Monoclonal Antibodies to the Low Density Lipoprotein Receptor as Probes for Study of Receptor-mediated Endocytosis and the Genetics of Familial Hypercholesterolemia*

(Received for publication, June 30, 1981)

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Monoclonal antibodies directed against the low density lipoprotein (LDL) receptor have been prepared by immunization of mice with a partially purified receptor from bovine adrenal cortex. Spleen cells from the mice were fused with the Sp2/0-Ag14 line of mouse myeloma cells. The most extensively studied monoclonal antibody, designated immunoglobulin-C7, reacts with the human and bovine LDL receptor, but not with receptors from the mouse, rat, Chinese hamster, rabbit, or dog. 125I-labeled monoclonal antibody binds to human fibroblasts in amounts that are equimolar to 125I-LDL. In fibroblasts from 6 of 8 patients with the receptor-negative form of homozygous familial hypercholesterolemia, which have less than 5% of normal LDL binding, the amount of monoclonal antibody binding was also less than 5% of normal. Fibroblasts from the other two receptor-negative homozygotes bound an amount of monoclonal antibody that was much greater than expected on the basis of LDL binding, suggesting that these two patients produce a structurally altered receptor that binds the antibody, but not LDL. In normal fibroblasts, the receptor-bound monoclonal antibody was taken up and degraded at 37 °C at a rapid rate similar to that for LDL. Fibroblasts from a patient with the internalization defective form of familial hypercholesterolemia bound the monoclonal antibody, but did not internalize or degrade it. The current data demonstrate the usefulness of monoclonal antibodies as probes for the study of the cellular and genetic factors involved in receptor-mediated endocytosis.

Many of the receptors on the surface of animal cells participate in receptor-mediated endocytosis. Ligands that bind to these receptors are rapidly taken into the cell when the patch of plasma membrane that contains the receptor folds inward and pinches off to form a vesicle. The ligand enclosed in the vesicle is delivered to intracellular sites, frequently to lysosomes, where the ligand is degraded. Receptor-mediated endocytosis has been studied through the use of ligands that are altered so as to permit detection by measurements of radioactivity, fluorescence, or appearance in the electron microscope. Ligands taken up through this route include plasma transport proteins, protein hormones, glycoproteins, toxins, viruses, and other plasma proteins. Many of these ligands enter cells because their receptors are clustered in discrete regions of the surface membrane called coated pits, which invaginate into the cell to form coated endocytic vesicles (reviewed in Refs. 1 and 2).

In one system of receptor-mediated endocytosis, namely the one for plasma low density lipoprotein, several naturally occurring mutations involving the structural gene for the receptor have been described in human cells (3). These mutations affect separately the ability of the receptor to bind LDL1 and its ability to be internalized after binding. Since the LDL receptor mediates the cellular uptake and degradation of plasma LDL, patients with mutations in the receptor develop high levels of plasma LDL, owing to impaired degradation, and exhibit a clinical syndrome called familial hypercholesterolemia (4).

To gain further insight into the receptor-mediated endocytosis of LDL and the genetics of this process, we have prepared monoclonal antibodies against the LDL receptor. Using technology originally described by Köhler and Milstein (5) and refined by Scharff and co-workers (6) and Kennett et al. (7), we have injected a partially purified receptor preparation into mice, fused spleen cells from immunized mice with a line of mouse myeloma cells, and obtained clones of hybrid cells that produce antibodies against the LDL receptor. Whereas monoclonal antibodies have been produced against a variety of cell surface proteins, they have not as yet been used as probes for the study of receptor-mediated endocytosis. The great advantage of a monoclonal antibody, as opposed to the polyclonal mixture of antibodies produced by animals, is its uniform composition. Inasmuch as a monoclonal antibody consists of a single molecular species that reacts with a single antigenic determinant on the receptor molecule, it can be used to assess quantitatively the number of receptors, as well as the movement of a receptor through various membrane compartments in the cell.

In the current paper, we show that a monoclonal antibody binds to the LDL receptor in an amount stoichiometric with that of LDL. The receptor-bound monoclonal antibody is internalized and degraded in lysosomes in a fashion similar to that of LDL. Moreover, mutant cells from patients with FH, which fail to bind or internalize LDL, also fail to bind or internalize the monoclonal antibody.

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* This research was supported by Research Grant HL-20948 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of a fellowship grant from Deutsche Forschungsgemeinschaft.

1 The abbreviations used are: LDL, low density lipoprotein; DMEM-G, Dulbecco's modified Eagle medium containing 4.5 g/liter of glucose; FCS, fetal calf serum; FH, familial hypercholesterolemia; HAT, selective growth medium for hybrid cells expressing hypoxanthine phosphoribosyltransferase activity; IgG, immunoglobulin; LPDS, lipoprotein-deficient serum; β-VLDL, β-migrating very low density lipoproteins; PBS, phosphate-buffered saline.
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EXPERIMENTAL PROCEDURES

Materials

Freund’s adjuvants (complete and incomplete) were purchased from DIFCO. [125I]iodide (carrier-free) was obtained from Amer sham/Searle. Protein A-Sepharose CL-4B was obtained from Pharmacia. Iso-Gen (1.3,4,8-tetrahydro-3a,6a-dihydroxy-9c-cyclononene) was purchased from Pierce. Fetal calf serum was purchased from Flow Laboratories. Medium NCTC-109 was obtained from Microbiological Associates. Dulbecco’s modified Eagle medium with 4.5 g/liter of glucose, powdered Eagle’s minimum essential medium, and Dulbecco’s phosphate-buffered saline were obtained from Grand Island Biological Company. Medium AT (medium containing bovine serum albumin). All reagents were of reagent grade or better.

Immunization—The LDL receptors used for immunization were solubilized from bovine adrenal cortex with octylglucoside, partially purified by DEAE-cellulose and agarose gel column chromatography, and precipitated with acetone in the presence of phosphatidylcholine as described (10). The final preparation bound -50 μg of [125I]LDL/mg of protein under standard assay conditions (10). The liposomes containing the receptor were suspended in buffer containing 20 mM Tris-chloride, 50 mM NaCl, and 1 mM CaCl2 (pH 8) to a final protein concentration of 50 μg/ml and were mixed with an equal volume of Freund’s adjuvant. Each mouse was immunized at intervals of three weeks with a series of three injections of LDL receptors (100 pg of protein of the DEAE-cellulose fraction was the source of the anti-receptor antibody used for the majority of the experiments in this paper, was cloned and subcloned. The H10 clone was cloned once. The cloned cells were grown in suspension in medium A. The cells were then expanded for collection of large volumes of antibody-containing ascites fluid by injection into pristane-primed BALB/C mice (7). A mouse injected with 107 cells of the C7 clone yielded, on average, -1 mg of ascites fluid containing 1.5 mg/L of IgG (see below).

Cell Fusion—Spleen cells from an immunized mouse were fused with myeloma cells according to standard methods (6, 7). In brief, the spleen lymphoid cells were collected by perfusing the spleen with medium B (Eagle’s minimal essential medium containing 20 mM N-2-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid at pH 7.4). Contaminating erythrocytes were removed by suspending the cells in ice-cold 0.17 M NH4Cl, followed by centrifugation (7). For each fusion, viable spleen lymphoid cells (2 × 108) and viable myeloma cells (4 × 107) were mixed in 0.5 ml of 30% polyethylene glycol 4000 in medium A adjusted to pH 8 and centrifuged for 6 min (7). The total exposure time to polyethylene glycol was 8 min. The cell pellet was diluted with 10 ml of medium B and again subjected to centrifugation.

The pellet was resuspended in HAT medium containing 200 μg per ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BrdG) and 1% FCS, pH 7.4, to a final concentration of 106 cells/ml. The suspension was divided into 100 μl aliquots and each aliquot was plated in 25-cm2 tissue culture flasks. After 2 days of incubation, the cells were removed by trypsinization and the cell number of each flask was determined by dilution and plating on noncoated tissue culture dishes. After an additional 1-2 days of growth, the cells were expanded to large flasks and harvested for antibody production.

Solid Phase Indirect Binding Assay—In the initial screening step, antibodies to the LDL receptor were detected by a solid-phase indirect binding assay. Aliquots (25 μl) of partially purified bovine adrenal LDL receptors (2.5 μg of protein of the DEAE-cellulose fraction (10) containing sufficient receptor to bind 25 ng of [125I]LDL) were plated into 96-well polyvinyl microtiter plates and dried overnight at 37 °C. Just prior to assay, the protein was fixed to the bottom of each well by addition of 100 μl of 50% methanol, followed by two washes of 100 μl of Buffer C (PBS containing 20 mg/ml of bovine serum albumin). Aliquots of medium from each growing culture (50-100 μl) were added to each precoated well and incubated for 2 h at room temperature. Each well was then washed 5 times with 100 μl of Buffer C, after which a saturating amount of [125I]labeled goat anti-mouse IgG (25 μg, 1.5 to 5 × 106 cpm) in 25 μl of PBS was added. After incubation for 2 h at room temperature, each well was washed 5 times with 100 μl of PBS. Blank binding values were determined by substituting fresh HAT medium for the culture supernates. All culture supernates that gave values for [125I]IgG binding that were at least 2-fold above the blank values (<1000 cpm/well) were submitted to a second screening step.

In the second screening step, the assay was identical to the first assay, except that 40 μg of protein of octylglucoside-solubilized extracts (10) from either normal or receptor-negative FH homozygote fibroblasts (SV-40 transformed) were fixed to each well and used as the solid-phase antigen in place of bovine adrenal LDL receptors. The LDL receptor activity of the soluble extracts from the normal and FH homozygote cells was 1.5 and <0.06 μg of [125I]LDL binding activity/mg of protein, respectively. Aliquots of medium from each hybridoma culture that was positive in the first screening step were assayed in pairs of wells precoated with normal and FH homozygote cell extracts. Nonspecific binding was <2500 cpm/well. Each culture supernate that gave values for [125I]IgG binding that were at least 2-fold lower in wells coated with normal fibroblast extracts as compared with wells coated with FH homozygote extracts were considered as positive hybridomas and were processed further as described below.

Cloning and Expansion of Hybridomas—Monoclonal hybridoma cell lines were cloned by limiting dilution (7). The C7 clone, which was the source of the anti-receptor antibody used for the majority of the experiments in this paper, was cloned and subcloned. The H10 clone was cloned once. The cloned cells were grown in suspension in medium A. The cells were then expanded for collection of large volumes of antibody-containing ascites fluid by injection into pristane-primed BALB/C mice (7). A mouse injected with 107 cells of the C7 clone yielded, on average, ~1 mg of ascites fluid containing 1.5 mg/L of IgG (see below).

Purification and Iodination of Monoclonal IgG—Cells were removed from the ascites fluid by centrifugation (18,000 rpm, 15 min, room temperature). An IgG fraction was isolated from the supernate on columns of Protein A-Sepharose CL-4B that were equilibrated with 0.1 M sodium phosphate (pH 7) (11). The IgG was eluted with a solution containing 1 M acetic acid and 0.1 M glycine (pH 3), after which the pH was raised immediately by addition of 1 M potassium phosphate (dibasic). The IgG fraction was dialyzed against Buffer D (10 mM sodium phosphate and 50 mM NaCl at pH 7.4) and stored at -70 °C at a concentration of 2-6 mg/ml. The class and subclass of the antibody produced by the C7 clone were determined by Ouchterlony immunodiffusion using antisera specific for the IgG subclasses which was kindly provided by Drs. Robert W. Mahley and Thomas Innerarity (Gladstone Foundation Laboratories, San Francisco, CA).

Lipoproteins

Human LDL (density, 1.019 to 1.063 g/ml) and lipoprotein-deficient serum (LpDS, density > 1.215 g/ml) were prepared from plasma of individual healthy subjects by ultracentrifugation (10). Human HDL was radioiodinated with [125I] by the iodine monochloride method (10). Rabbit β-VLDL was prepared as previously described (13). Canine apo E-HDL, was kindly provided by Drs. Robert W. Mahley and Thomas Innerarity (Gladstone Foundation Laboratories, San Francisco, CA).

Cultured Fibroblasts

SV-40-transformed skin fibroblasts from a control subject and from a patient with the receptor-negative form of homocystinuria FH were used as the source of fibroblast LDL receptors in the solid-phase binding assay for detection of anti-receptor antibodies. Nontransformed skin fibroblasts from control subjects and from patients with the heterozygous and homozygous forms of FH were used for all metabolic
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Hybridomas, 5 were positive when further screened for the presence of an antibody that would bind to solubilized extracts of hybridomas. About 12% of these wells (15/126) fused with the Sp2/0-Ag14 line of mouse myeloma cells. Approximately 8% (126/1680) of the wells produced viable hybridomas. For $^{125}$I-LDL and $^{125}$I-IgG-C7 binding experiments performed at 4°C, cell monolayers were placed for 30 min in a 4°C cold room, after which the medium was removed and replaced with 2 ml of ice-cold medium B containing 10% human LPDS (14).

Assays

The total cellular binding of $^{125}$I-LDL to fibroblast monolayers at 4°C was determined using the standard wash procedure as previously described (14). The total cellular binding of $^{125}$I-IgG-C7 to fibroblast monolayers at 4°C was determined exactly as described for $^{125}$I-LDL binding (14) except that the cells were washed 4 times rapidly, then incubated for 3 min in wash buffer, followed by a rapid final wash. The amounts of surface-bound and internalized $^{125}$I-LDL in fibroblast monolayers at 37°C were determined by the dextran sulfate release assay (14). The total cellular content (surface-bound plus internalized) of $^{125}$I-LDL and $^{125}$I-IgG-C7 at 37°C was determined as described for $^{125}$I-LDL (14) except that the wash procedure for $^{125}$I-IgG-C7 was modified as described above. The proteolytic degradation of $^{125}$I-LDL and $^{125}$I-IgG-C7 by fibroblast monolayers at 37°C was determined by measuring the amount of $^{125}$I-labeled trichloroacetic acid-soluble (noniodide) material formed by the cells and extracted into the culture medium (15). The protein content of lipoproteins, antibodies, and cell extracts was determined by the method of Lowry et al. (16) with bovine serum albumin as a standard. Unless otherwise stated in the legends, each data point represents the average of duplicate incubations.

Localization of Monoclonal Anti-receptor Binding Sites in Fibroblasts by Indirect Immunofluorescence—On Day 7 of cell growth, monolayers of fibroblasts grown on glass coverslips were exposed to 50 μg/ml (340 nm) of anti-receptor IgG-C7 for 1 h at either 4 or 37°C. The cells were then washed (17) and fixed for 15 min at room temperature in 3% paraformaldehyde in Buffer F (10 mM sodium phosphate, 0.15 M NaCl, and 2 mM MgCl₂ at pH 7.4), after which they were rinsed once with 2 ml of 50 mM NH₄Cl and twice with Buffer F (Fig. 7, A to D). Coverslips that had been incubated with IgG-C7 at 37°C (Fig. 7, E to G) were then treated with 3 ml of Buffer F containing 0.05% Triton X-100 for 10 min at −10°C. Both sets of coverslips (A to D) were then placed in a fresh Petri dish (cell side up) covered with 60 ml of goat anti-mouse IgG coupled to fluorescein (Cappel Laboratories). Both sets of coverslips (A to D) were washed at room temperature 4 times (15 min/wash) with 2 ml of Buffer F and mounted on glass slides with 90% glycerol in 0.1 M Tris-HCl at pH 9.4. The coverslips were viewed with a Zeiss photomicroscope III equipped with a 100-watt mercury epifluorescence light source and the appropriate filter package. Photographs were taken on Kodak Tri-X film at an ASA of 400 using the automatic photometer of the Zeiss instrument and were developed in Microdol X (Kodak) (17).

RESULTS

Preparation of Monoclonal Antibody, IgG-C7—Mice were immunized with a partially purified preparation of LDL receptors from both fetal and adult bovine adrenal cortex. The spleen cells were fused with the Sp2/0-Ag14 line of mouse myeloma cells. Approximately 8% (126/1680) of the wells produced viable clusters of hybridomas. About 12% of these wells (15/126) produced an antibody that bound to the partially purified LDL receptors from bovine adrenal cortex. Of these 15 positive hybridomas, 5 were positive when further screened for the presence of an antibody that would bind to solubilized extracts of plasma membranes from normal human fibroblasts, but not fibroblasts from a patient with receptor-negative homozygous FH. Antibody-producing clones from these 5 hybridomas were isolated as described under "Experimental Procedures." These clones were then expanded by injection into the peritoneal cavity of mice. Immunoglobulin G was isolated from the ascitic fluid on columns of Sepharose-bound Protein A from Staphylococcus aureus. The IgG was iodinated with $^{125}$I and used for further studies. The monoclonal antibody used for the current studies is designated IgG-C7. The antibody contains heavy chains of the IgG2b subclass.

Fig. 1A shows a saturation curve for the binding of $^{125}$I-IgG-C7 monolayers of normal human fibroblasts at 4°C. The curve had two components: a high affinity component that approached saturation at an IgG concentration of ~3 nM (0.45 μg/ml), and a nonspecific component that rose linearly at concentrations of $^{125}$I-IgG-C7 up to 16 nM. In cells from a patient with receptor-negative homozygous FH, no high affinity binding of the antibody was observed. There was only a linear binding that paralleled the nonspecific component in the normal cells. Previous studies have shown that the number of LDL receptors in normal fibroblasts is markedly reduced when the cells are grown in the presence of a mixture of 25-hydroxycholesterol plus cholesterol (4). Fig. 1B shows that the high affinity binding of $^{125}$I-IgG-C7 was also abolished by this sterol treatment.

Fig. 2 compares the saturation curves for binding of $^{125}$I-LDL (Fig. 2A) and $^{125}$I-IgG-C7 (Fig. 2B) to normal fibroblasts at 4°C. The binding experiments were performed in the absence or presence of an excess of unlabeled ligand. The difference between binding in the absence and presence of unlabeled ligand is the high affinity binding and it is indicated by the dashed lines in Fig. 2. Half-maximal binding for $^{125}$I-LDL to the high affinity receptor occurred at ~2 nM (Fig. 2A). Half-maximal binding for $^{125}$I-IgG-C7 occurred at ~1 nM (Fig. 2B). At saturation, the cells bound equimolar amounts of $^{125}$I-LDL and $^{125}$I-IgG-C7 (~175 fmol/mg of cell protein). Thus, in normal fibroblasts, there is one high affinity monoclonal antibody binding site for each high affinity LDL binding site.

We next performed a series of experiments in which parallel dishes of fibroblasts were incubated at 4°C with saturating...
levels of either $^{125}$I-LDL or $^{125}$I-IgG-C7 and the amount of high affinity binding of each ligand at saturation was measured (Fig. 3). One of the control cell strains was studied on 8 different occasions (closed circle with error bars). The maximal binding of $^{125}$I-IgG-C7 in this strain averaged 221 femtomoles/mg of cell protein. The maximal binding of $^{125}$I-LDL was similar, 258 femtomoles/mg. In cells from three other control subjects, there was also a close correlation between LDL and antibody binding (●). In cells from 2 patients with heterozygous FH, there was a proportionate reduction in the binding of both ligands (●). Cells from 8 patients with the receptor-negative form of homozygous FH showed less than 5% of the normal $^{125}$I-LDL binding activity. Six of these eight mutant strains also bound less than 5% of the normal amount of $^{125}$I-IgG-C7 (△). However, cells from two of the eight receptor-negative patients appeared to be different (indicated by asterisks). In these two subjects, the amount of $^{125}$I-IgG-C7 binding was much higher than would be expected on the basis of the small amount of $^{125}$I-LDL binding. We also studied fibroblasts from seven patients with the receptor-defective form of homozygous FH, whose cells bound 10–25% of the normal amount of $^{125}$I-LDL (△). In general, these cells also bound 10–25% of the normal amount of $^{125}$I-IgG-C7. However, there was one receptor-defective patient whose cells appeared to bind significantly more antibody than expected on the basis of the level of $^{125}$I-LDL binding (indicated by asterisks). It is possible that the individuals whose fibroblasts show inappropriately high antibody binding activity relative to LDL binding activity (indicated by asterisks in Fig. 3) represent patients whose mutations destroy the LDL binding site of the receptor, but leave a residual amount of antigenic activity (see “Discussion”).

Receptor-mediated Endocytosis of Monoclonal IgG-C7 in Fibroblasts—Fig. 4 shows a time course experiment designed to determine the fate of the receptor-bound $^{125}$I-IgG-C7 in intact normal fibroblasts at 37 °C. The cellular content of $^{125}$I-IgG-C7 reached a plateau by 1 h and remained constant over the ensuing 5 h. After a brief lag, degradation products appeared in the medium in the form of $^{125}$I-labeled acid-soluble noniodide radioactivity. This time course is similar to the time course previously observed for the degradation of receptor-bound $^{125}$I-LDL in fibroblasts (14, 15). By 6 h, ~4 times as much $^{125}$I-IgG-C7 had been degraded as was present in the cells in the steady state. Degradation, but not uptake, of the $^{125}$I-IgG-C7 was completely blocked by 75 μM chloroquine, an agent that disrupts lysosomal function (18), indicating that degradation occurred within lysosomes (data not shown).
TABLE 1

Metabolism of $^{125}$I-lgG-C7 by normal and FH homozygote fibroblasts at 37°C

On Day 7 of cell growth, each monolayer of the indicated fibroblast strain received 1.5 ml of medium E with 10% LPDS containing either 20 nM of $^{125}$I-lgG-C7 (21 cpm/fmol) in the absence or presence of 670 nM unlabeled IgG-C7 (Experiment A) or 34 nM of $^{125}$I-lgG-C7 (25 cpm/fmol) in the absence or presence of 1000 nM of unlabeled IgG-C7 (Experiment B). After incubation for 5 h at 37°C, the cellular content of $^{125}$I-lgG-C7 and the amount of $^{125}$I-lgG-C7 degraded by the cells and released into the medium were determined. Specific (or high affinity) values were calculated as described in the legend to Fig. 2.

| Cell strain             | Concentration of $^{125}$I-lgG-C7 in medium | Cellular content of $^{125}$I-lgG-C7 | Degradation of $^{125}$I-lgG-C7 |
|-------------------------|--------------------------------------------|-------------------------------------|---------------------------------|
|                         | Total High affinity                        | Total High affinity                 |                                 |
| Experiment A            |                                            |                                     |                                 |
| Normal                  | 20                                         | 780                                  | 1390                            |
| FH Homozygote, receptor-negative | 20                                         | 60                                   | 0.9                             |
| Experiment B            |                                            |                                     |                                 |
| Normal                  | 34                                         | 1150                                 | 2900                            |
| FH Homozygote, internalization-defective | 34                                         | 350                                  | 190                             |

As expected from their failure to bind $^{125}$I-lgG-C7 at 4°C, fibroblasts from a receptor-negative FH homozygote failed to take up significant amounts of $^{125}$I-lgG-C7 at 37°C or to degrade the antibody with high affinity (Table I, experiment A). Fibroblasts from a patient with the internalization-defective form of FH are known to bind 60 to 80% of the normal amount of $^{125}$I-LDL at the cell surface, but to internalize and degrade less than 10% of the normal amount (3, 19). When these cells were incubated with $^{125}$I-lgG-C7 at 37°C, the total cellular content of radioactivity (surface-bound plus internalized) was ~20% of normal (Table I, experiment B). The amount of high affinity degradation of the $^{125}$I-lgG-C7 was only 6% of normal. These findings are consistent with the notion that the internalization-defective cells bind the $^{125}$I-lgG-C7 at the receptor site, but fail to internalize or degrade it, just as they do for $^{125}$I-LDL.

To further probe the relation between metabolism of the $^{125}$I-lgG-C7 and $^{125}$I-LDL, we incubated normal fibroblasts with increasing concentrations of either ligand for 5 h at 37°C, after which the amount of $^{125}$I-ligand associated with the cells and the amount degraded were measured (Fig. 5). As previously reported (14, 15), the saturation curves for $^{125}$I-LDL uptake (Fig. 5A) and $^{125}$I-LDL degradation (Fig. 5B) were similar. On average, at each $^{125}$I-LDL concentration, 3-fold more $^{125}$I-LDL had been degraded than was associated with the cell. When the cells were treated with 25-hydroxycholesterol plus cholesterol, the high affinity uptake and degradation of $^{125}$I-LDL was abolished, and only a nonspecific linear uptake was obtained. The difference between the uptake or degradation of $^{125}$I-LDL in the absence and presence of sterols is equivalent to the specific receptor-mediated uptake or degradation and is shown by the dashed lines.

The results with $^{125}$I-lgG-C7 were similar to those obtained with $^{125}$I-LDL (Fig. 5, C and D). When the data for the sterol-treated cells were subtracted from the data from the untreated cells, the absolute value for high affinity uptake of $^{125}$I-lgG-C7 at saturation (dashed line) was similar to that for $^{125}$I-LDL (~1.8 pmol/mg of cell protein) (cf. Figs. 5A and 5C). Similarly, the amount of degradation at saturation was the same for $^{125}$I-lgG-C7.

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LDL and $^{125}$I-IgG-C7 (~6.5 pmol/mg) (cf. Figs. 5B and 5D).

Differences in Binding Affinity of Monoclonal Igg-C7 at 4 and 37 °C—Previous studies have shown that the affinity of the fibroblast LDL receptor for LDL is ~10-fold lower at 37 °C, as compared with 4 °C (14). A comparison of the 37 °C uptake data for $^{125}$I-LDL in Fig. 5 with the 4 °C binding data of Fig. 2 shows this 10-fold decrease in affinity at 37 °C, as compared with 4 °C. An even more striking difference was observed in the apparent affinity of the $^{125}$I-IgG-C7 for the receptor at the two temperatures. Half-maximal binding at 4°C was achieved at a $^{125}$I-IgG-C7 concentration of ~1 nM (Fig. 1), whereas a concentration of 75 nM was required for half-maximal uptake at 37 °C (Fig. 7 C and D), suggesting that the affinity of the receptor for the antibody was ~75-fold lower at 37 °C, as opposed to 4 °C.

One reason for the lower affinity of the antibody for the receptor at 37 °C was the rapid dissociation of the receptor-antibody complex at the higher temperature. Fig. 6 shows an experiment in which fibroblasts were incubated at 4 °C with $^{125}$I-IgG-C7, washed, and then incubated further at either 4 or 37 °C. When the normal cells were incubated at 4 °C, the dissociation of the $^{125}$I-IgG-C7 from the receptor was extremely slow (Fig. 7A). No detectable dissociation occurred over the first 60 min. At 37 °C, there was a biphasic loss of $^{125}$I-IgG-C7 from the cells. About one-third of the cell-bound radioactivity left the normal cell within the first 10 min and appeared in the medium in a form that was precipitable by trichloroacetic acid (Fig. 6B). Over the ensuing 2 h, there was a slight further loss of acid-precipitable radioactivity. A small amount of acid-soluble radioactivity also appeared in the medium.

To study the dissociation of cell-bound $^{125}$I-IgG-C7 in the absence of internalization, we performed the same experiment using fibroblasts from a patient with the internalization-defective form of FH. In these mutant cells, the amount of binding of $^{125}$I-IgG-C7 at 4 °C was ~50% of normal (compare Fig. 6C with Fig. 6A). At 4 °C, the rate of dissociation of the $^{125}$I-IgG-C7 from the receptor was slow, as in the normal cells. However, at 37 °C, most of the radioactivity left the cell within 15 min. This radioactivity appeared in the culture medium entirely as acid-precipitable radioactivity (Fig. 6D). No degradation occurred. Thus, in the internalization-defective FH cells, as well as in the normal cells, the rate of dissociation of the $^{125}$I-IgG-C7 from the receptor was much faster at 37 °C than at 4 °C.

Immunofluorescence Staining Pattern of Monoclonal IgG-C7 in Fibroblasts—Monolayers of fibroblasts were incubated with IgG-C7 for 1 h at 4 °C and then incubated with fluorescein-coupled goat anti-mouse IgG followed by fluorescein-coupled rabbit anti-goat IgG. The surface fluorescence appeared as discrete dots organized in linear arrays (Fig. 7A). This appearance is the same as that seen when the receptor is labeled directly with fluorescent reconstituted LDL (20). Only faint nonspecific binding was seen in cells from a receptor-negative FH homozygote (Fig. 7B). Another group of normal fibroblasts were incubated with the IgG-C7 for 1 h at 37 °C, then fixed and permeabilized with Triton X-100 prior to incubation with the fluorescein-coupled goat anti-mouse IgG (Fig. 7C). The IgG-C7 was localized within the cell in perinuclear vacuoles that correspond to the general distribution of lysosomes. Receptor-negative FH homozygote cells contained no detectable intracellular fluorescence after incubation with the IgG-C7 (Fig. 7D).

Competition between Monoclonal Antibody and Lipoproteins for Binding to the LDL Receptor—Unlabeled IgG-C7 competed with $^{125}$I-LDL for binding to the LDL receptor of intact fibroblasts at 4 °C (Fig. 8). Binding of $^{125}$I-LDL was reduced by 70% at an IgG-C7 concentration of 2 nM, which is in the same range as the concentration required for saturation of $^{125}$I-IgG-C7 binding at 4 °C (Fig. 2). Complete inhibition of $^{125}$I-LDL binding was not achieved, even when the IgG-C7 concentration was increased to 670 nM. At this concentration,
by an excess of unlabeled LDL reduced by a control mouse monoclonal antibody not directed against the LDL receptor.

In other experiments, we found that $^{125}$I-LDL binding was not specific in that it could be competitively reduced by a small amount of $^{125}$I-LDL binding persisted. This residual binding was specific in that it could be competitively reduced by an excess of unlabeled LDL ($\times$ on the ordinate of Fig. 8). In other experiments, we found that $^{125}$I-LDL binding was not reduced by a control mouse monoclonal antibody not directed against the LDL receptor.

At $37^\circ$C, the IgG-C7 reduced in parallel the binding, internalization, and degradation of $^{125}$I-LDL (Fig. 9). A 50% reduction occurred at an IgG-C7 concentration of $\sim 15$ nM, which is about one-fifth the concentration of $^{125}$I-IgG-C7 required for 50% maximal uptake at $37^\circ$C (see “Discussion”).

Although the IgG-C7 competitively reduced the binding of $^{125}$I-LDL to the cells at $4^\circ$C, the converse was not true. As shown in Table II, LDL did not reduce the binding of $^{125}$I-IgG-C7, even when the cells were incubated with the LDL at supersaturating levels for 1 h prior to addition of the $^{125}$I-labeled monoclonal antibody. We also tested two ligands that have extremely high affinities for the LDL receptor, rabbit $\beta$-VLDL ($K_d = \sim 0.4$ nm), and canine apo E-HDL ($K_d = \sim 0.1$ nm). Neither of these ligands reduced the binding of $^{125}$I-IgG-C7, even when added at concentrations 12 to 100-fold above their respective $K_d$s. To ensure that the $^{125}$I-IgG-C7 binding observed in this experiment was specific, we showed that the binding was reduced by an excess of unlabeled IgG-C7. Binding of $^{125}$I-IgG-C7 also was reduced by a monoclonal antibody derived from another clone, IgG-H10. Binding was not reduced by human IgG. In other experiments, we have found that the binding of $^{125}$I-IgG-C7 is not reduced by IgG isolated from the serum of nonimmunized mice or by a control mouse monoclonal antibody not directed against the LDL receptor (data not shown).

**TABLE II**

**Effect of prior incubation with unlabeled lipoproteins and IgG preparations in preventing the binding of $^{125}$I-labeled monoclonal anti-receptor IgG-C7 to human fibroblasts**

On Day 7 of cell growth, each monolayer of normal fibroblasts received 1.5 ml of medium B containing 10% LPDS and the indicated concentration of IgG-C7. After incubation for 30 min at $37^\circ$C, $^{125}$I-LDL was added to give a final concentration of 20 nM (10 pg/ml, 250 cpm/mg of protein). The cells were incubated for an additional 3 h at $37^\circ$C, after which the total amounts of surface-bound $^{125}$I-LDL (Fig. 8), internalized $^{125}$I-LDL (C), and $^{125}$I-LDL degradation products excreted into the medium (A) were determined. The “100% of control” values for surface-bound, internalized, and degraded $^{125}$I-LDL in the absence of IgG-C7 were 138, 882, and 2870 fmol/mg of protein, respectively. Each data point represents a single incubation except for the “100% of control” values, which represent the average of triplicate incubations.

![Graph](image)

**Fig. 9. Inhibition of the binding, internalization, and degradation of $^{125}$I-LDL in normal fibroblasts at $37^\circ$C by monoclonal anti-receptor IgG-C7.** On Day 7 of cell growth, each monolayer received 1.5 ml of medium B containing 10% LPDS and the indicated concentration of IgG-C7. After incubation for 30 min at $37^\circ$C, $^{125}$I-LDL was added to give a final concentration of 20 nM (10 pg/ml, 250 cpm/mg of protein). The cells were incubated for an additional 3 h at $37^\circ$C, after which the total amounts of surface-bound $^{125}$I-LDL (Fig. 8), internalized $^{125}$I-LDL (C), and $^{125}$I-LDL degradation products excreted into the medium (A) were determined. The “100% of control” values for surface-bound, internalized, and degraded $^{125}$I-LDL in the absence of IgG-C7 were 138, 882, and 2870 fmol/mg of protein, respectively. Each data point represents a single incubation except for the “100% of control” values, which represent the average of triplicate incubations.

**TABLE II**

**Effect of prior incubation with unlabeled lipoproteins and IgG preparations in preventing the binding of $^{125}$I-labeled monoclonal anti-receptor IgG-C7 to human fibroblasts**

On Day 7 of cell growth, each monolayer of normal fibroblasts received 1.5 ml of ice-cold medium B containing 10% LPDS and the indicated addition at the indicated concentration. After a prior incubation for 1 h at $4^\circ$C, 14 nM of $^{125}$I-IgG-C7 (62 cpm/fmol) was added to each dish. The cells were incubated further for 2 h at $4^\circ$C, after which the total amount of $^{125}$I-IgG-C7 bound to the cells was determined.

| Addition during prior incubation | Concentration of addition in medium | $^{125}$I-IgG-C7 bound to cells |
|----------------------------------|-----------------------------------|-------------------------------|
|                                  | $\mu$g protein/ml | nM (Kd) | fmol/mg protein |
| None                             | 0                  | 0     | 194 (100%)*    |
| Human LDL                        | 2                  | 4     | 154 (79%)     |
|                                  | 10                 | 20    | 154 (79%)     |
|                                  | 30                 | 60    | 154 (79%)     |
| Rabbit $\beta$-VLDL              | 0.5                | 0.25  | 174 (90%)     |
|                                  | 2                  | 1     | 161 (83%)     |
|                                  | 10                 | 5     | 154 (79%)     |
| Canine apo E-HDL                 | 0.2                | 0.4   | 181 (93%)     |
|                                  | 1                  | 2     | 174 (90%)     |
|                                  | 5                  | 10    | 174 (90%)     |
| Mouse monoclonal IgG-C7         | 5                  | 33    | 44 (23%)      |
|                                  | 20                 | 134   | 27 (14%)      |
|                                  | 100                | 670   | 21 (11%)      |
| Mouse monoclonal IgG-H10        | 5                  | 33    | 56 (29%)      |
|                                  | 20                 | 134   | 28 (14%)      |
|                                  | 90                 | 603   | 19 (10%)      |
| Human IgG                        | 5                  | 33    | 154 (79%)     |
|                                  | 20                 | 134   | 201 (103%)    |
|                                  | 100                | 670   | 194 (100%)    |

* The numbers in parentheses indicate the percentage of the control value, 194 fmol/mg of protein.

**DISCUSSION**

This paper describes the preparation of a monoclonal antibody against the LDL receptor and its use in the study of the genetics of receptor-mediated endocytosis of LDL. The first conclusion of these studies is that the monoclonal antibody, designated IgG-C7, binds to human fibroblasts in amounts that are equimolar to the binding of LDL. This suggests that each LDL receptor has one antibody binding site/LDL binding site. When the receptors are suppressed, as when the cells are treated with 25-hydroxycholesterol plus cholesterol, the binding of LDL and IgG-C7 are reduced proportionately. Moreover, when the receptor number is reduced by mutation, as occurs in FH heterozygotes and homozygotes, the binding of IgG-C7 is reduced proportionately (with certain notable exceptions, as discussed below).

After binding to the receptor, monoclonal IgG-C7 was rapidly internalized at $37^\circ$C by the cells and degraded in lysosomes in a manner similar to the receptor-mediated uptake of LDL. An unexpected finding was the observation that the monoclonal antibody apparently can be detached from the receptor within the cell, allowing the receptor to undergo its usual recycling process in which it returns to the surface and binds another molecule of antibody. This conclusion follows from the kinetic data showing that the cells continue to bind, internalize, and degrade the IgG-C7 at a steady rate without any depletion of receptors for at least 6 h at $37^\circ$C (Fig. 4). Continuous uptake of LDL in this fashion has been shown to be due to recycling of the LDL receptor to the surface after its dissociation from LDL within the cell (17, 21).

The ability of the LDL receptor to recycle in the presence of the monoclonal antibody may be dependent on its rapid dissociation from the receptor at $37^\circ$C (Fig. 6). If this dissociation occurs within the cell, it would liberate the receptor so that it could return to the surface and bind another molecule of antibody. Previously we have studied a polyclonal rabbit antibody to the LDL receptor (9). This antibody does not readily dissociate from the receptor at $37^\circ$C.2 Since dissociation of the rabbit antibody does not occur, the receptor cannot return to the surface. When fibroblasts are incubated with

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2 Unpublished observations.
this polyclonal antibody, the receptors on the surface immediately decline and remain low for several hours. Under these conditions, the receptors can be shown by indirect immunofluorescence to be localized within the cell in an intact form (21).

As a result of the rapid dissociation of the monoclonal antibody from the LDL receptor at 37 °C, its apparent affinity for the receptor is ~75-fold lower at 37 °C than at 4 °C. The affinity of the receptor for LDL is also ~10-fold lower at 37 °C, as opposed to 4 °C, and this may also be due, at least in part, to a more rapid dissociation at the higher temperature (19). In the case of LDL, the dissociation rate is further increased when the pH is lowered below 6 (22). The ability of the ligand (either LDL or IgG-C7) to dissociate from the receptor is crucial if recycling of the receptor is to occur.

Cells from a patient with the internalization-defective form of FH bound the monoclonal antibody but failed to degrade significant amounts of it. This failure of degradation is consistent with a failure to internalize the ligand. Also consistent with this interpretation was the finding that the amount of 125I-IgG-C7 bound to the surface of the internalization-defective cells at 4 °C was 50% of normal (Fig. 6C), whereas at 37 °C, the total cellular content of 125I-IgG-C7 (surface-bound plus internalized) was only 20% of normal (Table I). The greater degree of abnormality at 37 °C is presumably due to a lack of internalization. The rapid internalization of the monoclonal antibody in normal cells and the apparent lack of internalization in the internalization-defective FH cells strongly suggests that the IgG-C7 enters the cell through the normal LDL uptake mechanism, and not by inducing an abnormal internalization of the receptor. Studies with ferritin-labeled IgG-C7 are currently underway to confirm that this rapid internalization takes place through coated pits.

Although the LDL receptor has one IgG-C7 binding site for each LDL binding site, the two sites do not appear to be identical, as indicated by the following observations. Under conditions in which the receptor was fully occupied by prior incubation with LDL or with the higher affinity ligands β-VLDL and apo E-HDLa, the 125I-IgG-C7 was still able to bind in normal amounts. Moreover, when 125I-LDL was pre-bound to the receptor at 4 °C, the addition of a saturating amount of IgG-C7 did not cause the LDL to dissociate at 4 °C (data not shown). On the other hand, when the cells were first allowed to bind the IgG-C7 at 4 °C, the subsequent binding of 125I-LDL was reduced by ~80% (Fig. 8). Thus, although the IgG-C7 does not bind directly to the LDL binding site, the binding of the antibody may cause a structural change that occludes the LDL binding site. This could involve cross-linking of adjacent receptor molecules, a conformational change in the receptor, or simple steric hindrance by the antibody. It is of interest that these changes reduce the binding of 125I-LDL at 4 °C by a maximum of ~80%, but not by 100% (Fig. 8). At 37 °C, the monoclonal antibody completely blocks 125I-LDL binding, uptake, and degradation (Fig. 9). Half-maximal inhibition is achieved at an IgG-C7 concentration of ~15 nm, a concentration at which only ~20% of the receptors are occupied with antibody (Fig. 5). The complex stoichiometry of the interaction between the antibody binding site and the LDL binding site may be related to the possible organization of the LDL receptor in clusters of 4 binding sites (23, 24). Such an organization has been postulated on the basis of kinetic studies showing that 1 molecule of apoE-HDL, can bind to 4 LDL binding sites (23, 24). Further studies with monoclonal antibodies should allow testing of this hypothesis.

One of the major uses of the monoclonal antibody will be as a probe for study of possible mutations in the structural gene for the LDL receptor in subjects with FH. In most of the mutant cell strains so far studied, the observed reduction in LDL binding sites is associated with a parallel reduction in binding sites for 125I-IgG-C7. This reduction in antibody binding suggests one of two possibilities: 1) the absolute amount of receptor protein on the cell surface is drastically reduced, or 2) the receptor protein is present in near normal amounts, but is altered so as to lose its antigenic site, as well as its LDL binding site. We should be able to distinguish between these two possibilities by obtaining a variety of monoclonal antibodies that recognize different antigenic determinants (epitopes) on the receptor protein. Absence of multiple antigenic determinants would suggest an absence of the entire protein.

Fibroblasts from three FH homozygotes appear to possess at least one gene that produces a receptor that binds much more antibody than it does LDL. Two of these subjects are classified as receptor-negative with no significant LDL binding activity and one is receptor-defective with 25% of normal LDL binding activity. The findings in these 3 cell strains have been reproduced several times. Further studies are now in progress to determine whether these cells possess a structurally altered receptor with loss of the LDL binding site and preservation of the antibody binding site.

The monoclonal antibody used in the current studies was produced by immunizing a mouse with an LDL receptor partially purified from cows. We selected a clone of cells that produced an antibody that reacted with human fibroblasts. It is of interest that this antibody does not bind to the LDL receptor of cultured mouse 1 cells, mouse adrenal Y-1 cells, or Chinese hamster ovary cells (data not shown), even though these cells contain a receptor that binds human LDL. In addition, the IgG-C7 blocks binding of 125I-LDL to bovine adrenal membranes in vitro, but not to membranes from the adrenals of the mouse, rat, dog, or rabbit (data not shown). Thus, the antibody recognizes a determinant that is present on the LDL receptors of humans and cows, but not rats, mice, hamsters, dogs, and rabbits. The nature of this determinant is unknown, but its existence allows a clear differentiation between the human and bovine LDL receptors on the one hand and the receptors from the rodent and canine species on the other. The monoclonal antibody, therefore, will be useful for somatic cell genetic studies designed to determine whether a cultured rodent cell has acquired the human gene for the LDL receptor.

Acknowledgments—We gratefully acknowledge the generous help of Drs. Stella Robertson, Jack Kettman, Graham Smith, and Patrick Reynolds, who provided invaluable advice for setting up the hybridoma methodology. Debbie Noble and Gloria Brunschede provided excellent technical assistance.

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