A potential complication in the use of monoclonal antibodies: inhibition of apoB-mediated receptor binding by an anti-apoE antibody

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Abstract Monoclonal antibody (Mab) 1D7 is specific for human apolipoprotein (apo) E and blocks binding of lipid-associated apoE to the low density lipoprotein (LDL) receptor. We report here that 1D7 can also block the binding of apoE-free LDL to the LDL receptor. The inhibition of LDL-receptor binding is not due to immunological cross-reactivity between the anti-apoE Mab and apoB, the ligand responsible for the interaction of LDL with the LDL receptor: *1*) Mab 1D7 did not react with apoE-depleted LDL; *2*) the LDL receptor binding inhibitory activity of 1D7 immunoglobulin G (IgG) preparations could be dissociated from the anti-apoE activity; *3*) the inhibition was maintained when the fibroblasts were preincubated with the 1D7 IgG, extensively washed, and only then exposed to ^{125}I-labeled LDL. Rather, it appears that 1D7 recognizes mouse apoE, that mouse apoE-1D7 immune complexes contaminate 1D7 IgG preparations and that the contaminating mouse apoE can compete with ^{125}I-labeled LDL for the LDL receptor. We have demonstrated mouse apoE in IgG preparations of 1D7 but not in those of other anti-apoE Mabs that do not influence LDL-receptor binding. Precipitation of 1D7 IgG with NH_4SO_4 eliminates both apoE and the capacity of 1D7 to block LDL receptor binding. Finally, mouse apoE can be isolated by immunoaffinity chromatography of mouse serum on immobilized 1D7 Mab. As this is probably not a unique case, the observation has important implications for the use of Mabs as structural probes.—**Maurice, R., Y. L. Marcel, T. L. Innerarity, and R. W. Milne.** A potential complication in the use of monoclonal antibodies: inhibition of apoB-mediated receptor binding by an anti-apoE antibody. *J. Lipid Res.* 1989. **30:** 587–596.

Supplementary key words low density lipoproteins • LDL receptor • autoantibody • conserved epitope

The low density lipoprotein (LDL) receptor, present on the surface of most mammalian cell types, plays a key role in the maintenance of cholesterol homeostasis. In their lipid-bound forms, two apolipoproteins, apolipoprotein (apo) E and apoB can compete with each other for binding to the LDL receptor (1, 2). The receptor-binding domain of apoE has been localized to a region encompassing apoE residues 140 to 160, and an anti-apoE Mab, 1D7, whose epitope is situated between residues 139–169, blocks the binding of apoE-containing lipoproteins to the LDL receptor. While the receptor-binding domain of apoB has not been identified, Mabs that block LDL binding to the LDL receptor have epitopes that are clustered around a thrombin cleavage site at apoB residue 3249 (3–5). Analysis of the primary structure of apoE and apoB indicates little sequence homology between the two proteins. Nevertheless, common consensus sequences for the binding domains of apoB and apoE have been proposed (6, 7). These have been based on the identification of sequences that are similar in apoE and apoB with respect to the relative positions of basic amino acids. The basic amino acids in the consensus sequence could be approximately aligned with acidic residues in the putative ligand-binding domains of the LDL receptor.

In this study, we report that immunoglobulin G (IgG) preparations of the anti-apoE Mab 1D7 are capable of blocking the binding of apoE-free LDL to the LDL receptor. This observation has both practical and theoretical importance. In practical terms, the Mab 1D7 has been used to discriminate between apoE and apoB-mediated binding of lipoproteins to cell surface receptors (8–12) and as a probe for the identification of the receptor-binding (13) and heparin-binding (14) domains of apoE. It is therefore essential to determine the true specificity of this Mab. Furthermore, it would be of theoretical interest if it could

be shown that the inhibition of LDL binding by 1D7 was due to an epitope that is common to apoB and apoE. Such an immunological cross-reactivity may reflect a structural relationship between apoE and apoB that, in turn, could explain the dual specificity of the LDL receptor. In view of the potential significance of the observation, we have determined the mechanism by which Mab 1D7 inhibits LDL-receptor binding. The results have important implications for the use of Mabs as structural probes of protein function.

MATERIALS AND METHODS

Lipoproteins and antibodies

Blood from fasting normolipidemic subjects was collected in tubes containing EDTA, and red blood cells were removed by centrifugation. The plasma obtained was mixed with NaN_3 (0.02%) and phenylmethylsulfonyl fluoride (1 mM). Lipoprotein subfractions were prepared in a Beckman L8-M ultracentrifuge with a 50.2 Ti rotor (Beckman Instruments, Spinco Division, Palo Alto, CA). LDL was isolated at 10°C by successive preparative ultracentrifugations betweeen densities of 1.020 and 1.050 g/ml (15), dialyzed against phosphate-buffered saline (PBS) containing 1 mM EDTA and 0.02% NaN_3 (pH 7.4), sterilized by ultrafiltration, and stored at 4°C. Total mouse lipoproteins used as positive controls for Western blots were obtained at d < 1.210 g/ml.

Production and characterization of Mabs against human apoE and apoB have been described elsewhere (3, 16). The Mabs were isolated from a mixture of plasma and ascitic fluid from hybridoma-bearing mice by affinity chromatography on Protein A-Sepharose 4B (Pharmacia Inc., Uppsala, Sweden) (17). The five anti-human apoE Mabs used in this study are 1D7, 6C5, 3B7, 7C9, and 3H1. Mabs 6C5, 3B7, and 7C9 compete for either the same or closely situated antigenic determinants, which are located near the amino terminal of apoE, probably between amino acids 1 and 11 (14). The epitope of 3H1 is situated in the C-terminal part of apoE, probably between amino acids 243 and 272. The epitope of 1D7 is in the center of the molecule, between amino acids 139 and 169, and this Mab can inhibit apoE-mediated binding of lipoproteins to the LDL receptor of fibroblasts (13). One anti-human apoB Mab, 4G3, was used; its epitope is located between amino acids 3029 and 3132 of apoB (4). Mab 4G3 has been shown to inhibit the binding of LDL to the LDL receptor of cultured human fibroblasts (3).

The preparations of rabbit antibodies against rat apoA-I, apoA-IV, and apoB were a generous gift from Dr. Karl Weisgraber. The rat lipoproteins and apolipoproteins used for polyclonal antibody production were isolated as described (18). Rabbit anti-rat apoE was a generous gift from Drs. Renée LeBoeuf and John Elovson.

LDL binding to human fibroblasts

Human skin fibroblasts from a normal donor (strain GM0038, Human Genetic Mutant Cell Repository, Camden, NJ) were used in all experiments. The cells were maintained, as previously described (3), in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

For binding experiments, cells were plated in 9.5-cm² dishes in 2 ml of DMEM supplemented with 10% (v/v) fetal bovine serum. Before reaching confluency, the cells were washed with 2 ml of sterile PBS and exposed to DMEM supplemented with 10% human lipoprotein-deficient serum for 48 hr. Prior to the experiment, cells were placed at 4°C for 30 min; the medium was then removed and the cells were exposed to cold DMEM supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) and 0.5% bovine serum albumin (DMEM-BSA), as well as the reactants to be tested. Other experimental details have been described previously (3) or are given in the legends of the appropriate figures. Nonspecific binding was determined by adding 500 μg of unlabeled LDL/ml to the reaction mixture (19).

Immunoadsorbents and immunoaffinity chromatography

The procedure for coupling the Mabs to Sepharose 4B (Pharmacia Inc., Uppsala, Sweden) using CNBr-activated Sepharose has been described elsewhere (20). To obtain apoE-containing lipoproteins, variable amounts of normal mouse serum or normal human serum were applied to 10-ml columns of either 3B7, 1D7, 6C5, or 7C9 IgG covalently coupled to Sepharose (Mab-Sepharose). The column was washed with PBS, and bound lipoproteins that were eluted with 0.05 M citric acid (pH 2.5) containing 1 M NaCl were dialyzed against PBS that contained 1 mM EDTA and 0.02% NaN_3. ApoE-depleted LDL (LDL(apoE⁻)) or ¹²⁵I-labeled LDL(apoE⁻) were prepared by passing LDL or ¹²⁵I-labeled LDL over a 3-ml column of 3B7-Sepharose. In the case of ¹²⁵I-labeled LDL, the sample was diluted in PBS containing 1% BSA before application to the column to minimize nonspecific losses. ¹²⁵I-Labeled LDL that contain endogenous apoE (¹²⁵I-labeled LDL(apoE⁺)) were prepared by passing ¹²⁵I-labeled LDL over Sepharose that was coupled to an irrelevant Mab (anti-rat natriuretic factor) (21).

Analytical procedures

Electrophoresis of proteins and lipoproteins in the presence of sodium dodecyl sulfate on polyacrylamide gels (SDS-PAGE) was performed according to the method of Laemmli (22). Electrophoretic transfer of the migrated proteins to nitrocellulose paper (NCP) and immunodetection of the transferred proteins on the NCP replicas has been described previously (23).

To test for reactivity of anti-apoE Mabs with apoB, aliquots of dilutions of LDL or LDL(apoE⁻) were adsorbed to polystyrene wells (Dynatech Corp., Alexandria, VA) (16). After the wells were washed and then saturated with 1% BSA, they were exposed to the appropriately diluted Mab overnight and washed. The bound Mab was revealed by the addition of ^{125}I-labeled anti-mouse IgG. ApoE was measured as previously described (16). To measure the anti-apoE titre of IgG preparations, delipidated very low density lipoproteins (apoVLDL) (24) at a concentration of 5 μg/ml was adsorbed to polystyrene wells. Dilutions of the IgG to be tested were incubated with the immobilized apoVLVL and washed; the bound IgG was detected with ^{125}I-labeled anti-mouse IgG as described previously (3). At low dilutions of Mab, the bound radioactivity reached a plateau. The concentration of IgG that resulted in 50% of the plateau value of bound radioactivity was defined as the titre of the IgG preparation.

Protein was measured according to Lowry et al. (25) using BSA as standard.

Iodination of proteins and lipoproteins

Antibodies were labeled by a modification of the chloramine T method (26), while LDL was iodinated as described by Bilheimer, Eisenberg, and Levy (27). Specific activities obtained for the IgG were 5–10 μCi/μg of protein and 400–500 cpm/ng of protein for the LDL.

RESULTS

Inhibition of binding of LDL to the LDL receptor by anti-apoE Mabs

The ability of anti-apoE Mabs 1D7, and 6C5 and anti-apoB 4G3 to inhibit the binding of ^{125}I-labeled LDL to human fibroblasts is shown in **Fig. 1**. As expected, Mab 4G3 was able to inhibit 90% of the binding of LDL at a concentration of 10 μg/ml, whereas 6C5 displayed only a slight inhibition at 250 μg/ml. However, Mab 1D7 showed a concentration-dependent inhibition of LDL binding which, at 300 μg/ml, approached that of 4G3. As apoB is thought to be the ligand responsible for binding of LDL to the LDL receptor (1), this was a surprising and unexpected finding. Inhibition of binding by Mab 1D7 was at least as great with LDL that had been immunodepleted of apoE-containing particles (^{125}I-labeled LDL(apoE⁻)) as with ^{125}I-labeled LDL(apoE⁺). Thus, the ability of 1D7 to inhibit the binding of LDL is independent of the presence of apoE in the LDL. Contamination of the 1D7-IgG preparation with anti-apoB Mabs was excluded by antibody competition studies (data not shown).

Cross-reactivity of anti-apoE Mab with apoB

The inhibition of LDL binding shown in Fig. 1 could result from a low-affinity cross-reactivity of Mab 1D7 with the apoB of LDL. In our initial characterization of Mab
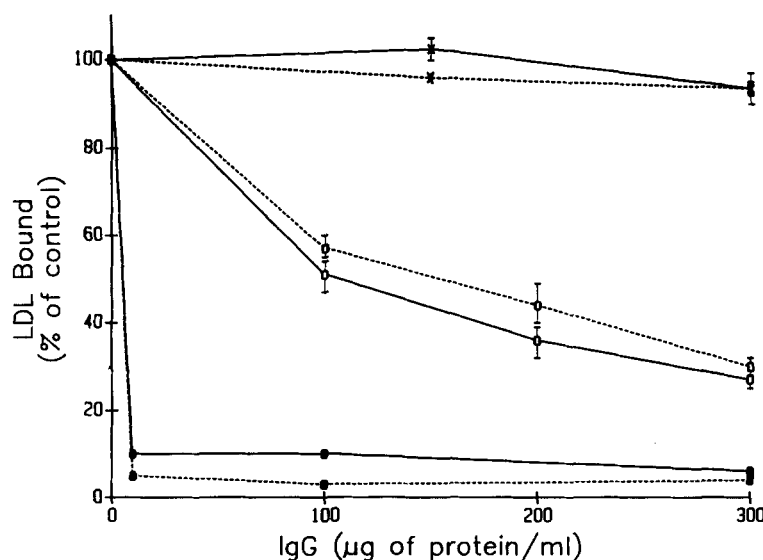

Fig. 1. Ability of anti-apoE Mabs 1D7 (○), 3B7 (X), and anti-apoB Mab 4G3 (●) to inhibit the specific binding of ^{125}I-labeled LDL(apoE⁺) (—) or ^{125}I-labeled LDL(apoE⁻) (---) to cultured human fibroblasts. Three μg of ^{125}I-labeled LDL in DMEM-BSA/ml was preincubated with the Mabs for 20 hr at 4°C. The mixture was then incubated with cultured human fibroblasts for 3 hr at 4°C and washed (3). The amount of ^{125}I-labeled LDL specifically bound to the cells was determined. The 100% control values for ^{125}I-labeled LDL(apoE⁺) or ^{125}I-labeled LDL(apoE⁻) binding to the LDL receptor were 220 and 145 ng of lipoprotein protein/mg of cellular protein, respectively. ^{125}I-labeled LDL(apoE⁺) and ^{125}I-labeled LDL(apoE⁻) were prepared as described in Materials and Methods. The standard deviation for Mab 4G3 was too small to be indicated in the figure. The characteristics of the Mabs used in these studies are given in Materials and Methods.

1D7, we reported that it did not cross-react with apoB (16). Because our conclusion was based on experiments in which LDL(apoE⁻) failed to compete with ^{125}I-labeled apoE in radioimmunoassay, we may not have detected a low-affinity cross-reactivity of Mab 1D7 with apoB. To test this possibility, we adsorbed dilutions of LDL or LDL(apoE⁻) to polystyrene and then tested the Mabs for their ability to directly bind to the immobilized lipoproteins. As can be seen in **Fig. 2**, binding to LDL of the anti-apoE Mabs, including 1D7, was due to the endogenous apoE in the LDL preparation.

Effect of time on the inhibition capacity of Mab 1D7

We have noted considerable variability amongst different preparations of 1D7 IgG with respect to their abilities to inhibit binding of LDL to the LDL receptor. When the results were compiled and analyzed, it became apparent that the loss of inhibitory activity appeared to be a function of the time elapsed between purification of the IgG and assessment of the ability of Mab 1D7 to block LDL binding (**Fig. 3**). In contrast to the loss in ability to block the interaction between LDL and its receptor, there was no consistent change with time in the anti-apoE titre of different preparations of 1D7 IgG (Fig. 3); as a consequence, it was possible to dissociate the anti-apoE activity in the 1D7 IgG preparation from the ability to block LDL-receptor binding. It was subsequently shown that the ability to block LDL-receptor binding could be maintained when the IgG preparations were either kept at 4°C in the presence of 1 mM EDTA or refrozen (data not shown).

Direct interaction of the 1D7 IgG preparation with the LDL receptor

As Mab 1D7 did not react with the ligand apoB, we next tested the possibility that the inhibition of LDL-receptor binding by Mab 1D7 results from a direct interaction of the IgG preparation with the LDL receptor. 1D7 IgG was added to cells for 2 hr, and the cells were then washed and exposed to ^{125}I-labeled LDL for an additional 2 hr. Maximum binding was determined by replacing the IgG preparation by 0.5% BSA. As shown in **Fig. 4**, it was possible, with this approach, to obtain the same inhibition of LDL binding to the fibroblasts as was seen when Mab 1D7 was preincubated with ^{125}I-labeled LDL prior to addition to the cells. In contrast, the anti-apoB Mab 4G3, which blocks LDL binding when added to cells in the presence of ^{125}I-labeled LDL, had no effect on binding if incubated with the cells prior to ^{125}I-labeled LDL. Thus, the inhibitory activity of 4G3 is due to an interaction of the Mab with the ligand apoB while the inhibition by 1D7 results from an interaction of the IgG preparation with the receptor itself. In subsequent experiments we were, however, unable to demonstrate anti-receptor antibody activity in the 1D7 IgG preparations (results not shown).

Contamination of anti-apoE Mab by apoE-containing lipoproteins

If 1D7 IgG preparations were contaminated with apoE- or apoB-containing lipoproteins of mouse origin, these murine lipoproteins could potentially compete with human LDL for the LDL receptor. IgG prepared by affinity chro-
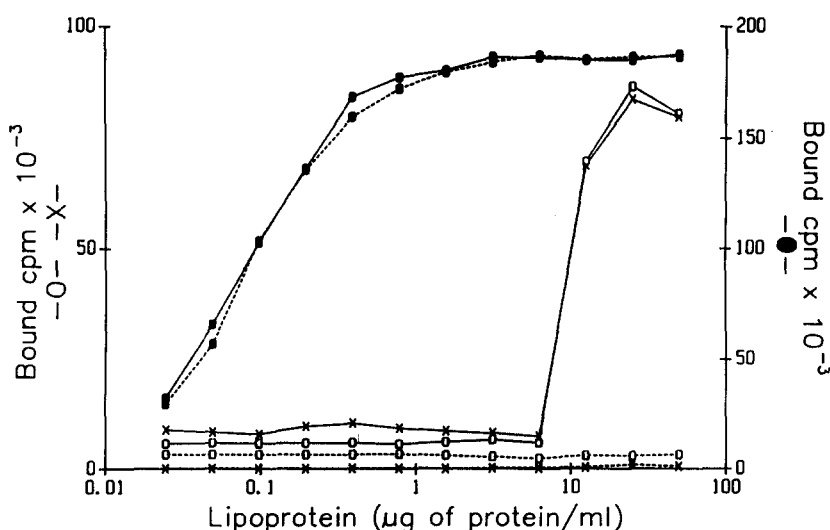

Fig. 2. Reactivity of anti-apoE Mabs 1D7 (○), 3B7 (X), and anti-apoB Mab 4G3 (●) with apoB. Total LDL (—) or LDL(apoE⁻) (---) were diluted and adsorbed to polystyrene, and Mabs were added to immobilized LDL as described in Materials and Methods. Bound antibodies were revealed by adding labeled anti-mouse IgG to the well. Results are expressed as bound radioactivity, in counts per min (cpm), per well. Each value represents the average of duplicate determinations.
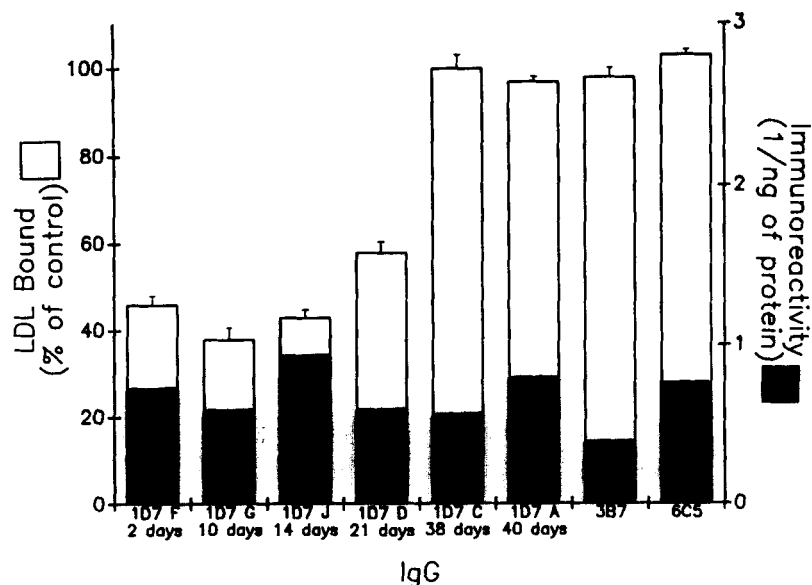
Fig. 3. Anti-apoE reactivity (solid bars) of individual 1D7 IgG preparations and their respective abilities to inhibit specific binding of ^{125}I-labeled LDL to cultured human fibroblasts (open bars). Different IgG preparations were purified from the same pool of ascites by affinity chromatography on Protein-A Sepharose and are referred to as 1D7 A, 1D7 C, 1D7 D, 1D7 F, 1D7 G, and 1D7 J. The preparations differed with respect to the time, in days, between the purification of the IgG and the evaluation of the IgG for its ability to influence LDL-receptor binding (see text). Purified IgG were kept at 4°C without EDTA until they were assessed in the binding study (see technical details in Fig. 1). Specific binding of ^{125}I-labeled LDL to the LDL receptor was determined, and the results are shown in open bars. The 100% control value for the binding of ^{125}I-labeled LDL was 100 ng of lipoprotein protein/mg cellular protein. Each value represents the mean of triplicate determinations. The immunoreactivity of the Mabs was determined by direct fixation of diluted Mabs to fixed amounts of immobilized apoVLDL, and results are expressed as the reciprocal of the anti-apoE titer (closed bars). Bound IgG was revealed with labeled anti-mouse IgG. Each value represents the average of duplicate determinations.
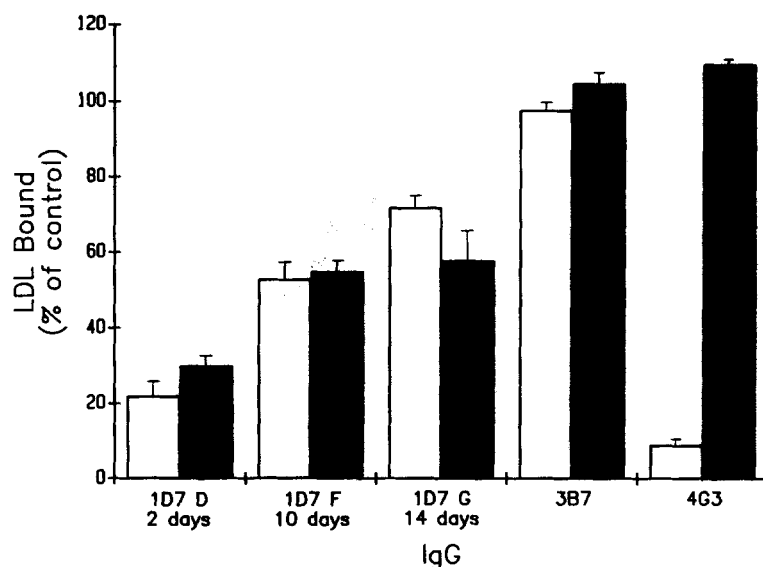

Fig. 4. Ability of anti-apoB Mabs 1D7, 3B7 and anti-apoB Mab 4G3 to inhibit the specific binding of ^{125}I-labeled LDL to the LDL receptor by an interaction of the IgG preparation with ^{125}I-labeled LDL (open bars) or with cultured human fibroblasts (solid bars). For experiments in which the cells were pre-exposed to Mab before addition of ^{125}I-labeled LDL, 200 μg of purified IgG/ml in 0.75 ml of DMEM-BSA was added to cultured human fibroblasts for 2 hr at 4°C. The cells were then washed (3) and incubated with 3 μg of ^{125}I-labeled LDL in DMEM-BSA/ml for another 2 hr (solid bars). Maximum binding was determined by omitting the Mab during the first incubation. The inhibition of binding of ^{125}I-labeled LDL to the LDL receptor following preincubation of Mabs with ^{125}I-labeled LDL (open bars) was done as in Fig. 1 except for a 2-hr incubation of the reaction mixture with the cells. Maximum binding values were 75 ng and 130 ng of lipoprotein protein/mg of cellular protein for the experiments represented by solid and open bars, respectively. Each value represents the average of triplicate determinations.

matography on Protein A-Sepharose from ascites containing the anti-apoE Mabs 1D7, 3B7, 6C5, 7C9, and 3H1 were separated by SDS-PAGE, and the migrated proteins were electrophoretically transferred to NCP. The NCP replicas were exposed first to a rabbit anti-rat apoE antiserum, which shows a strong cross-reactivity with mouse apoE (R. LeBoeuf, personal communication) and then to ^{125}I-labeled anti-rabbit IgG. The immunoblots revealed that mouse apoE was present in the preparation of Mab 1D7 but was absent from the IgG preparations of the other anti-apoE Mabs, 6C5, 7C9, 3H1, and 3B7 (**Fig. 5**). These results strongly suggest that apoE-containing lipoproteins from mouse origin co-purify with the Mab 1D7 during its isolation from mouse ascites on a Protein A-Sepharose column. In addition to the 35-kDa band of apoE, the heavy and light chains of Mabs (50 kDa and 25 kDa, respectively) were revealed because of a cross-reactivity of the ^{125}I-labeled anti-rabbit IgG with mouse IgG.

We have similarly tested 1D7 IgG preparations for the presence of other mouse apolipoproteins using anti-sera to rat apoA-I, apoA-IV, and apoB. No mouse apolipoproteins, with the exception of apoE, were detected (data not shown).

Purification of apoE-containing lipoproteins from mouse serum by immunoaffinity chromatography on insolubilized anti-human apoE Mab 1D7

The fact that mouse apoE was present in the 1D7 preparation and not in other anti-apoE Mab IgG preparations could indicate a cross-reactivity of 1D7 with mouse apoE. To confirm this, we coupled Mabs 1D7, 6C5, 7C9, and 3B7 to Sepharose and tested the insolubilized Mabs for their ability to purify apoE-containing lipoproteins from mouse
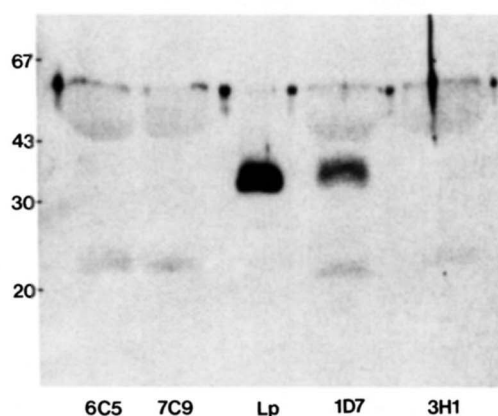

67 -
43 -
30 -
20 -

6C5   7C9   Lp   1D7   3H1

Fig. 5.   Presence of mouse apoE in 1D7 IgG preparations. Monoclonal anti-human apoE IgG preparations were reduced and subjected to 12% SDS-PAGE, transferred to NCP, then incubated with a diluted rabbit anti-rat apoE serum followed by radioiodinated goat anti-rabbit IgG, and autoradiographed. Two hundred micrograms of Mab protein and 100 µg of mouse lipoproteins were reduced with β-mercaptoethanol and applied to the gel. From left to right, anti-apoE Mab 6C5; anti-apoE Mab 7C9; density < 1.210 g/ml mouse lipoproteins; anti-apoE Mab 1D7; and anti-apoE Mab 3H1. Molecular weight markers are indicated in kDa.

serum. Each immunoadsorbant was first tested for its ability to bind apoE-containing lipoproteins from human serum. Measurement of apoE by radioimmunoassay in the retained and nonretained fractions showed that the respective capacities of the four immunoadsorbants were similar for binding of human apoE (data not shown). Each immunoadsorbant was then tested for its ability to bind mouse serum apoE. Immunoblots of the retained fractions obtained from 3B7- and 1D7-Sepharose are shown in **Fig. 6**. A 35-kDa band is present in the 1D7-Sepharose retained fraction. This band was absent from the 3B7-Sepharose retained fraction. This band was also absent from the 6C5- and 7C9-Sepharose retained fractions (data not shown).

We have tested the retained fractions of human or mouse serum that had been passed over 1D7- or 3B7-Sepharose columns in competition with human ^{125}I-labeled LDL for binding to the LDL receptor of cultured human fibroblasts. The retained human serum fractions from both immunoadsorbents competed with ^{125}I-labeled LDL for binding to the LDL receptor, whereas only the retained mouse serum fraction from the 1D7-Sepharose competed effectively (**Fig. 7**).

Elimination of apoE-containing lipoproteins from 1D7 IgG preparations

Mab 1D7 has been used to differentiate between the apoE and apoB components of lipoprotein binding to cell surface receptors. While the concentrations of Mab 1D7 required to totally eliminate apoE-mediated binding are normally sufficiently low so that effect of mouse apoE in 1D7 IgG preparation would be minimal, 1D7 IgG free of contaminating mouse apoE would certainly be preferable for use in receptor binding studies.

It is well known that IgG precipitates in 33% saturated ammonium sulfate solutions (28). 1D7 ascites was therefore subjected to precipitation by 33% saturated ammonium sulfate and the pellet was redissolved and dialyzed. The IgG was then purified by affinity chromatography on Protein-A Sepharose and tested for the presence of mouse apoE (**Fig. 8**) and for its capacity to inhibit binding of ^{125}I-labeled LDL to the LDL receptor (**Fig. 9**). Unlike the IgG prepared from 1D7 ascites not precipitated with ammonium sulfate that contains a 35-kDa protein which reacts with anti-rat apoE (lane 2, Fig. 8), the IgG fraction of ammonium sulfate-precipitated ascites contains no immunoreactive apoE (lane 4, Fig. 8). Likewise, the IgG from the non-precipitated ascites inhibits LDL binding to the LDL receptor, whereas there is no inhibition with 1D7 IgG prepared from precipitated ascites (Fig. 9). The precipitation does not change the anti-apoE titer of the purified IgG and the precipitated 1D7 continues to inhibit apoE-mediated lipoprotein binding to the LDL receptor (data not shown). These results, on the one hand, indicate again that it is the mouse apoE that is responsible for the inhibition of LDL-receptor binding and, on the other hand, provide a method to eliminate contaminating apoE from 1D7 IgG prepara-
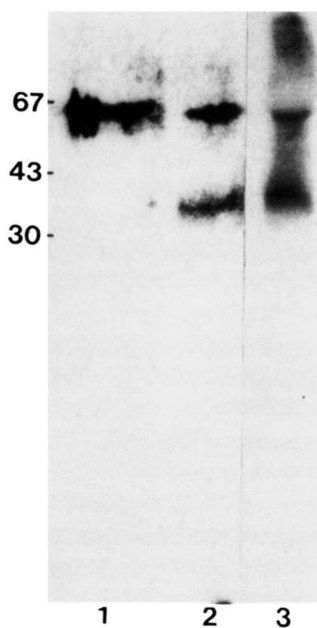
Fig. 6. Presence of apoE in the retained fraction of normal mouse serum passed over a 1D7 immunoaffinity column. Mouse serum was passed over 1D7- or 3B7-Sepharose and the retained fraction eluted at pH 2.5 was immunoblotted as described in the legend to Fig. 5. The quantity of protein applied to the gel was 15 μg of the retained fractions and 100 μg for d < 1.210 g/ml mouse lipoprotein. Lane 1, retained fraction from the 3B7-Sepharose column; lane 2, retained fraction from the 1D7-Sepharose column; and lane 3, d < 1.210 g/ml mouse lipoprotein. Molecular weight markers are indicated in kDa.

tions. Precipitation of the purified 1D7 IgG preparation is equally effective (data not shown).

DISCUSSION

When we first observed that the anti-apoE Mab 1D7 was able to inhibit the binding of ^{125}I-labeled LDL to the LDL receptor, our initial interpretation was that the Mab was cross-reacting with apoB, the major protein of LDL and known to be the ligand responsible for LDL-receptor binding. This was an attractive hypothesis in that it would provide experimental evidence for a common structure on apoE and apoB which could account for the dual specificity of the LDL receptor. Moreover, if Mab 1D7 did recognize the binding domains of both apoE and apoB, identification of the corresponding epitope in apoB would also be an identification of the apoB receptor-binding domain. We have subsequently shown, however, that the ability of the 1D7 IgG preparation to block LDL-receptor binding is, in fact, due to a cross-reactivity with autologous mouse apoE rather than with human apoB, as we originally thought. Mab 1D7 binds mouse apoE and the resulting immune complexes of apoE-1D7 are co-purified with 1D7 IgG by Protein A-Sepharose immunoaffinity chromatography. The apoE present in the 1D7 IgG preparations can then compete with ^{125}I-labeled LDL for the LDL receptor. We have shown that a 35-kDa protein that is immunoreactive
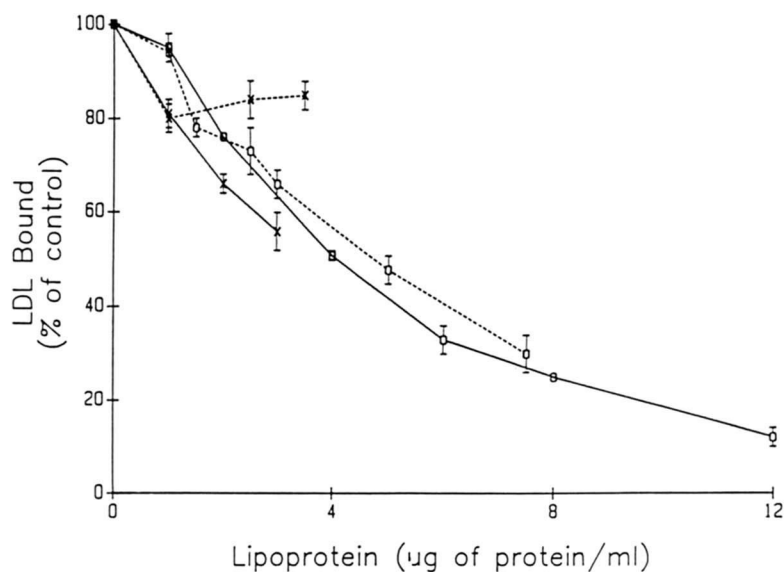

Fig. 7. Competition between human ^{125}I-labeled LDL and the retained fractions of human (——) or mouse serum (----) that had been passed over 1D7 (O) or 3B7 (X) immunoadsorbents for the binding to cultured human fibroblasts. The retained fractions from human serum or mouse serum were obtained as described in Materials and Methods. Increasing concentrations of the retained fractions were added to 3 μg of ^{125}I-labeled LDL/ml in 0.75 ml of DMEM-BSA and mixed with cultured human fibroblasts for 3 hr at 4°C. The 100% control value was 78 ng of lipoprotein protein/mg of cellular protein. Each value represents triplicate determinations.
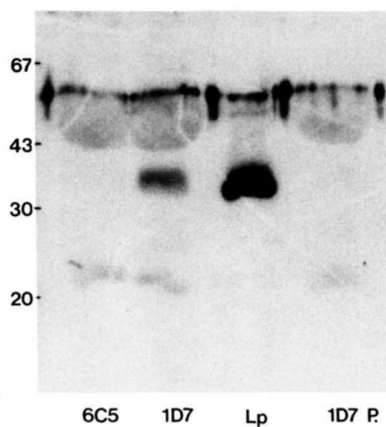
Fig. 8. Elimination of apoE from 1D7 IgG preparations by precipitation with ammonium sulfate. Mouse IgG preparations, IgG that had been ammonium sulfate-precipitated, and mouse lipoproteins were immunoblotted as described in Fig. 5. From left to right, anti-apoE Mab 6C5; anti-apoE Mab 1D7; d < 1.210 g/ml mouse lipoproteins; and anti-apoE Mab 1D7 prepared from ascites which had been precipitated at 33% ammonium sulfate (1D7 P). Molecular weight markers are indicated in kDa.

with anti-rat apoE is present in the IgG fractions of 1D7 but not in the IgG preparations of other anti-apoE Mabs which do not influence LDL-receptor binding. According to all evidence, this protein is mouse apoE. Precipitation of 1D7 ascites at 33% saturated ammonium sulfate eliminates both the mouse apoE and the capacity of the IgG to inhibit LDL-receptor binding. Finally, when mouse serum is passed over 1D7-Sepharose, the retained fraction

contains mouse apoE and is capable of blocking binding of LDL to the LDL receptor.

It is known that lipid-free apoE is not recognized by the LDL receptor (29). The fact that the mouse apoE in the 1D7 IgG preparation can compete with human LDL for the LDL receptor led us to assume that it is an apoE-containing lipoprotein that is isolated with 1D7 IgG. We did not, however, detect mouse apoA-I, apoA-IV, or apoB in 1D7 IgG that contained both immunoreactive mouse apoE and activity that inhibited LDL-receptor binding. HDL_1, having apoE as the only detectable apolipoprotein, has been shown to represent a major lipoprotein subclass in mouse plasma (T. Innerarity, unpublished results). It may be such a lipoprotein that is the major contaminant of the 1D7 IgG preparations.

The cross-reactivity of Mab 1D7 with mouse apoE is probably of low affinity. Mab 1D7 apparently does not block binding of mouse apoE-containing lipoproteins to the human LDL receptor, presumably because the affinity of Mab 1D7 for mouse apoE is less than the affinity of mouse apoE-containing lipoproteins for the LDL receptor. The low affinity of Mab 1D7 for autologous mouse apoE could explain why we were unable to demonstrate 1D7 IgG in the serum lipoprotein fraction (d < 1.210 g/ml) of hybridoma-bearing mice or to show binding of Mab 1D7 to mouse apoE on Western blots (unpublished results). Nevertheless, the affinity is sufficient to allow the co-purification of mouse apoE with 1D7 IgG during affinity chromatography on Protein A-Sepharose.

The lack of species specificity in the interaction of lipoproteins with the LDL receptor would indicate that both
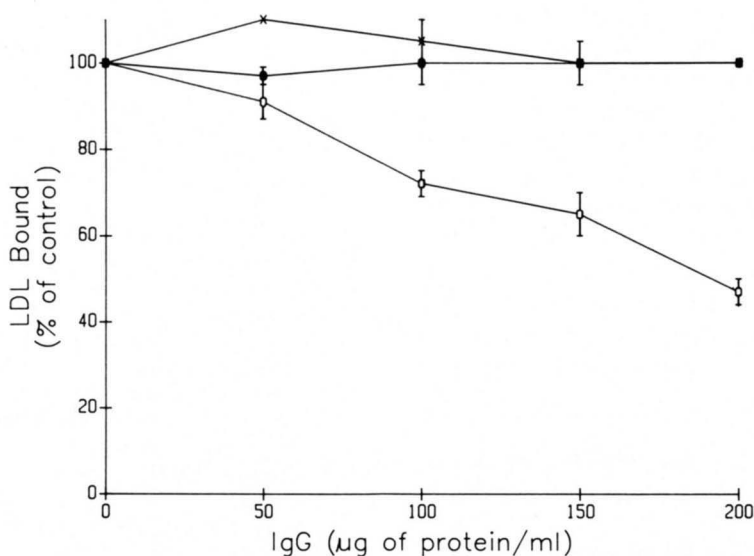

Fig. 9. Inhibition of specific binding of ^{125}I-labeled LDL to cultured human fibroblasts by anti-apoE Mab 1D7 (○), anti-apoE Mab 1D7 prepared from ascites that had been precipitated with ammonium sulfate (●), or anti-apoE Mab 6C5 (X). Binding conditions were as in Fig. 1. The 100% control value for the binding of ^{125}I-labeled LDL to the LDL receptor was 105 ng of lipoprotein protein/mg of cellular protein.

the ligand-binding domain of the LDL receptor and the receptor-binding domains of apoE and apoB have been maintained during mammalian evolution (30, 31). Mabs against human apoB that block the LDL binding to the LDL receptor have been shown to cross-react with a large panel of heterologous LDL, whereas cross-reactivity was much more limited with Mabs that failed to block LDL receptor binding (32, 33). It is, therefore, probably not by chance that Mab 1D7, which reacts with the apoE receptor-binding domain, cross-reacts with mouse apoE and that Mabs specific for other regions of the protein do not show such cross-reactivity.

A question that naturally arises from these observations concerns the generality of the phenomenon and the conditions under which one might anticipate its occurrence. As the Mab forms an immune complex with the self antigen, the problem would presumably be restricted to Mabs specific for antigens that would be present in the ascitic fluid, e.g., plasma proteins or proteins produced locally in the peritoneal cavity. Secondly, antibodies directed against conserved regions of the antigen (e.g., functional domains) would be those most likely to detect a cross-reactivity with the equivalent mouse antigen, as was the case for apoE. While antibodies tend to be produced against epitopes that are not shared between the immunogen and the host, the production of antibodies with reactivity against self-components following immunization with self-antigens in adjuvant or with cross-reacting antigens does occur. Furthermore, it should be emphasized that the reactivity of 1D7 with murine apoE was apparently of very low affinity; nevertheless, it resulted in contamination of the IgG preparations with mouse apoE.

We have demonstrated here that a Mab that detects an immunological cross-reactivity between the immunogen and the equivalent mouse protein can lead to the co-purification of the mouse protein with the Mab. Furthermore, we have shown that, when one does not have the means to distinguish between the effects attributable to the Mab and those to the contaminating mouse antigen, this can complicate the interpretation of experiments in which the Mab is used as a probe of protein function. At present, there is little reason to believe that the phenomenon described here represents a unique case. However, until IgG preparations of Mabs are systematically tested for the presence of mouse antigen, it is difficult to predict how prevalent it may be. ■

REFERENCES

1. Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science.* **232:** 34–47.
2. Innerarity, T. L., K. H. Weisgraber, S. C. Rall, Jr., and R. W. Mahley. 1987. Functional domains of apolipoprotein E and apolipoprotein B. *Acta Med. Scand. Suppl.* **715:** 51–59.
3. Milne, R. W., R. Théolis, Jr., R. B. Verdery, and Y. L. Marcel. 1983. Characterization of monoclonal antibodies against human low density lipoproteins. *Arteriosclerosis.* **3:** 23–30.
4. Knott, T. J., R. J. Pease, L. M. Powell, S. C. Wallis, S. C. Rall, Jr., T. L. Innerarity, B. Blackhart, W. H. Taylor, Y. Marcel, R. Milne, D. Johnson, M. Fuller, A. J. Lusis, B. J. McCarthy, R. W. Mahley, B. Levy-Wilson, and J. Scott. 1986. Complete protein sequence and identification of structural domains of human apolipoprotein B. *Nature.* **323:** 734–738.
5. Marcel, Y. L., T. L. Innerarity, C. Spilman, R. W. Mahley, A. A. Protter, and R. W. Milne. 1987. Mapping of human apolipoprotein B antigenic determinants. *Arteriosclerosis.* **7:** 166–175.
6. Knott, T. J., S. C. Rall, Jr., T. L. Innerarity, S. F. Jacobson, M. S. Urdea, B. Levy-Wilson, L. M. Powell, R. S. Pease, R. Eddy, H. Nakai, M. Byers, L. M. Priestly, E. Robertson, L. B. Rall, C. Betsholtz, T. B. Shows, R. W. Mahley, and J. Scott. 1985. Human apolipoprotein B: carboxyl-terminal domains, sites of gene expression, and chromosomal localization. *Science.* **230:** 37–43.
7. Hospattankar, A. V., S. W. Law, K. Lackner, and H. B. Brewer. 1986. Identification of low density lipoprotein receptor binding domains of human apolipoprotein B-100: a proposed consensus LDL receptor binding sequence of apoB-100. *Biochem. Biophys. Res. Commun.* **139:** 1078–1085.
8. Hui, D. Y., T. L. Innerarity, and R. W. Mahley. 1984. Defective hepatic lipoprotein receptor binding of β-very low density lipoproteins from type III hyperlipoproteinemic patients. Importance of apolipoprotein E. *J. Biol. Chem.* **259:** 860–869.
9. Hui, D. Y., T. L. Innerarity, R. W. Milne, Y. L. Marcel, and R. W. Mahley. 1984. Binding of chylomicron remnants and β-very low density lipoproteins to hepatic and extrahepatic lipoprotein receptors: a process independent of apolipoprotein B48. *J. Biol. Chem.* **259:** 15060–15068.
10. D. A. Chappell. 1988. Pre-β-very density lipoproteins as precursors of β-very low density lipoproteins. A model for the pathogenesis of familial dysbetalipoproteinemia (type III hyperlipoproteinemia). *J. Clin. Invest.* **82:** 628–639.
11. Sachs, F. M., and G. P. Krukonis. 1987. Subtypes of normolipidemic VLDL, which has the receptor binding region of apolipoprotein E exposed, causes most of the uptake of VLDL by U937 human macrophages. *Arteriosclerosis.* **7:** 544a.
12. Friedman, G., S. Eisenberg, D. Gavish, T. Vogel, and Y. Marcel. 1987. Effects of exogenous apo E3 on the cellular metabolism of human intermediate density lipoproteins. *Arteriosclerosis.* **7:** 501a.
13. Weisgraber, K. H., T. L. Innerarity, K. J. Harder, R. W. Mahley, R. W. Milne, Y. L. Marcel, and J. T. Sparrow. 1983. The receptor-binding domain of human apolipoprotein E:

monoclonal antibody inhibition of binding. *J. Biol. Chem.* **258:** 12348–12354.

14. Weisgraber, K. H., S. C. Rall, Jr., R. W. Mahley, R. W. Milne, Y. L. Marcel, and J. T. Sparrow. 1986. Human apolipoprotein E. Determination of the heparin binding sites of apolipoprotein E3. *J. Biol. Chem.* **261:** 2068–2076.

15. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1355.

16. Milne, R. W., P. Douste-Blazy, Y. L. Marcel, and L. Retegui. 1981. Characterization of monoclonal antibodies against human apolipoprotein E. *J. Clin. Invest.* **68:** 111–117.

17. Ey, P. L., S. J. Prowse, and Jenkin. 1978. Isolation of pure IgG1, IgG2 and IgG2b immunoglobulin from mouse serum using protein-A Sepharose. *Immunochemistry.* **15:** 429–436.

18. Weisgraber, K. H., and R. W. Mahley. 1983. Lipoproteins of non-human species. *In* CRC Handbook of Electrophoresis. Vol. IV. L. A. Lewis and K. H. Naito, editors. CRC Press, Boca Raton. 101–103.

19. Brown, M. S., and J. L. Goldstein. 1974. Familial hypercholesterolemia: defective binding of lipoproteins to cultured fibroblasts associated with impaired regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. *Proc. Natl. Acad. Sci. USA.* **71:** 788–792.

20. Milne, R. W., P. K. Weech, L. Blanchette, J. Davigon, P. Alaupovic, and Y. L. Marcel. 1984. Isolation and characterization of apolipoprotein B-48 and B-100 very low density lipoproteins from type III hyperlipoproteinemic subjects. *J. Clin. Invest.* **73:** 816–823.

21. Milne, R. W., J. Gutkowska, G. Thibault, P. Schiller, C. Charbonneau, J. Genest, and M. Cantin. 1987. A murine monoclonal antibody against rat atrial natriuretic factor (ANF) which cross-reacts with mouse ANF. *Molec. Immunol.* **24:** 127–132.

22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.

23. Théolis, R., Jr., P. K. Weech, Y. L. Marcel, and R. W. Milne. 1984. Characterization of antigenic determinants of human apolipoprotein B. Their distribution on tryptic fragments of low density lipoprotein. *Arteriosclerosis.* **4:** 498–509.

24. Olofsson, S. O., W. J. McConathy, and P. Alaupovic. 1978. Isolation and partial characterization of a new acidic apolipoprotein (apolipoprotein F) from high density lipoproteins of human plasma. *Biochemistry.* **17:** 1032–1036.

25. Lowry, O. H., N. J. Rosebrough, A. J. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.

26. Mellman, I. S., and J. Unkeless. 1980. Purification of a functional mouse Fc receptor through the use of a monoclonal antibody. *J. Exp. Med.* **152:** 1048–1069.

27. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoproteins. 1. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–221.

28. Hurn, B. A. L., and S. M. Chantler. 1980. Production of reagent antibodies. *Methods Enzymol.* **70:** 104–142.

29. Innerarity, T. L., R. E. Pitas, and R. W. Mahley. 1979. Binding of arginine-rich(E) apoprotein after recombination with phospholipid vesicles to the low density lipoprotein receptors of fibroblasts. *J. Biol. Chem.* **254:** 4186–4190.

30. Innerarity, T. L., R. E. Pitas, and R. W. Mahley. 1980. Disparities in the interaction of rat and human lipoproteins with cultured rat fibroblasts and smooth muscle cells. *J. Biol. Chem.* **255:** 11163–11172.

31. Drevon, C. A., A. D. Attie, S. H. Pangburn, and D. Steinberg. 1981. Metabolism of homologous and heterologous lipoproteins by cultured rat and human skin fibroblasts. *J. Lipid Res.* **22:** 37–46.

32. Young, S. G., J. L. Witztum, D. C. Casal, L. K. Curtiss, and S. Bernstein. 1986. Conservation of the low density lipoprotein receptor-binding domain of apolipoprotein B. *Arteriosclerosis.* **6:** 178–188.

33. Nelson, C. A., M. A. Tasch, M. Tikkanen, R. Darger, and G. Schonfeld. 1984. Evolution of low density lipoprotein structure probed with monoclonal antibodies. *J. Lipid Res.* **25:** 821–830.