphthol (Sigma, C8890) 4 g/l in PBS, in the presence of 0.005 M H_2O_2.

Iodination of LDL

LDL were radioiodinated with $Na^{125}I$ according to a modification of McFarlane's procedure as previously described (33). The specific activity of ^{125}I-labeled LDL was 0.45 μCi/μg.

Solid phase radioimmunoassay

To determine whether the Mabs bind all forms of the antigen with the same affinity, we compared competitive displacement curves obtained with different competitors (VLDL, IDL, LDL, and two types of plasma, normo- and hypertriglyceridemic). Three partial delipidation procedures were performed on the hypertriglyceridemic sample. In the first it was treated with a lipolytic enzyme (pancreatic triglyceride lipase) (EC 3.1.1.3., Boehringer Mannheim GmbH), in the second with n-butanol–diisopropyl ether 40:60 (v/v) (34), and in the third with a detergent solution such as SDS (35). The reactivities of the native and delipidated plasma were compared as above to test the role of lipids in the expression of antigenic determinants recognized by our antibodies.

Microtiter wells were coated with 100 μl of each Mab solution (20 μg/ml of PBS) overnight at room temperature. After emptying, the wells were washed three times with PBS and then incubated with PBS containing 10 g of bovine serum albumin (BSA) per liter (PBS-BSA) for 1 h at room temperature to saturate extra binding sites on the plastic. After removal of this blocking solution, increasing amounts of each competitor (expressed as protein apoB content) were added in 50 μl PBS-BSA together with a constant amount of ^{125}I-labeled LDL (45,500 cpm containing 100 ng of protein).

After incubation overnight at room temperature, the wells were emptied and the contents were washed three times with PBS, sliced, and counted in a gamma counter. The results were plotted as logit (B/B_o = log B/B_o/ 1 – (B/B_o) versus log of the concentration of competitor added, where B and B_o are specific cpm bound in the presence and absence of competitor, respectively (36). The slopes of the displacement curves were compared by a test for heterogeneity of slopes based on the general linear models procedure (37).

Immunoprecipitation of ^{125}I-labeled LDL with monoclonal antibodies

Immunoprecipitation was carried out under equilibrium conditions using the principle of double antibody

precipitation in a fluid phase. Triplicate assays were performed in Eppendorf tubes and all dilutions were made in PBS-BSA. To 100 μl of ^{125}I-labeled LDL (containing 100 ng of protein), 100 μl of different dilutions of Mabs was added. After overnight incubation of these mixtures at 4°C, 100 μl of precipitating second antibody (rabbit anti-rat IgG(H + L), Miles Laboratories, Ref. 65-128), diluted to give a slight antibody excess, was added. Following a 2-h incubation at room temperature, 1 ml of polyethyleneglycol (PEG) 6000 solution, 3 g/l, was added and the tubes were centrifuged at 4000 *g* for 15 min.

The supernatant was removed and the pellet was counted. The maximum precipitable radioactivity (X) was determined by replacing the second antibody with trichloroacetic acid (TCA, 200 g/l). The minimum precipitable radioactivity, or zero binding control (B), was determined by replacing the Mabs dilution with PBS-BSA. Results were expressed as percentage of radioactivity bound: % ^{125}I-labeled LDL bound = X – B/TCA-B × 100, where X = mean radioactivity precipitated in the presence of a given amount of specific antibody.

Determination of affinity constants

Competition between ^{125}I-labeled LDL and LDL was used to determine the affinity constants of Mabs. Each sample contained 50 μl of serial triplicate dilutions of unlabeled LDL, 100 μl of ^{125}I-labeled LDL (100 ng of protein), and 50 μl of the Mab at the dilution required for approximately 50% maximum binding. Maximum binding in the absence of competing antigen was also determined. The tubes were incubated for 18 h at 4°C. Then, 100 μl of rabbit anti-rat IgG was added. After a further 2 h at 4°C, the assay tubes were centrifuged (4000 *g*, 15 min). The radioactivity was counted in the pellet as above. The affinity constant (K_a) of the antibodies was calculated from the formula (38): $K_a = 1/(C - L)(1 - 1.5\,b + 0.5\,b^2)$ where C is the molar concentration of competing antigen required for 50% inhibition of ^{125}I-labeled LDL binding to the antibody, L is the molar concentration of ^{125}I-labeled LDL added, and b is the maximum binding of ^{125}I-labeled LDL in the absence of competitor calculated as the proportion of radioactivity bound, B/B_0 as above. The molar concentration of LDL was calculated from the protein mass of LDL (5×10^5 g/mol).

Reactivity of Mabs with apoB thrombolytic fragments

Purified apoB thrombolytic fragments T1, T2, T3, and T4 (39) were adsorbed to plastic Removawells (Dynatech, Alexandria, VA) and then exposed sequentially to Mab and ^{125}I-labeled anti-mouse IgG (40).

Mapping of epitopes using trypsin-digested LDL

The conditions for digestion of LDL with trypsin have been described (40), and digestion was allowed to proceed for 30 min using a protein-to-enzyme ratio of 50. Peptides in tryptic digest were subjected to SDS electrophoresis with linear gradient gels of 5–15% acrylamide and then transferred to nitrocellulose paper (40). The replicas were then incubated with either Mabs and ^{125}I-labeled anti-mouse IgG or with rabbit antisera prepared against apoB synthetic peptides (41) and ^{125}I-labeled anti-rabbit IgG. The conditions for the immunological detection of protein on the nitrocellulose paper have been described (40). Autoradiography was performed on KAR-5 Kodak films with an intensifier screen (Cronex, Dupont). The limits of the tryptic fragments with respect to apoB primary structure were estimated (as in ref. 40).

Inhibition of binding of human ^{125}I-labeled LDL to HeLa LDL receptor by rat Mabs

HeLa cells Williams were grown in culture medium DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) of fetal calf serum, penicillin (100 IU/ml), and streptomycin sulfate (100 μg/ml).

Uptake and degradation of ^{125}I-labeled LDL by cells was carried out for 4 h at 37°C. HeLa cells were plated in 35-mm diameter dishes, cultured for 2 days in 10% fetal-calf serum in DMEM and for 2 days in DMEM containing 10% (v/v) lipoprotein-deficient serum (LPDS-DMEM), and then incubated for 4 h at 37°C (as detailed in the legend to Fig. 1) with either mixtures of ^{125}I- labeled LDL and unlabeled LDL or ^{125}I-labeled LDL and Mabs preincubated overnight at 4°C in LPDS-DMEM.

Immunoprecipitation in gel

Double immunodiffusion in 10 g/l Indubiose A-37 in veronal-Tris buffer, pH 9.2, ionic strength 0.05 was carried out by the technique of Ouchterlony (32).

LDL and each Mab (corresponding to about 10 μg of protein) were applied to wells in 10-μl aliquots and allowed to diffuse for 18 h at room temperature in a damp chamber.

RESULTS

Production of Mabs

From three fusions (FTh 5, 6, and 14), eight cloned hybridoma lines producing antibodies to human LDL were selected and characterized. These are listed in **Table 1**. The ascitic fluids generated from intraperitoneal growth of each cloned hybridoma line were purified by immunoaffinity chromatography. The usual recovery of ascitic fluid from one hybridoma rat was 30–50 ml. Depending on the hybridoma cells, 1–5 mg of antibody per ml of ascitic fluid could be obtained, amounting to 30–250 mg of antibody per rat.

Two of the antibodies (B_6 and BA_{11}) had an IgG2a isotype while the others were all of the IgG1 isotype. All had a kappa light chain.

TABLE 1. Characterization of the rat Mabs to human LDL (isotype, specificity, maximum binding and affinity)

Mabs	Chain Type	Specificity	Affinity Constant (K_a) for LDL[a]	Maximum Binding for LDL[a]
			l/mol × 10^8	%
B_1	IgG1, -1a	B-100, B-48, B-26	3.6 ± 0.35	85 ± 2.31
B_2	IgG1, -1a	B-100, B-74	1.4 ± 0.12	89 ± 2.57
B_3	IgG1, -1a	B-100, B-74	1.6 ± 0.13	77 ± 1.55
B_4	IgG1, -1a	B-100, B-74, B-48	4.6 ± 0.42	76 ± 2.08
B_5	IgG1, -1a	B-100, B-74, B-48	28.0 ± 3.1	69 ± 0.70
B_6	IgG2a, -1a	B-100, B-74	1.9 ± 0.2	89 ± 1.97
DA_7	IgG1, -1a	B-100, B-74	1.9 ± 0.15	99 ± 0.53
BA_{11}	IgG2a, -1a	B-100, B-74	0.8 ± 0.09	85 ± 1.27

[a]Mean of three determinations ± SD.

The isotype was determined by double immunodiffusion using mouse antibodies to immunoglobulin isotypes. The specificity for the different forms of apoB was defined by antibody blots. Maximum binding values and antibody affinity constants (K_a) for LDL were obtained from fluid-phase radioimmunoassays using a second antibody as described.

Western blot analysis

The specificity of the Mabs for the different forms of apoB was defined by antibody blots, and the data are summarized in Table 1. All bound apo B-100 and apoB-74 except for B_1, which was the only Mab to recognize apoB-26. Mabs B_4 and B_5 as well as B_1 reacted with an epitope in the region common to B-48 and B-100.

Inhibition of binding of ^{125}I-labeled LDL to the LDL receptor by Mabs

All eight Mabs were selected for studies on their ability to interfere with the binding of ^{125}I-labeled LDL to its receptor. HeLa cells were used in the study since they were defined to possess LDL receptors (42). These Mabs were purified and incubated at several concentrations with a fixed concentration of ^{125}I-labeled LDL. These mixtures were then incubated with HeLa cells at 37°C and the amount of ^{125}I-labeled LDL bound, internalized, and degraded in the presence or absence of Mab was determined (**Fig. 1 a and b**). Mab BA_{11} was the only Mab able to reduce the uptake of ^{125}I-labeled LDL (50% inhibition at a 50-fold-molar excess of Mab). The same inhibition of LDL degradation was obtained in these experimental conditions.

None of the other Mabs inhibited the binding of human ^{125}I-labeled LDL to the LDL receptor, even at high Mab/^{125}I-labeled LDL molar ratios, suggesting they were not directed to the binding site for the LDL receptor (results not shown). This is in agreement with the mapping of the epitopes detailed above.

Mapping determinants of apoB

B_2 to B_6 as well as DA_7 reacted with T1 and with the T1 secondary fragment T3, whereas B_1 reacted with T1

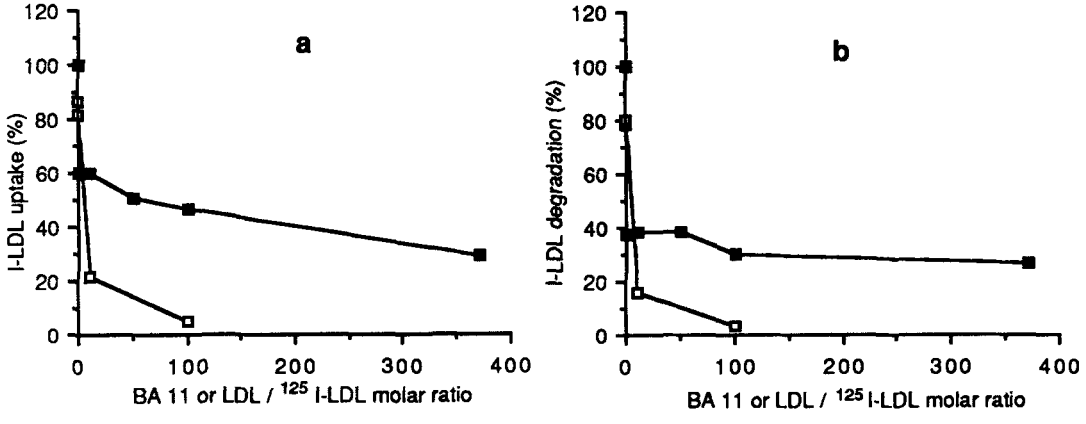

Fig. 1. Uptake (a) and degradation (b) of human ^{125}I-labeled LDL to HeLa cells LDL receptor inhibition by Mab BA11. HeLa cells were cultured and then incubated with unlabeled LDL (□) and 10 μg of ^{125}I-labeled LDL (molar ratios from 0:1 to 100:1) or with Mabs (■) and ^{125}I-labeled LDL (molar ratios from 1:1 to 370:1) for 4 h at 37°C. Bound and internalized ^{125}I was measured after solubilization of cell proteins with 0.1 N NaOH. Degradation was measured as acid-soluble noniodide catabolic products present in the medium after incubation. Results are expressed in ng of LDL bound, internalized, or degraded per mg of cell protein.

and T4. BA_{11} did not bind any of the purified and immobilized apoB thrombolytic fragments (results not shown). As can be seen in **Fig. 2** (lanes 2 and 3) B_1 reacted with an apoB tryptic fragment of about 30 kD, which also included residues 399–415. Mabs B_4 and B_5 (which compete for binding to immobilized LDL, results not shown) reacted with a fragment of about 28 kD, which was also recognized by an antiserum prepared against apoB residues 2000–2025 (lanes 4 and 5). The epitopes for B_2, B_3, and B_6 were present on fragments having apparent molecular masses of 52 and 57 kD, which include residues 2000–2025, 2301–2325, and 2481–2499 (lanes 5 to 9). The three epitopes were also on a 30 kD fragment that reacted with the antiserum against 2301–2325. With certain antibodies, a single band was observed (e.g., lanes 2 and 3) whereas for other antibodies there were numerous bands. This may have been due to differential exposure of cleavage sites in LDL and a variable recognition of the fragments depending upon the Mabs. The positions of the epitopes are shown in a theoretical linear map (**Fig. 3**). The position of the epitopes has also been confirmed by the reactivities of the Mabs with apoB-galactosidase fusion proteins produced in a bacterial expression system (R. Pease, R. Milne, W. Jessup, A. Law, P. Provost, J. C. Fruchart, R. Dean, Y. Marcel, and J. Scott, unpublished results). Mabs B_2 and B_3 reacted with two proteins including residues 2148–2375 and residues 2240–2658, respectively, thus situating the epitope position between residues 2240 and 2375. Mabs B_4 and B_5 reacted with two proteins including residues 1480–2240 and residues 1693–2148, respectively, thus localizing the epitope position between residues 1693 and 2240.

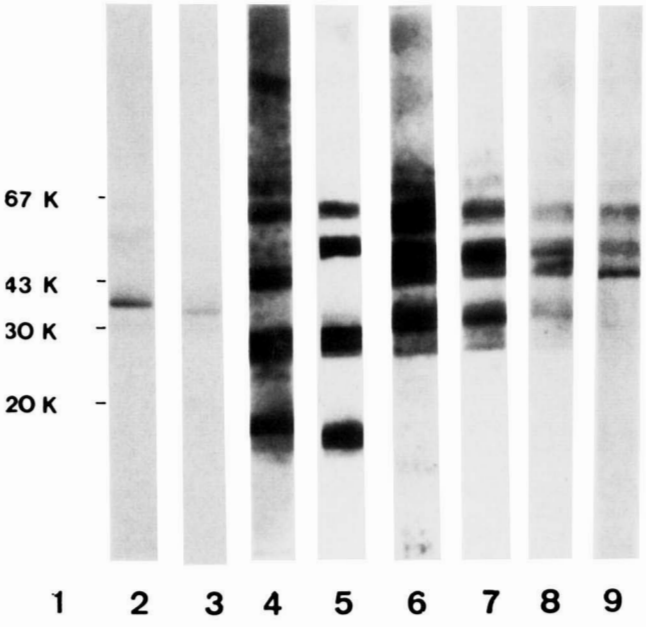

Fig. 2. Immunoblots of the 30-min tryptic digest of LDL apoB, separated by SDS-gel electrophoresis. Lane 1, ^{125}I-labeled molecular mass standards (67, 43, 30, 20 kDa); lanes 2–9, Immunoreaction with antiserum to peptide 399–415, B_1, B_5, antiserum to peptide 2000–2025, B_2, B_6, antiserum to peptide 2301–2325, and antiserum to peptide 2481–2499, respectively.

Antibody affinities and immunoprecipitation of ^{125}I-labeled LDL (Table 1)

All Mabs had affinity constants for apoB epitopes on intact LDL that ranged from 0.8 to 28 $\times$ 10^8 l/mol. The maximum amount of ^{125}I-labeled LDL that could be bound at 4°C by each of the individual Mabs varied from 69 to 99%. A complete binding of ^{125}I-labeled LDL was not observed for antibodies, suggesting that epitopes defined by some Mabs were not expressed by certain subsets of ^{125}I-labeled LDL.

Apolipoprotein B expression by LDL, VLDL, IDL, and two types of plasma (normo- and hyperlipemic)

ApoB is present, in various amounts, in plasma, VLDL, and IDL as well as LDL. Complete competition was observed with each antibody at high concentration of competitors, indicating that each of the apoB epitopes was expressed by the competitors. For each Mab, the slopes of the LDL dose titration regression lines were compared statistically to the slopes of the regression lines obtained for each heterologous competitor (**Table 2**). For a given Mab, a significant difference between two slopes indicated that the apoB epitopes recognized by this Mab, on the different apoB-containing lipoprotein particles, were not identical with the apoB epitopes expressed on LDL; and a steeper slope of the LDL regression line compared with the slopes of the competitors indicated that this epitope on LDL had a higher apparent affinity for the antibody than the epitope on the competitors.

Antibodies B_4, B_5, DA_7, and BA_{11} exhibited regression lines for the different competitors IDL and VLDL which did not differ significantly from the slopes of those for LDL, thus indicating that each of these Mabs recognized one specific apoB epitope expressed equally by all apoB-containing lipoprotein fractions we tested.

Three Mabs, B_1, B_2, and B_6, identified another category of epitope expression, exhibiting regression lines with similar slopes only for LDL and IDL. The slope of the VLDL dose titration was different and significantly lower. The epitopes recognized by theses antibodies were expressed in a similar manner only on IDL and LDL. They were also present on VLDL, but were not identical, having lower apparent affinities. The remaining antibody B_3 appeared to define a third different pattern of epitope expression and the dose titration competitive slopes for VLDL and IDL were not parallel to the slope for LDL. This indicated that the apoB epitope recognized by this Mab on VLDL and IDL was not identical with the apoB epitope expressed on LDL. The steeper slope value of the IDL regression line compared with the slope of LDL indi-

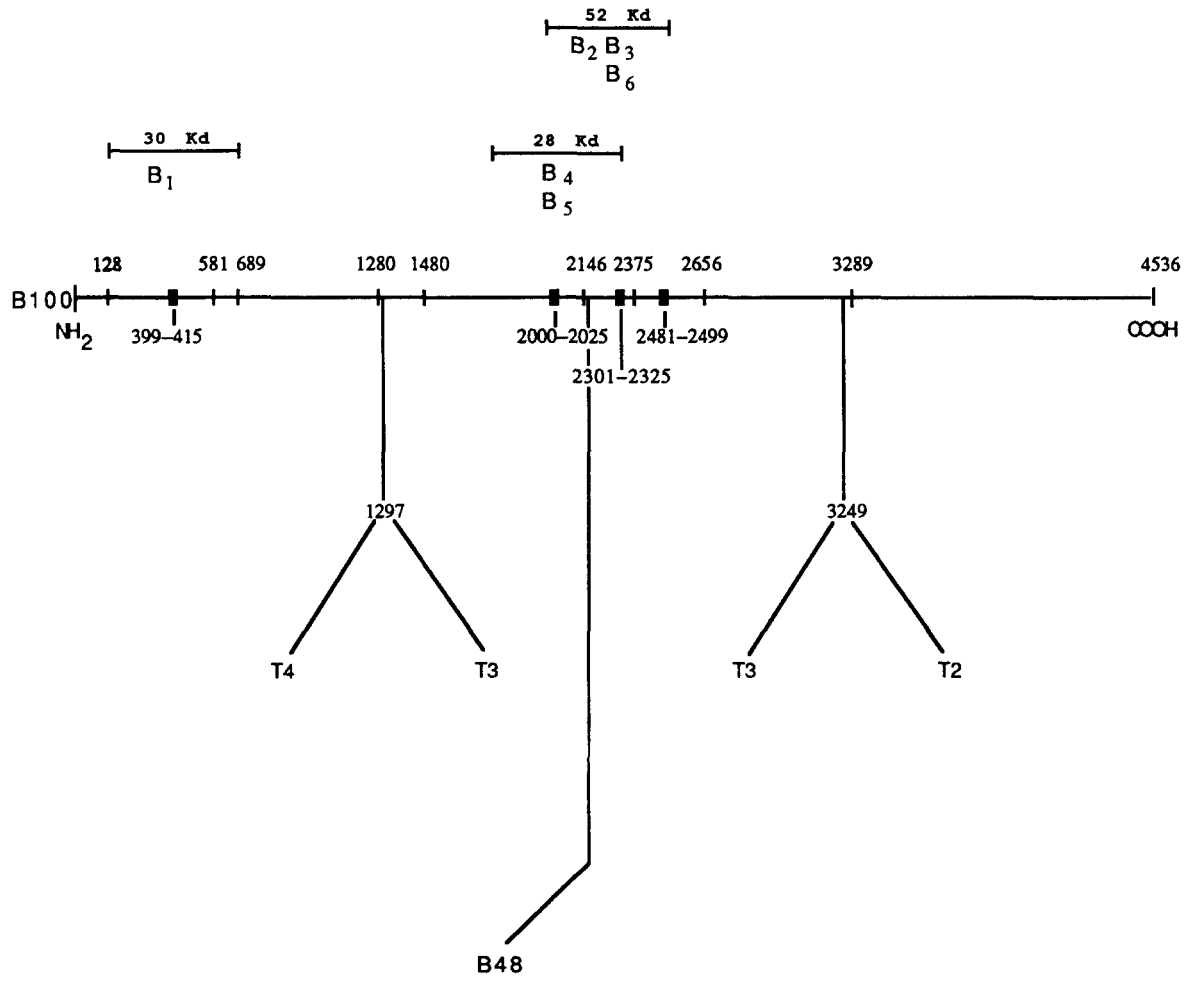

Fig. 3. Theoretical linear map of the location of epitopes on apoB-100. The boxes on the linear representation of the apoB sequence indicate the position of the partial synthetic peptides used. The numbers below the linear sequence identify the residues of the synthetic peptides and the T_4/T_3 cleavage site relative to the N-terminus and the T_3/T_2 cleavage site relative to the C-terminus. The epitope clusters recognized by groups of close Mabs are linked by a horizontal bracket.

cated that this B_3 epitope on LDL had a lower affinity for the antibody than the epitope on IDL. However, this epitope had a higher affinity for the antibody on LDL than the related epitope on VLDL.

Slopes of the purified LDL regression lines were the same as those obtained with native LDL in both types of plasma for B_3, B_5, DA_7, and BA_{11}, and so these specific epitopes were accessible in the same manner. The slight difference in the hyperlipemic plasma for B_2 might be related to the lower apparent affinity observed for this Mab in VLDL. Antibodies B_1, B_4, and B_6 showed significant different apparent affinities for their epitopes in both types of plasma and purified LDL, and these discrepancies could be explained by a preservation of the immunological properties in native LDL in plasma but not in isolated LDL.

Role of lipids in the expression of antigenic determinants

To assess any changes in immunoreactivity caused by selective removal of lipids, we treated hyperlipemic sera with delipidating agents (see Materials and Methods) and used them as competitors in competition assays. Partial delipidation with SDS or diisopropyl ether–butanol resulted in total losses of immunoreactivity for all eight Mabs. This reduction in binding may have been caused by alteration in secondary structure of apoB resulting in variable destruction of epitopes.

A more physiological type of delipidation was achieved by incubating the plasma with a lipase. The slopes of regression lines obtained with the pretreated plasma were compared to those obtained with the same untreated plasma. No statistical differences were obtained for Mabs B_1

TABLE 2. A comparison of the expression of LDL apoB epitopes on VLDL, IDL and two types of plasma

Antibody	Slope Analysis				
	Homologous Competitor	Heterologous Competitor			
	LDL	VLDL	IDL	Normolipemic Plasma	Hyperlipemic Plasma
B_1	−2	-0.75^{***}	-1.80^{NS}	-0.86^{***}	-1^{***}
B_2	−2.5	-1.76^{***}	-2.57^{NS}	-2.12^{NS}	-2.04^{*}
B_3	−2.06	-1.49^{***}	-2.30^{**}	-2.15^{NS}	-2.13^{NS}
B_4	−3.7	-2.99^{NS}	-3.43^{NS}	-2.40^{***}	-2.59^{**}
B_5	−1.94	-2.04^{NS}	-2.41^{NS}	-2.02^{NS}	-1.70^{NS}
B_6	−1.29	-0.99^{***}	-1.02^{NS}	-1.00^{***}	-1.02^{***}
DA_7	−1.88	-1.96^{NS}	-2.11^{NS}	-2.03^{NS}	-1.9^{NS}
BA_{11}	−1.91	-1.71^{NS}	-2.02^{NS}	-1.44^{NS}	-1.61^{NS}

Differences in the expression of LDL apoB epitopes on VLDL, IDL, and plasma (normo- and hyperlipemic) were analyzed by solid phase double antigen competitive radioimmunoassays. The slope of the linear regression line of the homologous competitor (LDL) was compared for each antibody with the slopes of the heterologous competitors VLDL, IDL, and the two types of plasma.

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

to B_6, suggesting that these epitopes were confined to a stable domain of apoB that was not altered by interactions with the lipids. However, after incubation of plasma with lipase, the apparent affinity of DA_7 and BA_{11} was different, and there was no competition. This may reflect a dependence of apoB conformation on the physical state of the associated lipids.

Immunoprecipitation of apoB by Mabs

Fig. 4 illustrates the immunodiffusion patterns of LDL reacting with immunoglobulins purified from ascitic fluids. A clear precipitation line was observed in the reaction of LDL with Mabs B_3, B_4, B_5, or B_6.

DISCUSSION

Many research groups have already generated and characterized murine anti-human apoB Mabs and in some cases their epitopes have been mapped (1,40,43). In the present report, we describe a new series of Mabs against human apoB. What distinguishes this panel of antibodies from previously described Mabs is that they are produced by hybridomas that are the products of a fusion of rat splenocytes and rat myeloma cells. During preparation of this paper, another group reported the production and characterization of rat anti-human apoB Mabs (44).

An obvious advantage of production of Mabs using rat hybridomas is the ease of producing large quantities of the antibody. A second potential advantage is that the antibody specificities generated in the rat may be different from those of the mouse. Both intrinsic and extrinsic factors determine the immunogenicity of a given protein (45). The intrinsic factors include structural characteristics of the antigen itself whereas, among the extrinsic factors, are the degree of cross-reactivity between the antigen and the equivalent host protein and the repertoire of the host immune response and antibody structure genes.

Based on the intramolecular specificities of the murine Mabs described to date, it would appear that the T3 segment of human apoB is poorly immunogenic for the mouse compared to T2 and T4 (43). In contrast, six of the eight rat Mabs described here recognize epitopes in T3. Moreover, based on competition studies (results not shown), these Mabs are specific for epitopes that are distinct from at least three different T3 epitopes defined by mouse Mabs (2D8, 3F5, and anti B_{sol} 4, 5, 6, and 8) (40). In the search for useful structural probes and immunoassay reagents, it is of interest to have a large choice of different antibody specificities. The use of rat Mabs may be useful in this choice.

One Mab, BA_{11}, was capable of interfering with the binding of human ^{125}I-labeled LDL to the LDL receptor. Unfortunately, it was the Mab which had a lower affinity for LDL and this can explain the partial inhibition of LDL binding to the receptor. While BA_{11} failed to react with any of the purified thrombolytic apoB fragments, it competed for binding to immobilized LDL with murine Mabs MB47, 5E11, 3A10 and 4F6 (R. Milne, R. Theolis,

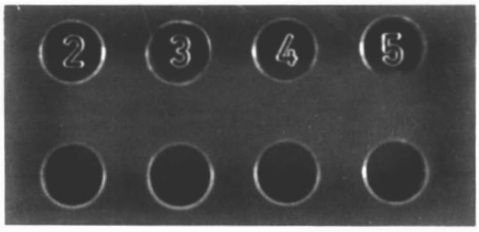

Fig. 4. Immunoprecipitation of apoB LDL by Mabs B_3, B_4, B_5, or B_6. LDL and Mabs were allowed to diffuse for 18 h in a 1% Indubiose A-37 gel. B_3, B_4, B_5, and B_6 correspond, respectively, to holes 2 to 5.

R. Maurice, R. Pease, P. Weech, E. Rassart, J. C. Fruchart, J. Scott, and Y. Marcel, unpublished observations) which also blocked LDL-receptor binding and whose epitopes have been mapped to T2 near the T2/T3 junction (19,40). It is therefore probable that the epitope of BA_{11} is also in this region. Since we can obtain very precise maps of epitopes recognized by Mabs (using fusion proteins always more numerous), such antibodies may prove useful in identifying the LDL-receptor binding site on apoB and define the minimal sequence of apoB required for binding to the receptor.

The expression of apoB epitopes is modulated by the lipid composition and/or the size and density of the lipoprotein in which the apoB is found (43, 46–48). Thus individual epitopes may be expressed differently in chylomicrons, VLDL, IDL, and LDL. This immunochemical heterogeneity due to apoB conformation is superimposed upon that which results from apoB-100/apoB-48 structural heterogeneity. Marcel et al. (9) have demonstrated that there are differences in the requirement for lipids for conformation of antigenic determinants that would appear to be a function of the position of the epitope in the apoB primary structure. Epitopes near the T4/T3 junction appear to have less stringent lipid requirements than epitopes near the putative LDL receptor-binding domain (overlapping the T3/T4 junction). It is therefore expected that the location of Mabs B_1 to B_6 in a domain of apoB that is thermodynamically stable in the presence of lipids is unaffected by the different lipids or by the size of the particle in which apoB is present. Mab BA_{11} recognizes an epitope near the putative LDL receptor-binding domain and, as for other determinants in the same region, it has stringent lipid requirements for its expression.

The existence of different subsets, even within a lipoprotein as homogeneous as LDL, is clearly indicated by the observation that not all LDL can be bound by a number of our Mabs. Maximum binding of ^{125}I-labeled LDL varied from 69 to 99% under conditions of Mab excess, which indicates that some epitopes are not expressed at the surface of all apoB-containing LDL particles. One of the Mabs, DA_7, may be considered to be a pan-LDL Mab as it bound 99% of LDL, which would indicate that its epitope is expressed on all LDL particles of density 1.019–1.063 kg/l. This was the only antibody that precipitated all of the LDL. The expression of the DA_7 epitope on other lipoprotein classes is currently being investigated to determine whether DA_7 is a true pan-apoB Mab. The other Mabs are unlikely to be useful reagents for quantifying total plasma apoB but may selectively detect subpopulations of apoB-containing particles of clinical interest. We are now testing in an immunoenzymatic assay such potential correlation with atherosclerosis.

Due to their specificity for unique epitopes on the antigen, individual Mabs are generally thought to be incapable of forming classic immune precipitates with simple antigens, i.e., antigens not composed of repetitive subunits. It was, therefore, surprising that several of the Mabs formed precipitating lines with LDL in double radial immunodiffusion. This is a novel observation. While some internal homology in apoB primary structure has been detected (49), it is questionable whether it is sufficient to produce more than one identical epitope/apoB molecule. Moreover, we saw no evidence in the mapping studies that the epitopes of the precipitating Mabs were repeated in the apoB primary structure. As apoB contains about 5% carbohydrate, these Mabs could be specific for carbohydrate chains that are present in several copies in apoB. This, however, is unlikely as these Mabs can bind apoB fusion proteins produced in bacterial expression systems where one would not expect normal glycosylation of apoB. While the theoretical basis of the immunoprecipitation by these Mabs remains to be elucidated, this characteristic may, nevertheless, enable these antibodies to be used as reagents in immunoprecipitation-based apoB assays such as immunodiffusion or electroimmunoassay.

Finally, the use of anti-lipoprotein polyclonal antibodies in therapeutic interventions to lower plasma LDL levels has been reported (50). Highly specific Mabs may represent further improvement to the approach and, because of increased yield, Mabs of rat origin have a definite advantage.

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