Conservation of the Low Density Lipoprotein Receptor-Binding Domain of Apoprotein B

Demonstration by a New Monoclonal Antibody, MB47

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The fact that low density lipoprotein (LDL) from multiple animal species binds to the human LDL receptor suggested that the LDL-receptor binding domain of apoprotein (apo) B must be evolutionarily conserved. To determine if a common receptor domain epitope existed on apo B, we generated a monoclonal antibody that was specific for the LDL-receptor domain of apo B. This was accomplished by using a screening procedure that selected for a hybridoma supernatant that could block specific cellular uptake and degradation of LDL. Western blots showed that this antibody, termed MB47, was specific for apo B-100. Fluid phase assays indicated a high binding affinity ($K_a = 4 \times 10^9 M^{-1}$) and demonstrated that all human LDL particles expressed the MB47 epitope. Scatchard analysis indicated that a maximum of one MB47 molecule bound to each LDL particle. In solid phase assays, antibody MB47 bound to plasma or LDL of multiple mammalian species, including guinea pig, rabbit, pig, dog, cat, seal, whale, bear, and lion, but it did not bind to mouse or rat LDL. In contrast, a rabbit antiserum to LDL and two other anti-apo B monoclonal antibodies, MB3 and MB19, which do not bind to the receptor domain, were specific only for human LDL. LDL from multiple species, including mouse LDL, competed effectively with $^{125}$I-human LDL for binding to human fibroblasts. MB47 effectively inhibited uptake and degradation of labeled human, guinea pig, and rabbit LDL by both human and guinea pig fibroblasts. We conclude that antibody MB47 binds to a single receptor domain on LDL and identifies a vital region conserved through mammalian evolution.

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Methods
Preparation of Human and Heterologous Lipoproteins
Fresh plasmas were obtained from humans and various animal species. Asian lion and spectacled bear plasmas were a generous gift of Kurt Benirschke, University of California, San Diego. Shark, seal, whale, and tuna plasmas were obtained from Jeff Graham and Randy Davis at Scripps Institute of Oceanography, La Jolla, California. In addition, hemolymph was isolated from a lobster.

Human and animal lipoprotein fractions were isolated at the following densities: very low density lipoprotein (VLDL) \( d < 1.006 \) g/ml; LDL, \( d = 1.025-1.050 \) g/ml; high density lipoprotein (HDL) \( d = 1.070-1.21 \) g/ml, and LDS (\( d > 1.21 \) g/ml). In some cases, human LDL was also isolated at \( d = 1.019-1.063 \) g/ml or at \( 1.045-1.065 \) g/ml. The protein concentration of plasma and each fraction was determined by the Lowry technique with use of a BSA standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the animal LDL fractions indicated that over 85% of the protein was apo B. For each LDL and VLDL fraction, estimations of apo B content were also made following precipitation of apo B with tetramethylurea or isocryl alcohol. Human apo E and apo E-DMPC liposomes were kindly supplied by Thomas Innerarity of the Gladstone Laboratories.

Modification of Low Density Lipoprotein
Methyalted, reductively glucosylated, acetylated, carbamylated, and cyclohexanedione modified LDL were prepared as described. Extensive derivation of each preparation was confirmed by a trinitrobenzene-sulfonic acid assay. The ability of native and modified LDLs to compete with native LDL for binding to MB47 was assessed in a competitive solid phase radioimmunoassay (RIA).

Monoclonal Antibody Screening and Production
Balb/C mice were immunized with a narrow density fraction of human LDL (\( d = 1.045-1.065 \) g/ml) isolated from a single donor. SDS-PAGE of the immunizing LDL revealed only apo B-100. This narrow density fraction was used because we were simultaneously attempting to develop monoclonal antibodies that selectively recognized different density fractions of LDL (data not reported here). A single fusion was performed with the P3 Ag 8.653.1 myeloma according to previously described techniques. Primary screening of hybridoma supernatants was performed by using microtiter plates coated with human LDL. We then assessed the ability of those supernatants containing anti-LDL antibodies to inhibit degradation of \( ^{125} \)I-LDL by human fibroblasts. LDL was iodinated with \( ^{125} \)I (specific activity, 200 cpm/ng) by using the iodine monochloride assay. The ability of native and modified LDLs to compete with native LDL for binding to MB47 was assessed in a competitive solid phase radioimmunoassay (RIA).

Fluid Phase Radiolmmunoassays
To determine the fraction of \( ^{125} \)I-LDL particles bound by antibody MB47, a fluid phase RIA was utilized. Two different LDL (\( d = 1.019-1.063 \) g/ml) preparations were tested, one isolated from the pooled plasma of 10 normal subjects and one isolated from the plasma of one normal subject. \( ^{125} \)I-LDL (2000 cpm/ng) prepared with the iodogen (Pierce Chemical Company) technique was >90% tri-chloroacetate acid (TCA) precipitable. It was diluted in 9% bovine serum albumin (BSA) and spun at 30,000 g for 15 minutes before each assay to remove the aggregated material. The assays were performed in 12 × 75 mm glass tubes in triplicate in 55 mM barbital buffer, pH 8, containing 150 mM NaCl, 0.02% Na Azide, 3% BSA, and 1.5 mM Na-EDTA. To 0.1 ml of \( ^{125} \)I-LDL (containing 20 ng LDL protein) was added 0.1 ml of buffer or competing antigen and 0.1 ml of increasing concentrations of immunopurified antibody MB47 diluted in the BSA-barbitol buffer. After 18 hours at 4°C, 0.1 ml of IgSORB (The Enzyme Company, Boston, Massachusetts) was added. After 2 hours, 2 ml of BSA-free barbitol buffer was added, and the tubes were immediately spun at 1500 g for 60 minutes. The precipitates were washed twice with barbitol buffer. The maximum precipitability was determined by replacing the IgSORB with 100% TCA. The minimum precipitable radioactivity
was determined in the absence of antibody MB47. The percent 125-LDL bound was then calculated as previously described.8

**Determination of Stoichiometry of MB47 Binding**

Immunopurified antibody MB47 was iodinated with 125I by the iodogen technique (specific activity, 3000 cpm/ng). Following extensive dialysis against PBS, over 95% of the radioactivity was precipitable by 10% TCA. More than 98% of the 125I-MB47 bound to an LDL column. Assays were performed in triplicate in 10 × 75 mm silicon-coated glass tubes. Increasing concentrations of 125I-MB47 in 0.1 ml of BSA-barbital buffer were added to 100 ng of pooled, normal human LDL diluted in 0.2 ml of BSA-barbital buffer. Each tube contained 182 fmol of LDL apo B (based on an apo B MW of 550,000). After incubation for 16 hours at 4°C, the LDL was quantitatively precipitated by a rabbit antiserum specific for human LDL. (Only the d > 1.21 g/ml fraction of the rabbit antiserum was used, as antibody MB47 binds rabbit apo B). Preliminary experiments established a concentration of delipidated rabbit antiserum that precipitated more than 98% of 100 ng of 125I-human LDL. After the addition of the rabbit antibody, the tubes were incubated for 16 hours at 4°C, and then spun at 1500 g for 60 minutes at 4°C. The supernatants were removed, and the pellets were washed twice with 2 ml of ice-cold barbital buffer. Nonspecific binding and precipitation were determined in two sets of parallel tubes. In the first set, no human LDL was added to the initial incubation, but the same amount of rabbit second antibody was added. In the second set of tubes, nonimmune rabbit serum (d > 1.21 g/ml fraction) was substituted for the immune rabbit serum. Both methods yielded identical values for nonspecific binding, which was linear with increasing concentrations of 125I-MB47 antibody, and in all cases was less than 1% of the total counts added. Specific 125I-MB47 binding to LDL was obtained by subtracting nonspecific binding from total binding. Binding data was analyzed by a linear regression program for Scatchard analysis of ligand binding systems, which provided an estimate of the antibody affinity constant (Ka) and the receptor or epitope concentration.28

**LDL Binding to an MB47-Sepharose 4B Column**

Antibody MB47 (50 mg, purified on a protein A column) was bound to 12 ml of activated Sepharose 4B, according to the instructions of the manufacturer (Pharmacia Fine Chemicals). Subsequent studies showed that the MB47-Sepharose 4B immunoadsorbant column bound 3 mg of LDL/ml of gel.

**Solid Phase Radioimmunoassays**

Competitive solid phase RIAs for human apo B were performed with antibody MB47, the rabbit antiserum to human LDL, and two previously characterized mouse monoclonal antibodies against human apo B, B3 and B19.8,27 In this report, B3 and B19 are termed MB3 and MB19, respectively. Polyvinyl chloride wells were coated with 0.05 ml of human LDL diluted to 10 µg/ml in PBS (pH 7.3), and incubated for 2 hours at 37°C; the remaining binding sites were blocked by coating with 5% BSA in PBS for 30 minutes at room temperature. The plates were then washed with PBS washing buffer containing 0.1% BSA, 0.01% azide, and 0.05% Tween-20. Fresh human LDL, prepared from pooled normal plasma, was used for the standard curve in dilutions ranging from 0.4 µg/ml to 97.2 µg/ml. All dilutions were made in a PBS buffer containing 3% BSA, 0.01% Na azide, and 0.05% Tween-20. The standard LDL or competitors (0.025 ml) were added to the LDL-coated wells followed by 0.025 ml of buffer containing a fixed, limiting amount of monoclonal antibody (ascites fluid) or rabbit antiserum. The optimal final concentration of antibody was determined from preliminary antibody dilution experiments and was chosen as the amount of antibody resulting in 50% of maximum binding. The plates were incubated for 18 hours at 4°C, then washed with the PBS washing buffer. Mouse or rabbit antibody binding was then quantitated by adding 0.05 ml of 125I-immunopurified goat antirabbit Ig or 125I goat antimouse Ig (40 ng/well, 8000 cpm/ng). After 4 hours of incubation at 4°C, the plates were washed and the individual wells were counted. The results were plotted as B/Bo vs log standard concentration, where B and Bo are specific counts bound in the presence and absence of competitor.

To check for the ability of rat or mouse plasma to bind antibody MB47, a solid phase assay was used in which antibody MB47 was bound to the plastic wells. Wells were coated with 0.05 ml of immunopurified antibody MB47 (10 µg/ml in PBS); the remaining binding sites were coated with 5% BSA in PBS. Competitor and human 125I-LDL (10 ng, 5000 cpm/ng) were added simultaneously to the MB47-coated wells. After 16 hours of incubation, the wells were washed, and the wells were counted.

**Studies with Cultured Fibroblasts**

Using established techniques, we tested the ability of antibody MB47 and MB47 Fab fragments to block specific LDL binding, internalization, and degradation. Studies were also performed to test the ability of antibody MB47 to inhibit binding and degradation of iodinated rabbit, guinea pig, and human LDL to both guinea pig fibroblasts and human fibroblasts. Using the same techniques, we performed studies to test the ability of human LDL and heterologous LDL samples to compete with human 125I-LDL for uptake by human fibroblasts.

**Results**

**Antibody Screening and Antibody Specificity**

Previously, Curtiss and Edgington reported the production and characterization of 11 apo B-specific monoclonal antibodies. None of these antibodies inhibited 125I-LDL uptake and degradation by cultured fibroblasts (S. G. Young and T. Innerarity, personal communications). In this study, we report a screening technique for identifying apo B-specific hybridomas that secrete antibodies with the ability to block 125I-LDL uptake by fibroblasts. From a single fusion, we identified 48 hybridoma colonies that produced antibodies that bound LDL. When directly tested for their ability to inhibit LDL uptake, several supernatants produced minor degrees of inhibition of LDL uptake (Figure 1). However, only one supernatant, No. 47, produced marked inhibition of LDL uptake and degradation. Following cloning, the specific activity of antibody MB47 was demonstrated by Western blot analysis.8 antibody MB47 bound apo B-100 of human LDL and VLDL but not to apo B-48 of chylomicrons. Western blots also showed that Antibody MB47 reacted with the high molecular weight (MW) apo B of dog, pig, guinea pig, and rabbit, but not with apo B of the mouse.
Characterization of MB47 Binding to LDL

Previous studies suggested that apo B epitopes recognized by some monoclonals are not expressed by all LDL particles. An excess of certain monoclonal antibodies does not result in complete binding of all 125I-LDL particles in a fluid phase RIA. To test whether the epitope(s) recognized by antibody MB47 was uniformly expressed by all 125I-LDL particles, we assayed the ability of immunopurified MB47 to bind 125I-LDL in a fluid phase RIA (Figure 2). The LDL isolated from pooled plasma of 10 normal subjects and from a single normal subject were tested. The maximal amount of 125I-LDL bound in the presence of an excess of antibody MB47 was assessed by precipitation with IgSORB and was expressed as a percentage of 125I-LDL precipitable by TCA (Figure 2). In antibody excess, essentially all 125I-LDL was bound, indicating that the receptor domain epitope(s) identified by antibody MB47 was expressed by all LDL particles.

To assess the average affinity constant of antibody MB47 for LDL, competitive equilibrium fluid phase RIAs were performed. Homologous unlabeled LDL produced full displacement of 125I-LDL, and the calculated affinity constant of antibody MB47 for LDL was 4.06 x 10^9 M^-1, somewhat higher than the values reported by Tsao et al. for 11 other apo B-specific monoclonal antibodies.

We also used a MB47-Sepharose 4B immunoabsorbant column as another means to assess the percentage of LDL particles that expressed the MB47 epitope. The column had a total LDL binding capacity of greater than 36 mg of LDL protein. When 10 mg of LDL protein was added to the column and incubated overnight at 4°C, over 99% of the added LDL protein was bound.

Stoichiometry of MB47 Binding to LDL

To determine the number of antibody binding sites per apo B molecule, a different fluid phase assay was used in which an increasing amount of immunopurified 125I-MB47 antibody was added to a fixed concentration of LDL. The amount of 125I-MB47 binding to LDL was assessed by quantitative precipitation of LDL with a rabbit antiserum specific for human LDL. Specific binding of 125I-MB47 antibody was saturable (Figure 3 A), and a Scatchard plot of the binding data was linear (Figure 3 B), suggesting the uniformity of MB47 binding sites on LDL particles. The affinity constant (Ka) determined in this assay was 2.70 x 10^9 M^-1. A maximum of 212 fmol of 125I-MB47 antibody bound to 182 fmol of LDL, indicating that only one MB47 molecule binds to each molecule of apo B.

MB47 Binding to Modified LDLs and Apo E

Using solid phase competitive RIAs, we assessed the ability of chemically modified LDLs to bind to antibody MB47. Compared to equivalent amounts of native LDL, methylated LDL showed essentially no immunoreactivity to antibody MB47. Acetyl LDL, glucosylated LDL, and carbonyl LDL showed a reduced ability to bind to antibody MB47. Cyclohexanone modification of LDL did not alter immunoreactivity to antibody MB47.

Because apo E also binds to the LDL receptor, we tested whether MB47 might bind to apo E. However, when tested in a competitive RIA, neither apo E nor apo E-DMPC liposomes (even when added at 0.2 mg/ml) competed with LDL for binding to antibody MB47. A similar result was seen when using the rabbit antiserum as antibody source.

Figure 1. Ability of 48 hybridoma culture supernatants containing anti-LDL antibodies to inhibit 125I-LDL degradation by human fibroblasts. To each 10 mm well of cultured fibroblasts was added 0.4 ml of Dulbecco's modified Eagle medium containing 2.5 mg/ml of lipoprotein-deficient serum (LDS) and 2.5 uM of 125I-LDL and 0.1 ml of hybridoma culture supernatant. 125I-LDL degradation was expressed as a percentage of control degradation in the presence of supernatants from hybridoma colonies negative for anti-LDL antibodies. 500 Atg/ml of unlabeled LDL inhibited LDL degradation by 95%. Only supernatant 47 (△) produced marked inhibition (95%) of 125 I degradation.

Figure 2. The percentage of 125I-LDL particles bound by increasing concentrations of antibody MB47 in a fluid phase radioimmunoassay (RIA). In each tube a fixed amount of 125I-LDL was incubated for 18 hours at 4°C with the indicated amounts of immunopurified MB47. The amount of 125I-LDL bound was then determined by precipitation of antibody MB47 with IgSORB. LDL was prepared from pooled plasma of 10 subjects (○) or from a normolipidemic individual (△). Each point represents the mean of triplicate determinations.
**MB47 Binding to Heterologous Plasma and Lipoprotein Fractions**

Using a solid phase RIA, we tested the ability of heterologous whole plasma to compete with human LDL for antibody MB47 binding (Figure 4). All mammalian plasmas tested, except for mouse and rat, effectively competed for MB47 binding. Whale plasma was also a good competitor (data not shown). The spectacled bear plasma was the best competitor, due to its high lipoprotein levels (total cholesterol 402 mg/dl, triglycerides 1013 mg/dl) and strong reactivity of antibody MB47 for bear apo B (Figure 5). Lobster hemolymph, shark plasma, and plasma from three species of tuna showed no reactivity with MB47. However, undiluted plasma from one species of tuna consistently showed approximately 30% inhibition of MB47 binding to coated human LDL. None of the heterologous lipoprotein-deficient plasmas showed competition. When similar RIAs were performed by using either of two previously described apo B-specific monoclonal antibodies, MB3 and MB19, or a polyclonal rabbit antiserum specific for human LDL, no competition was seen with the heterologous plasmas (Figure 6). Thus, the human apo B epitopes recognized by antibodies MB3 and MB19, and the rabbit antibodies are unique to human apo B.

The ability of isolated lipoproteins from multiple species to compete with human LDL for antibody MB47 binding was also tested. The LDL of each species tested, except for the mouse, was an effective competitor (Figure 5).

**Figure 3.** Binding of $^{125}$I-MB47 antibody to LDL in a fluid phase radioimmunoassay (RIA). Increasing amounts of immunopurified and iodinated antibody MB47 were incubated for 16 hours at 4°C with 100 ng of pooled human LDL. The amount of antibody MB47 bound was determined by quantitative precipitation of LDL with use of a rabbit antiserum specific for human LDL. A shows specific binding of $^{125}$I-MB47 antibody. Each point represents the mean of triplicate determinations. B shows a Scatchard analysis of the data. The Ka of $^{125}$I-MB47 for LDL was 2.70 $\times$ 10$^8$ M$^{-1}$. Extrapolation of the line to conditions of infinite antibody excess yielded an estimate of 212 fmol (35 ng) of $^{125}$I-MB47 bound by 182 fmol (100 ng) of LDL (based on a molecular weight of apo B = 550,000).

**Figure 4.** Ability of human and heterologous plasmas (A) and lipoprotein-depleted plasmas (B) to compete with human LDL for binding to Antibody MB47 in a solid phase radioimmunoassay (RIA). Microtiter wells were coated with pooled human LDL, and MB47 ascites fluid was used at 1:20,000 dilution. The amount of antibody MB47 bound to the solid phase LDL was quantitated with a subsequent incubation with $^{125}$I-goat antimouse IgG. Each lipoprotein-deficient plasma was diluted to the same protein concentration as the plasma from which it was isolated. For reference, the competition curve of human plasma is also shown in B.
Whale LDL also was an effective competitor, but rat LDL was not (data not shown). Although LDL of most species did not compete as well as human LDL, bear LDL was a better competitor. As expected, heterologous LDL fractions did not compete in similar assays when using MB3, MB19, or the polyclonal rabbit antiserum.

Human VLDL as well as heterologous VLDL samples were effective competitors for binding to antibody MB47. Figure 7 compares competition curves for human and dog lipoprotein fractions in RIAs when using antibodies MB47 and MB3. Human and dog VLDL-apo B preparations bound MB47 nearly as well as the respective LDL (Figure 7 A, B). In contrast, human VLDL and LDL were effective competitors for binding to antibody MB3, but dog VLDL and LDL were not. As expected, neither dog nor human HDL competed with human LDL for MB47 or MB3 binding. Similar data were obtained for the lipoprotein fractions of the rabbit, pig, guinea pig, bear, seal, and whale. antibody MB47 bound LDL and VLDL of each of these species, whereas antibodies MB3 and MB19 did not.

**MB47 Inhibition of Cellular LDL Uptake and Degradation**

Antibody MB47 was selected in the initial screening studies because it inhibited fibroblast uptake of $^{125}$I-LDL. Further studies performed after the hybridoma-producing antibody MB47 was cloned demonstrated that this effect was due to marked inhibition of specific $^{125}$I-LDL binding (Figure 8). Inhibition of specific binding, internalization, and degradation by antibody MB47 was comparable to that produced by a 200-fold excess of unlabeled LDL.

It is possible that antibody MB47 binds to an epitope adjacent to the apo B receptor domain, and because of its size, sterically hinders the binding of apo B to the LDL receptor. Therefore, the capacity of Fab fragments of antibody MB47 to inhibit human LDL uptake and degradation were also tested. MB47-Fab fragments completely blocked specific cellular binding and degradation of LDL (Figure 9).

Next, the concentration of antibody MB47 required for maximal inhibition of LDL degradation was assessed. Since antibody MB47 bound heterologous LDL, we also tested whether MB47 would block the uptake and degradation of heterologous LDL by heterologous fibroblasts. This was accomplished by testing the ability of immunopurified antibody MB47 to inhibit cellular uptake and degradation of iodinated human, rabbit, and guinea pig LDL preparations on human as well as guinea pig fibroblasts. At a molar ratio of IgG to LDL of 1, there was nearly complete inhibition of $^{125}$I-human LDL degradation by human fibroblasts (Figure 10 A). The molar ratio required for maximal inhibition of rabbit LDL or guinea pig LDL uptake and degradation was 4:1 to 8:1, consistent with the solid phase RIA data, which showed that antibody MB47 bound human LDL better than guinea pig and rabbit LDL. The maximal degree of inhibition caused by high MB47 concentrations was less for the guinea pig and rabbit LDL preparations than for the human LDL. Antibody MB47 inhibited human

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**Figure 5.** Ability of human and heterologous plasmas to compete with human LDL for antibody binding in solid phase radioimmunoassays (RIAs) with the rabbit antiserum specific for human LDL (A), apo B-specific monoclonal antibodies MB19 (B), and MB3 (C). The conditions for the RIAs are as indicated in the legend to Figure 4. Antibody dilutions for the rabbit antiserum were 1:50,000; for MB19, 1:45,000; and for MB3, 1:20,000.

**Figure 6.** Ability of human and heterologous LDL fractions to compete with human LDL for binding to antibody MB47 binding in a solid phase RIA. Conditions for the assay are defined in legend to Figure 4. LDL fractions were added to the assay on the basis of their protein content, corrected for the percent precipitable by tetramethyurea.
LDL degradation by human fibroblasts by 95%, but the maximal inhibition of rabbit and guinea pig LDL degradation was 55% and 75%, respectively. A 200-fold excess of unlabeled human LDL inhibited uptake and degradation of all three tracers by over 95%. In part, the inability of antibody MB47 to completely inhibit specific degradation of guinea pig and rabbit LDL preparations may have been due to the presence of apo E-containing lipoproteins in the LDL density range in these animals. When subjected to SDS-PAGE, a small band consistent with apo E was seen in both guinea pig and rabbit LDL preparations.

Parallel studies on guinea pig fibroblasts with use of the same iodinated LDL preparations showed similar results (Figure 10 B). Again, a 1:1 molar ratio of antibody MB47/LDL-apo B resulted in nearly complete inhibition of human LDL degradation, whereas higher antibody concentrations were required to achieve similar inhibition with rabbit LDL preparations. These results suggest that apo E may play a role in the uptake and degradation of LDL in both species.

Figure 7. Ability of human and dog VLDL, LDL, and HDL fractions to compete for antibody binding in the MB47 assay (A and B) and in the MB3 assay (C and D). Assays of human lipoproteins are shown in A and C; assays of dog lipoproteins are shown in B and D. Conditions for the assays are given in the legends to Figures 4 and 5. LDL protein was determined by the Lowry technique, while VLDL and HDL proteins were added on the basis of tetramethylurea-insoluble protein concentrations (•, human HDL; ▲, human VLDL; △, human LDL; ○, dog HDL; ●, dog VLDL; ●, dog LDL).

Figure 8. Inhibition by antibody MB47 and excess unlabeled human LDL of 125I-human LDL binding, internalization, and degradation by cultured human fibroblasts. Fibroblast monolayers were grown in 35 mm wells in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum. Fibroblast LDL-receptors were stimulated by a 24-hour preincubation with DMEM containing 2.5 mg/ml of lipoprotein-deficient serum (LDS). DMEM-LDS containing 2.5 µg/ml of 125I-LDL and either 20% of MB47 hybridoma culture supernatant (vol/vol) or a 200-fold excess of unlabeled LDL (final concentration, 500 µg/ml) were preincubated for 16 hours at 4°C before being placed on the fibroblast monolayers. Determinations of binding, internalization, and degradation were performed in triplicate, and expressed as a percentage of control values determined in the absence of antibody MB47. The baseline values (100%) for binding, internalization, and degradation were 2.5 µg/ml 125I-LDL were 23 ng/mg cell protein, 0.1 µg/mg cell protein, and 0.5 µg/mg cell protein, respectively.
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Figure 9. Ability of Fab fragments of MB47 to inhibit $^{125}$I-human LDL binding and degradation by human fibroblasts. Individual media containing increasing amounts of MB47-Fab fragments and a constant amount of $^{125}$I-LDL (2.5 μg/ml) were preincubated for 16 hours at 4°C before being placed on the fibroblast monolayers. Fab concentration is expressed as the molar ratio of Fab/LDL present in each media (based on a MW of Fab of 40,000 and of apo B of 550,000). Binding and degradation are expressed as the percentage of control values in the absence of MB47-Fab fragments. All determinations were performed in duplicate. Excess unlabeled LDL (final concentration, 500 μg/ml) produced greater than 95% inhibition of $^{125}$I-LDL binding and degradation. Baseline values (100%) for binding and degradation of 2.5 μg/ml of $^{125}$I-LDL were 20 ng/mg cell protein and 1 μg/mg cell protein, respectively. Similar results were obtained in three experiments with different Fab preparations.

centrations were required for maximal inhibition of guinea pig and rabbit LDL degradation.

Because antibody MB47 bound all LDLS except for mouse and rat, we tested whether human and mouse LDL-apo B shared a common region recognized by the human LDL receptor. We assessed the ability of human LDL and mouse LDL (and other heterologous LDL preparations) to inhibit $^{125}$I-human LDL binding and uptake by human fibroblasts. The concentration of $^{125}$I-human LDL in the fibroblast media was kept constant, and increasing amounts of human or heterologous LDL were added (Figure 11). Human and pig LDL were the best competitors. Mouse LDL competed as well as rabbit, guinea pig, or dog LDL, although not as well as human LDL. Interestingly, the ability of guinea pig, rabbit, and dog LDL-apo B to compete with $^{125}$I-human LDL for cellular binding roughly correlated with their ability to compete with human LDL for MB47 binding in the radioimmunoassays (Figure 6). Pig LDL was a better competitor in the fibroblast system than in the RIA.

Discussion

We have demonstrated that LDL isolated from multiple animal species will bind specifically to the human LDL-receptor (Figure 11) and, in turn, that human LDL will bind to specific LDL-receptors present on heterologous fibroblasts (Figure 10 B). These observations are in agreement with the results of others and suggest that apo B must contain a highly conserved domain that mediates binding to the cellular LDL-receptor. However, we (Figure 5 A) and others have noted that rabbit antisera generated against human LDL have little reactivity toward heterologous LDL in RIAs, suggesting that few antibodies in the antisera are directed toward common, conserved epitopes. Presumably this occurs because the rabbit, when immunized with human LDL, makes few antibodies against the receptor domain of apo B, which is conserved, but makes many antibodies to nonhomologous domains, which are present in portions of apo B not involved in receptor binding. Indeed, when using MB3 and MB19 (Figure 5) we found that

Figure 10. Ability of immunopurified Antibody MB47 to inhibit degradation of $^{125}$I-human LDL (△), $^{125}$I-rabbit LDL (●), and $^{125}$I-guinea pig LDL (○) by normal human fibroblasts (A) and normal guinea pig fibroblasts (B). Individual media containing increasing amounts of antibody MB47 and a constant amount of $^{125}$I-LDL were preincubated for 16 hours at 4°C before being placed on fibroblast monolayers for incubation at 37°C for 6 hours. The concentration of MB47 in the media is expressed as the molar ratio of MB47/tetramethylurea-insoluble $^{125}$I-LDL added (based on a MW of apo 550,000 and MW of MB47 of 166,000). Degradation of each LDL is expressed as the percentage of a control value in the absence of MB47. Each point is the mean of triplicate determinations. On human fibroblasts, the baseline (100%) values for degradation of 2.5 μg/ml of $^{125}$I-LDL were 0.75 μg/mg cell protein for the human LDL, 0.1 μg/mg cell protein for the guinea pig LDL, and 0.15 μg/mg cell protein for the rabbit LDL. Similar values were obtained with guinea pig fibroblasts. Binding studies were also performed and showed identical patterns of inhibition. On both types of fibroblasts and for all three iodinated LDL preparations, excess unlabeled human LDL (500 μg/ml) inhibited over 95% of LDL binding and degradation.
antibodies that did not bind to the LDL-receptor binding domain (i.e., they did not block $^{125}$I-LDL binding to fibroblasts) did not bind to LDL of other species. Similar results were obtained with two other previously characterized antibodies against apo B (B11 and B20), which do not bind to the LDL-receptor binding domain (data not shown). This evidence suggests that domains not involved in receptor binding are less likely to be conserved, but it is certainly possible that some such domain may be conserved.

For reasons noted above, we postulated that a monoclonal antibody specifically selected because it was directed against the LDL-receptor binding domain of apo B would be likely to bind to LDL of many species. Therefore, we developed an appropriate screening procedure and succeeded in isolating one such LDL-receptor domain monoclonal antibody, MB47. We showed that this antibody binds to a highly conserved apo B epitope.

Antibody MB47 specifically bound apo B-100 in human LDL and VLDL, but did not bind to apo B-48. Its apparent affinity constant (Ka) for human LDL, assessed by competitive RIA, was $4 \times 10^9$ M$^{-1}$. Scatchard analysis indicated that there was only one high affinity epitope expressed per LDL particle, consistent with previous reports. This suggests that there is only one LDL-receptor binding domain per LDL particle (if we assume that the MW of apo B is 550,000). Furthermore, all binding sites appeared to be of similar configuration (i.e., the slope of the Scatchard plot was linear).

Several investigators have demonstrated substantial immunological heterogeneity in LDL. That is, some apo B epitopes are not expressed by all LDL particles. If the LDL-receptor binding domain of apo B were not expressed on every LDL particle, it would suggest the possibility that LDL might be metabolically heterogeneous. However, using either fluid phase RIAs or immunofluorescence chromatography, we found that all $^{125}$I-LDL particles in solution expressed the MB47 epitope. This suggests that MB47 should be a useful monoclonal antibody for a RIA designed to quantitate apo B in plasma. Indeed, preliminary studies with over 100 plasma samples indicated that MB47 accurately measures total plasma apo B in all of them.

Two previously described monoclonal antibodies that did not block cellular uptake of LDL, MB3 and MB19, bound only to human LDL and not to heterologous LDLs. Thus, it appears that epitopes not critically involved in binding to the cellular LDL-receptor may not be conserved.

Similarly, Mao et al. found that their mouse monoclonal antibodies against human apo B did not bind pig LDL. Whether these monoclonals bound to the apo B receptor domain was not reported. Interestingly, they did find that a mouse antiserum against human LDL recognized some shared epitopes in pig and human LDL. Our rabbit antiserum against human LDL showed little, if any, ability to bind epitopes present in heterologous plasmas (Figure 3A). However, in separate experiments (data not shown), we have found that the polyclonal rabbit antiserum does compete with $^{125}$I-MB47 antibody for LDL binding in a solid phase RIA, but only under conditions of marked rabbit antibody excess. This suggests that the polyclonal antisemur does contain a few antibodies against the conserved receptor domain of apo B. Obviously, the predominant populations of antibodies in the rabbit antiserum were directed against the regions of apo B not conserved and not directly involved in LDL-receptor binding. Similarly, we found that only one of the 48 mouse hybridoma supernatants producing anti-LDL antibodies had a significant ability to markedly inhibit LDL uptake by cultured fibroblasts. Previously, Curtiss and Edgington developed 13 monoclonal antibodies specific for apo B, and none bound to a receptor domain epitope. In contrast, two of seven monoclonals developed by Tikkanen et al. and five of seven analyzed by Milne et al. were capable of blocking LDL uptake by cultured fibroblasts.

Antibody MB47 completely inhibited receptor-mediated binding, internalization, and degradation of human LDL by human fibroblasts (Figure 8). This inhibition was complete when the antibody MB47/LDL-apo B molar ratio in the fibroblast media was 1:1 (Figure 10). Fab fragments of MB47 were also effective in totally blocking receptor-mediated LDL uptake (Figure 9), although the molar ratio of Fab fragments to LDL-apo B in the fibroblast media required for complete inhibition of LDL uptake was 4:1 to 8:1, higher than that for intact antibody MB47 (Figure 10). This is most likely due to a lower affinity of Fab fragments for LDL compared to that of intact immunoglobulin. Milne et al. have previously reported that Fab fragments of certain anti-apo B monoclonal antibodies may block binding of LDL.

Antibody MB47 bound to the plasma, or isolated LDL or VLDL, of each mammalian species tested, except for the

**Figure 11.** Ability of unlabeled human and heterologous LDL preparations to compete with $^{125}$I-human LDL for binding (A) and degradation (B) on human fibroblast monolayers. To each 35 mm dish of fibroblasts was added $^{125}$I-human LDL (2.5 $\mu$g/ml) and the indicated concentration of unlabeled LDL competitor, added on the basis of tetramethylurea precipitable protein. The binding and degradation data are expressed as the percentage of control values obtained in the absence of competitor. The baseline values (100%) for binding and degradation of 2.5 $\mu$g/ml of $^{125}$I-LDL were 26 ng/mg cell protein and 0.75 $\mu$g/mg cell protein, respectively. Each point is the mean of duplicate determinations. • = rabbit LDL; ▲ = mouse LDL; ◇ = guinea pig LDL; □ = dog LDL; △ = human LDL; ● = pig LDL.
mouse and a closely related species, the rat (Figures 4, 6, 7). No immunoreactivity was observed in the plasma of more primitive species, such as shark, lobster, or tuna fish. Western blots confirmed that antibody MB47 bound to apo B-100 (or an analogous high MW protein) of dog, pig, guinea pig, and rabbit, but not mouse. Antibody MB47 was also capable of inhibiting the cellular uptake of heterologous LDLs by either human or guinea pig fibroblasts.

The fact that monoclonal antibody MB47, as well as Fab fragments of MB47, bind to a conserved region of apo B, and the fact that it also blocks the cellular uptake of both human and heterologous LDL samples strongly support the suggestion that antibody MB47 binds to the receptor domain of apo B-100. However, our present data cannot exclude the possibility that antibody MB47 binds to a conserved epitope adjacent to, or even distant from, the receptor domain and blocks LDL binding to cells by steric hindrance or by inducing a conformational change in apo B. If MB47 binds to the LDL-receptor binding domain of apo B-100, how does one explain the observation that both mouse and rat apo B bind to the human LDL receptor, although neither reacts with antibody MB47? It is likely that subtle structural differences exist between the receptor domain of mouse and human apo B, which are insufficient to interfere with recognition by the receptor but can be distinguished immunochemically. In fact, this would explain why we and others have been able to develop mouse monoclonal antibodies against the receptor domain of human apo B. Although the ability of monoclonal antibodies to detect such subtle differences in antigenic structure is well known.

Because a conserved region of apo B exists and because heterologous LDLs bind to the cellular LDL-receptor of multiple species, it follows that a significant conservation of the LDL-binding domain of the LDL-receptor must also exist. Biesiegel et al. and Huettliger et al. have provided immunohistochemical evidence for conservation of portions of the cellular LDL receptor using a rabbit antisem for the bovine LDL receptor and specific mouse monoclonal antibodies. One mouse monoclonal antibody, IgG-C7 detected an epitope shared by human and bovine LDL receptors, and it effectively competed with human LDL for cellular LDL-receptor binding. However, this mouse monoclonal antibody did not bind to the LDL-receptor of mouse cells, even though these cells contained a receptor that bound human LDL-apo B. In an analogous fashion, antibody MB47 did not bind mouse LDL, although mouse LDL bound to the human LDL receptor.

Further evidence that proteins with important roles in lipoprotein metabolism contain conserved sequences of importance in biologic function are found in recent studies by Weisgraber and colleagues. They demonstrated conservation of the binding domain of apo E, another ligand that binds to the LDL-receptor. Using mouse monoclonal antibodies developed by Milne et al., they showed that an antibody against the binding receptor domain of human apo E also bound to canine apo E.

While this manuscript was in preparation, Schonfeld and colleagues reported similar findings. They studied the ability of seven previously characterized anti-apo B monoclonal antibodies to bind to LDL isolated from a number of animal species. Two of the seven antibodies, which had previously been shown to block LDL uptake on fibroblasts, bound LDL of other animal species. Each antibody bound to one epitope on LDL. The five monoclonals that did not block LDL uptake and degradation showed little reactivity with heterologous LDL.

Acknowledgments

We thank Daniel Steinberg for his ongoing support and advice. We thank Susan Butler, Kethi Bergmann, Sandra Bertics, and Frank Peralta for excellent technical help and Anita Fargo for manuscript preparation. We also thank Larry Mahan for assistance in determining the stoichiometry of MB47-LDL binding.

References

24. Fraker PJ, Speck JC. Protein and cell membrane iodinations with a sparingly soluble chloroamide 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. Biochem Biophys Res Comm 1978; 80:849–857
38. Tikkanen M, Schoenfeld G. The recognition domain for the low density lipoprotein cellular receptor is expressed once on each lipoprotein particle. Biochem Biophys Res Comm 1985; 126:773–777

Index Terms: monoclonal antibodies  •  apoprotein B  •  low density lipoprotein  •  radioimmunoassay
Conservation of the low density lipoprotein receptor-binding domain of apoprotein B. Demonstration by a new monoclonal antibody, MB47.
S G Young, J L Witztum, D C Casal, L K Curtiss and S Bernstein

doi: 10.1161/01.ATV.6.2.178
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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