

Surface Distribution and Recycling of the Low Density Lipoprotein Receptor as Visualized with Antireceptor Antibodies

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For more than 45 years biologists have known that cells internalize extracellular fluid and its contents by fluid-phase endocytosis, an inefficient process for the uptake of molecules that are present at low concentrations. More recently, animal cells have been found to express surface receptors that bind, and thereby concentrate, certain molecules so that they can be taken up in large amounts even when they are present at low concentrations outside the cell. Ligands that are internalized in this way include plasma transport proteins, hormones, and other macromolecules that fulfill nutritional and regulatory roles in cells. In many cases this process, called receptor-mediated endocytosis, takes place in specialized regions of the plasma membrane called coated pits (reviewed in references 1 and 2).

One of the most extensively studied model systems for receptor-mediated endocytosis is the low density lipoprotein (LDL) receptor system. LDL, the major cholesterol carrying protein in human plasma, delivers cholesterol to cells (3). Delivery is accomplished by cell surface receptors that bind LDL and lead to its internalization by receptor-mediated endocytosis. The LDL is carried to lysosomes where it is degraded to liberate cholesterol, which is used for the synthesis of plasma membranes (most cells), steroid hormones (steroid-secreting cells), and bile acids (hepatocytes).

In 1976 we described the mechanism of internalization of LDL in human fibroblasts. Using LDL that was covalently coupled to ferritin as a specific electron microscopic probe, we showed that LDL binds to receptors that are associated with coated pits on the cell surface (4). Coated pits (5), which represent 2% of the cell surface, contain about 70% of the LDL receptors (4, 6). Immediately after binding, these pits invaginate and the LDL-ferritin is internalized into coated endocytic vesicles (6). Within 1 min, the endocytic vesicles lose their coat and become larger and irregularly shaped, apparently as a

result of fusion with other endocytic vesicles. We called these newly formed vesicles endocytic vesicles or endosomes (6); others have used the terms sorting endocytic vesicles (7) or receptosomes (8). After 5 to 10 min, LDL-ferritin can be detected in lysosomes. By quantitative analysis, the kinetics of binding and uptake of LDL-ferritin are the same as those observed with ^{125}I -LDL, a biochemical probe for binding, internalization, and degradation (9).

On the basis of the combined morphological and biochemical studies, we concluded that the rapid and efficient internalization of LDL, which is completed within 15 min, is due to the association of the LDL receptors with coated pits (6). This conclusion was supported by the discovery of a naturally occurring mutation in human fibroblasts in which the genetically altered LDL receptors bind but do not internalize LDL (10). In these internalization-defective cells, receptor-bound LDL is not internalized because the LDL receptors are not clustered in coated pits but instead are scattered on the cell surface (11). The lesson learned from this mutant is that the receptor must be able to associate with coated pits for LDL internalization to take place (9).

Over the past few years, many laboratories have demonstrated that other macromolecules are internalized by processes that share many of the features of the LDL uptake mechanism. The list of molecules taken up in this fashion now includes the following: transport proteins (such as LDL, chylomicron remnants, yolk proteins, transferrin, transcobalamin II); protein hormones (such as epidermal growth factor, insulin, chorionic gonadotropin, nerve growth factor, β -melanotropin); glycoproteins (such as asialoglycoproteins and lysosomal enzymes); other plasma proteins (such as α -2-macroglobulin and maternal immunoglobulins); and certain viruses and toxins (such as Semliki Forest virus, diphtheria toxin, pseudomonas toxin, and ricin) (for review, see references 1, 2, and 12).

Although the receptors for all of the above ligands appear to function similarly in that they transport ligands into the cell, a few differences have been found. Among these differences are two that will be addressed here: (a) whereas some receptors

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seem to migrate to coated pits spontaneously, others do so only after binding their ligands; and (b) whereas some receptors are destroyed with their ligands after entering the cell, other receptors are disengaged from the ligand whereupon they return to the surface—a process termed receptor recycling. Early and indirect studies of the LDL receptor led to the suggestion that the receptor moved to coated pits spontaneously (4, 6) and that it recycled (1, 13). During the past year, we have been able to test these suggestions directly. Our current studies have been made possible by the development of polyclonal and monoclonal antibodies to the receptor that permit us to study its distribution on the surface in the absence of ligand binding (14, 15) and by the use of agents that inhibit receptor recycling (16). The results of these studies are reviewed below.

Antibodies to the LDL Receptor

Schneider et al. have recently purified the LDL receptor from bovine adrenal cortex (17, 18). The receptor is a glycoprotein with a molecular weight of 164,000 and an acidic isoelectric point of 4.6. Beisiegel et al. have used partially purified preparations of bovine LDL receptor to obtain two kinds of antibodies: (a) a polyclonal rabbit anti-LDL receptor (14); and (b) a monoclonal mouse anti-LDL receptor (15). Both types of antibodies cross-react with the LDL receptors of human cells.

The specificity of the monoclonal and polyclonal antibodies has been studied by immunoblotting techniques. When partially purified bovine adrenal receptors are subjected to electrophoresis on SDS polyacrylamide gels, multiple bands are visible by Coomassie Blue staining (Fig. 1, left), which indicates that these preparations contain many proteins. When the proteins are electrophoretically transferred from the SDS gel to nitrocellulose paper and incubated with the antireceptor monoclonal antibody (Fig. 1, middle) or the antireceptor polyclonal antibody (Fig. 1, right; and Fig. 2, right) followed by an ^{125}I -labeled second antibody, one band at $M_r = 164,000$ is observed.

Fig. 2 (left, *N*) shows that the polyclonal antibody also stains one protein, $M_r = 164,000$, in membranes from normal human fibroblasts. Furthermore, when membranes from mutant receptor-negative human fibroblasts derived from a patient with the disease homozygous familial hypercholesterolemia (FH) were subjected to electrophoresis and immunoblotting, no immunoreactive band was observed (Fig. 2, left, *FH*). In studies not shown, we have determined that the monoclonal antibody and the polyclonal antibodies stain the same protein in extracts of normal human fibroblasts. Thus, both types of antibodies recognize a single species of protein with a $M_r = 164,000$ that appears in normal human fibroblasts and bovine adrenal membrane preparations but not in fibroblasts from patients who lack LDL receptors. This 164,000-dalton protein has been purified to homogeneity from the bovine adrenal gland and has been shown to be the LDL receptor (18).

Native Distribution of Surface LDL Receptors

When the LDL receptor is visualized with LDL-ferritin at 4°C or after prefixation with formaldehyde, about 70% of the LDL receptors are associated with coated pits (4, 6). These observations have been interpreted to mean that the unoccupied receptors cluster spontaneously in coated pits before binding LDL (1). Evidence from other laboratories has suggested that some receptors involved in receptor-mediated endocytosis (e.g., α -2-macroglobulin [19], epidermal growth factor [20], and

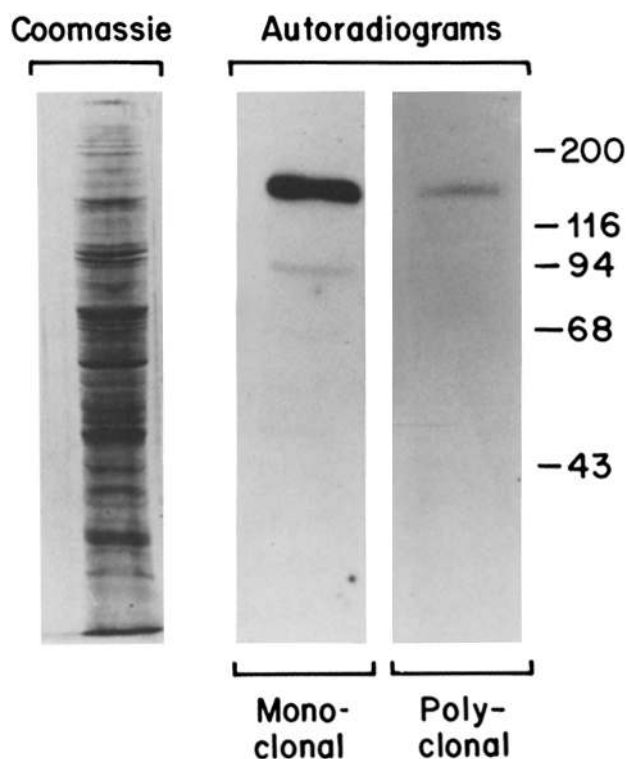


FIGURE 1 Electrophoretic transfer and immunoblotting of LDL receptor from bovine adrenal cortex. LDL receptors from bovine adrenal cortex were solubilized with detergent and partially purified on DEAE-cellulose (18). Replicate samples (100 μg protein) were subjected to electrophoresis in 7% slab polyacrylamide gels containing 0.1% SDS (27). After electrophoresis, one lane of the gel was stained with Coomassie Brilliant Blue and the other two were used for immunoblotting using a modification of the procedure of Burnett (28). For immunoblotting, the proteins from the SDS gel were transferred electrophoretically to BA85 nitrocellulose filters and incubated with either monoclonal antireceptor IgG (1 $\mu\text{g}/\text{ml}$ of mouse IgG-C7) or polyclonal antireceptor IgG (10 $\mu\text{g}/\text{ml}$ of rabbit IgG) as indicated. The filters were washed (28), followed by a second incubation with 1 $\mu\text{g}/\text{ml}$ of ^{125}I -labeled second antibody, either goat anti-mouse IgG (6×10^5 cpm/ μg) for monoclonal lane or goat anti-rabbit IgG (3×10^5 cpm/ μg) for polyclonal lane. The dried filters were exposed for ~ 16 h to Kodak XAR-5 film with enhancing screens (29). M_r ($\times 10^{-3}$) standards are indicated.

Semliki forest virus [12]) are randomly distributed on the cell surface and that the ligand must bind to the receptor before it and the receptor become associated with coated pits. It is conceivable that neither 4°C nor formaldehyde fixation entirely immobilizes the LDL receptor and that this receptor, too, has become clustered as a result of LDL-ferritin binding. To clarify this point, we have used the antireceptor antibodies to study receptor distribution in cells that have been thoroughly fixed so as to prevent receptor mobility in the plane of the membrane. To do these studies, we have adapted the antibodies for use as visual probes.

The monoclonal antibody against the LDL receptor was covalently coupled to ferritin by the method of Kishida et al. (21) and then used to determine LDL receptor distribution in monolayers of cultured human fibroblasts. Fig. 3 compares the distribution at 4°C of LDL-ferritin (Fig. 3A) and ferritin-labeled monoclonal antibody, designated IgG-C7-ferritin (Fig. 3B). It is apparent that both probes are clustered preferentially in coated pits, and this visual impression was confirmed by a quantitative analysis of ferritin distribution.

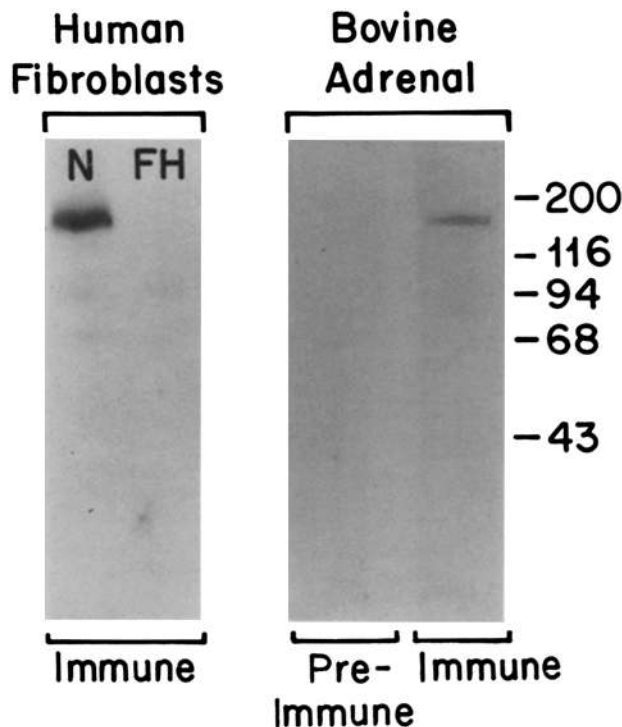


FIGURE 2 Electrophoretic transfer and immunoblotting of LDL receptors from human fibroblasts and bovine adrenal cortex. Human fibroblasts from a normal subject (*N*) and from a patient with the receptor-negative form of homozygous familial hypercholesterolemia (*FH*) were grown in lipoprotein-deficient serum before harvest (15), scraped from the petri dishes, and solubilized with octylglucoside (16). The extracts were centrifuged at 100,000 *g* for 1 h at 4°C, and the supernate was used for electrophoresis. Bovine adrenal cortex membranes were solubilized and partially purified as described in the legend to Fig. 1. Samples of normal (*N*) and mutant (*FH*) fibroblast extracts (200 μ g protein) and bovine adrenal cortex extracts (100 μ g protein) were subjected to electrophoresis on SDS polyacrylamide gels and transferred electrophoretically to BA85 nitrocellulose filters as described in the legend to Fig. 1. The filters were incubated with 10 μ g/ml of either preimmune rabbit IgG or immune rabbit polyclonal antireceptor IgG as indicated. The filters were washed (28), followed by a second incubation with 1 μ g/ml of 125 I-labeled goat anti-rabbit IgG (3×10^5 cpm/ μ g). The dried filters were exposed for ~16 h to Kodak XAR-5 filters with enhancing screens (29). *M_r* ($\times 10^{-3}$) standards are indicated.

With the light microscope, indirect immunofluorescence has been used to show that both the polyclonal antibody (14) and the monoclonal antibody (15) have the same binding distribution on the surface of human fibroblasts as does LDL (22, 23). The antibodies and LDL all bind in discrete clusters that are usually linearly arranged on the cell surface. In the mutant internalization-defective cells in which the receptor is excluded from coated pits, the antireceptor antibodies and LDL appear as smaller foci that are not linearly arranged (23). We concluded from these observations that the punctate linear distribution in normal cells is attributable to the association of receptors with coated pits (22, 23). Consistent with this hypothesis was the finding that an antibody to clathrin, the structural protein of coated pits, also binds in a linear, punctate pattern to permeabilized human fibroblasts (23). In fibroblasts this type of punctate linear staining appears to be diagnostic for surface receptors that are associated with coated pits; it is distinct from the distribution of other cell surface determinants such as β -2-microglobulin, aminopeptidase, and Na^+ , K^+ ATP-

ase, which are randomly arranged unless they are aggregated together by incubation with bivalent antibody at 37°C (24).

To confirm that ligand binding does not induce movement of the LDL receptor to coated pits, we took advantage of the observation that the antireceptor antibodies will bind to the LDL receptor even after the cells have been fixed with concentrations of glutaraldehyde that prevent protein movement in the plane of the membrane. Cells were fixed with glutaraldehyde plus formaldehyde and then incubated with antireceptor antibody followed by horseradish peroxidase conjugated anti-rabbit IgG. Fig. 4*A* shows that the polyclonal antibody bound to glutaraldehyde-fixed normal human fibroblasts. Numerous punctate clusters of reaction product were found on the cell surface, and many of these were organized into a linear pattern (Fig. 4*A*, note linear dots between two arrowheads). Thus, even in glutaraldehyde-fixed fibroblasts, the receptor is distributed in clusters that are suggestive of the organization of coated pits. The receptor-negative human fibroblasts did not bind the polyclonal antibody (Fig. 4*B*).

Previous studies have shown that detergent treatment of formaldehyde-fixed cells removes much of the plasma membrane without removing coated pits (22). If the unoccupied LDL receptors are clustered in coated pits, then formaldehyde-fixed, detergent-treated cells should display clustered receptors on the cell surface because the coated pits are retained in these cells. With the surrounding membranes disrupted, it would be unlikely that ligand binding could induce receptor movement into coated pits. To test for this possibility, we fixed normal human fibroblasts with formaldehyde, treated them with or without Triton X-100, and incubated them with antireceptor antibody and then with fluorescein-isothiocyanate (FITC)-conjugated anti-rabbit IgG (Fig. 5). As indicated by the clusters of fluorescence, polyclonal antibody bound to formaldehyde-fixed, detergent-treated fibroblasts in the punctate linear pattern which is consistent with a localization in coated pits (compare formaldehyde-fixed, detergent-treated cells in Fig. 5*B* with control fixed cells in Fig. 5*A*).

Electron microscope studies of formaldehyde-fixed, detergent-treated cells confirmed that the antibody was binding to clustered receptors in coated pits. Fig. 6 shows two coated pits from formaldehyde-fixed, detergent-treated cells that were incubated with monoclonal antibody conjugated to ferritin. Each coated pit contains several ferritin particles. By quantitative analysis, the distribution and amount of binding was similar to that seen in formaldehyde-fixed, nondetergent-treated cells (data not shown). With the noncoated plasma membrane removed by the detergent, it would not have been possible for the ligand to induce the movement of the receptor into the coated pits. Therefore, in the case of the LDL receptor, ligand binding is not required to achieve this organizational pattern.

Receptor Recycling

Cells that take up molecules by receptor-mediated endocytosis often do so at a continuous rate without any apparent depletion of receptors from the cell surface. This means that each time an occupied receptor internalizes its ligand, the receptor must either return to the cell surface in an unoccupied state or a newly synthesized receptor must be recruited to the cell surface to replace the receptor removed during internalization. In the case of the LDL receptor, we know that the receptor is recycled because inhibitors of protein synthesis do not affect internalization for at least 12 h (25). During this time, each receptor is able to internalize more than 100 LDL

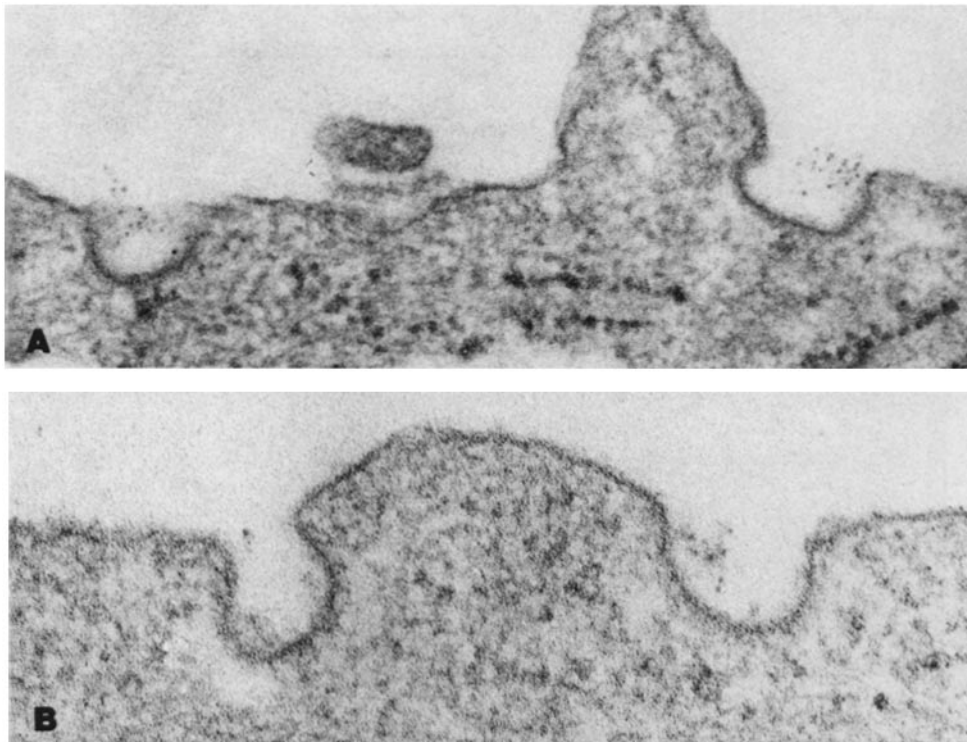


FIGURE 3 Electron micrographs showing the distribution of LDL receptors on the surface of human fibroblasts as detected with LDL-ferritin (A) and ferritin-labeled monoclonal antireceptor antibody (B). Normal human fibroblasts were grown in monolayer culture and incubated in lipoprotein-deficient serum as previously described (15). Monolayers were chilled to 4°C and incubated for 2 h with 50 µg/ml of LDL-ferritin (A) or 50 µg/ml of IgG-C7-ferritin (B). The cells were washed to remove nonspecifically bound ligand (6), fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate at pH 7.3 for 5 min at 4°C followed by further fixation for 25 min at 23°C, and embedded as a monolayer in Epon as previously described (6). A, × 92,000; B, × 125,500.

particles.

The process of receptor recycling depends upon the receptor becoming separated from the LDL at some step during internalization so that the receptor can return to the cell surface and the LDL can be degraded in the lysosome. Fig. 7 outlines three possible sites where this segregation event might take place. The first possibility is that during internalization the receptors move laterally out of the coated pit and the LDL is trapped in an endocytic vesicle that is carried to the lysosome. The receptors then move laterally into new coated pits without ever leaving the cell surface. A second possibility is that LDL and the receptors are both internalized into an endocytic vesicle and it is within this compartment that LDL and receptors segregate. The LDL is diverted into the lysosome and the receptors return to the cell surface. The third possibility is that both the receptors and LDL reach the lysosome. Within the lysosome, the receptor and LDL segregate into separate compartments, which allows the receptor to escape degradation. The LDL is degraded and the receptors return to the cell surface.

Several questions about this recycling process immediately come to mind. Can the receptor travel its normal route and recycle effectively if it carries a molecule other than LDL? Can this recycling process be inhibited? Which one of the pathways shown in Fig. 7 is the actual mode of LDL receptor recycling? Let us consider the first question: Can a non-LDL molecule, such as the antireceptor antibody, be transported and disengaged from the receptor in the same way as LDL?

Internalization of the receptor-bound monoclonal antibody was demonstrated by indirect immunofluorescence studies, which showed that the monoclonal antibody, designated IgG-C7, is internalized into vesicles that are located in the perinuclear area of the cell, where lysosomes normally reside (15). Biochemical studies show that at saturation the LDL receptors are able to bind, internalize, and degrade the same amount of ¹²⁵I-labeled monoclonal antibody (Fig. 8, right) as ¹²⁵I-labeled

LDL (Fig. 8, left). This uptake and degradation is mediated by the LDL receptor since the uptake of both LDL and monoclonal antibody is suppressed in parallel in cells that have been previously treated with sterols (25-hydroxy-cholesterol plus cholesterol) to lower the number of surface receptors (3). These studies indicate that the receptor internalizes the monoclonal antibody in the same way that it does LDL.

After the monoclonal antibody has been internalized, the LDL receptor dissociates from it and returns to the surface. This conclusion is based on experiments such as that of Fig. 8. In this experiment cells were incubated with the ¹²⁵I-labeled antibody at 37°C for 5 h, during which time more than 20 rounds of internalization had occurred. Since there was no apparent decrease in the rate of internalization of monoclonal antibody during this interval, the number of surface receptors must have remained constant during this time. This means that receptor-ligand segregation into separate compartments, a prerequisite for receptor recycling, does not depend on a unique biochemical characteristic of LDL but rather upon a feature that is also expressed by the antibody.

The increased efficiency of cellular uptake consequent upon receptor binding is illustrated dramatically by the experiment of Fig. 9 in which the cellular degradation of the monoclonal antibody against the LDL receptor (IgG-C7) is compared with that of a monoclonal antibody (IgG-2001) directed against an irrelevant antigen (*Hemophilus influenzae*, type B). At low concentrations the antireceptor antibody is taken up and degraded 50-fold more rapidly than the irrelevant antibody in normal fibroblasts (Fig. 9A). In receptor-negative familial hypercholesterolemia homozygote cells, the two antibodies are degraded at the same low rate (Fig. 9B).

Further study of the steps in receptor recycling would be facilitated by the availability of agents that specifically inhibit this process. One such inhibitor is monensin (16), a carboxylic ionophore that catalyzes the exchange of Na⁺ and H⁺ across biological membranes (26). In the presence of this ionophore,

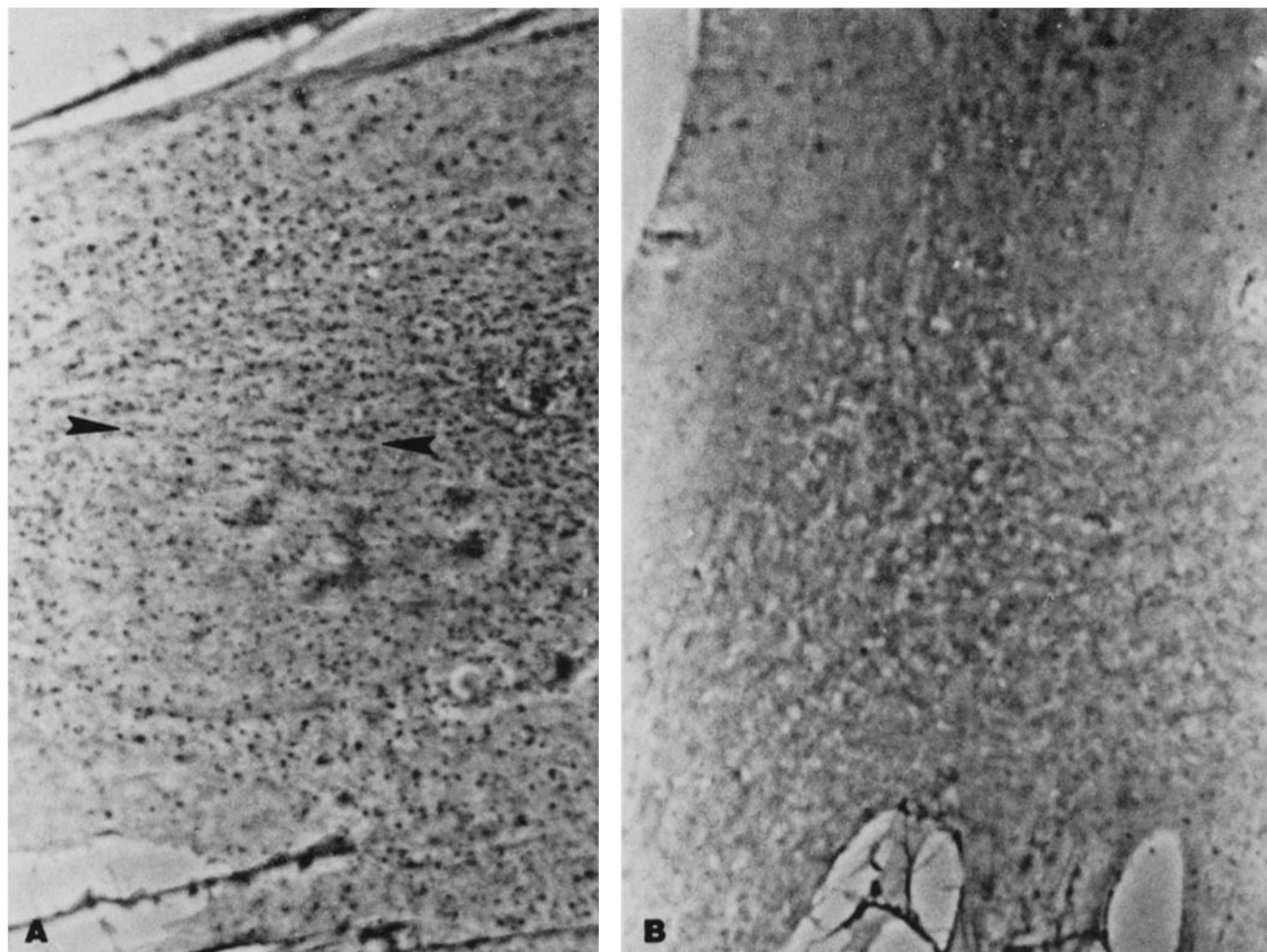


FIGURE 4 Binding of polyclonal antireceptor antibody to glutaraldehyde-fixed human fibroblasts from a normal subject (A) and from a patient with receptor-negative homozygous familial hypercholesterolemia (B). Fibroblasts were grown as described in the legend to Fig. 3. Cells were fixed for 15 min at 23°C in 0.5% glutaraldehyde plus 1% formaldehyde in phosphate-buffered saline (PBS) (10 mM sodium phosphate, 0.15 M NaCl, and 2 mM MgCl₂ at pH 7.4). Cells were then washed and incubated with 0.2 mg/ml of rabbit antireceptor IgG for 1 h at 37°C. Cells were washed and treated with 1 mg/ml of horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h at 37°C. The cells were then processed to visualize peroxidase reaction product (22). A, × 1,000; B, × 1,000.

LDL receptors are unable to return to the cell surface after internalization. When cells are incubated with monensin in the absence of LDL, there is a rapid decrease in the number of receptors, reaching ~50% at 15 min (16). The time of this loss coincides with the normal rate of entry of receptors into the cell. By immunofluorescence, the LDL receptors that are lost from the surface can be found in vesicles within the cell (16, 25). These data suggest that receptors enter the cell normally in the presence of monensin but are unable to recycle to the surface. If monensin is added to cells together with LDL, the loss of surface receptors is equally rapid and even more extensive—>80% of the receptors disappear from the surface within 15 min.

The studies with monensin have clarified several aspects of LDL receptor recycling (reviewed in reference 25). First, coated pits are required for receptor entry, because monensin does not cause a loss of cell surface receptors from the mutant internalization-defective cells whose receptors are not associated with coated pits (11). Second, when fluorescent LDL is internalized in the presence of monensin, it accumulates in the

same vesicular compartment as the receptors (25), which can be visualized by staining with antireceptor antibody after the cells are permeabilized. Therefore, the ionophore prevents segregation of LDL and the receptor in separate compartments. Finally, receptor entry and recycling must occur continuously because monensin causes 50% of the receptors to be trapped inside the cell within 15 min even when LDL is not present. We do not know why the other 50% of receptors remain on the surface in the presence of monensin. These receptors are potentially functional because they enter the cell normally and become trapped if LDL is subsequently added in the continued presence of monensin (16). It may be that only half of the receptors normally enter the cell and recycle in the absence of LDL. Alternatively, all of the receptors may continuously recycle, and monensin may reduce the rate at which the receptors return to the surface. This slowing would distort the steady-state distribution between external and internal receptors, causing 50% of the receptors to be inside the cell at any instant in time. The receptors that remain on the surface after monensin treatment are localized in coated pits and they have

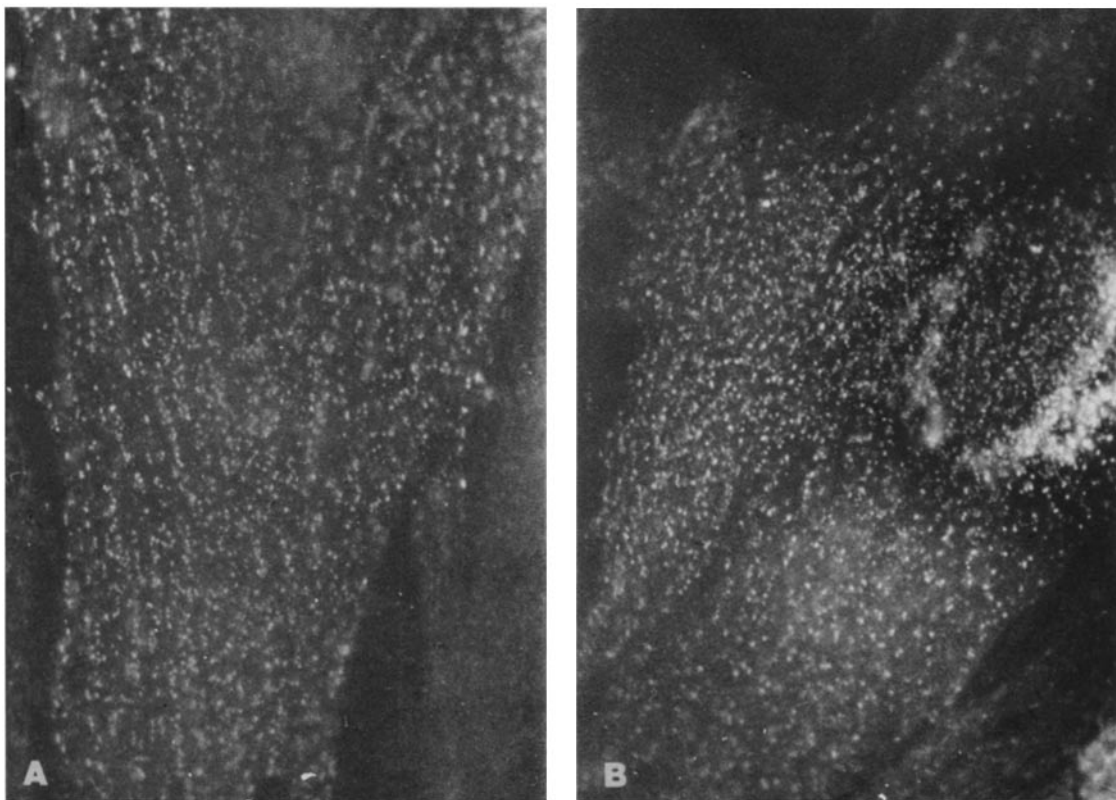
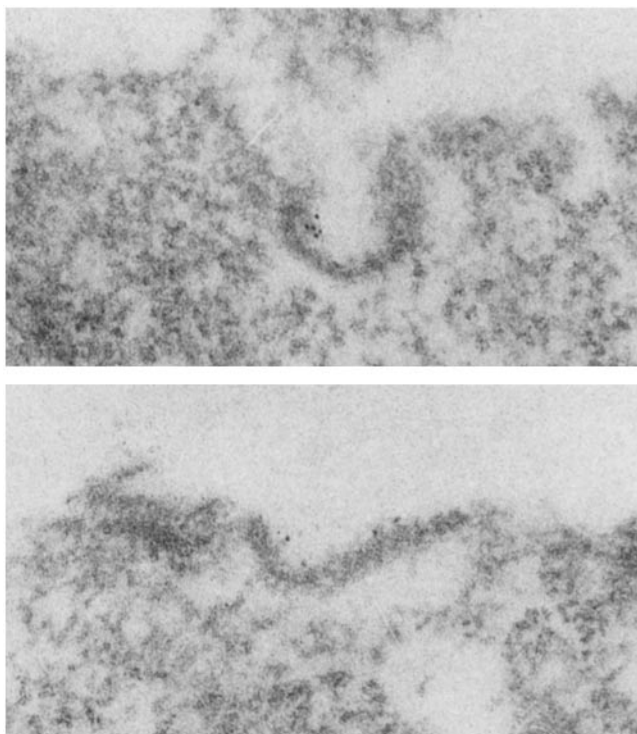


FIGURE 5 Binding of polyclonal antireceptor antibody to formaldehyde-fixed normal fibroblasts before (A) and after (B) treatment with detergent. Normal human fibroblasts were grown in monolayer as described in the legend to Fig. 3. Cells were fixed with 3% formaldehyde in PBS for 15 min at 23°C. One set of cells (B) was then treated with 0.05% Triton X-100 for 10 min at -10°C, after which both sets of cells (A and B) were incubated with 0.2 mg/ml of rabbit antireceptor IgG for 1 h at 37°C. The cells were washed and then treated for 1 h at 37°C with 0.5 mg/ml of FITC-conjugated goat anti-rabbit IgG. After washing, the cells were mounted in buffer containing 90% glycerol and 10% Tris-HCL at pH 9.4 and viewed with a Zeiss photomicroscope III (23). A, $\times 900$; B, $\times 900$.



a normal affinity for ^{125}I -LDL as well as for the ^{125}I -labeled monoclonal antireceptor antibody (unpublished observations). Thus, they do not seem to constitute a distinct class of receptors.

The recycling model outlined in Fig. 7 raises the possibility that inhibition of the segregation of receptor and ligand into separate compartments might prevent return of the receptor to the surface. We have recently found that the polyclonal antibody against the LDL receptor may behave in this way. In contrast to the monoclonal antibody, the polyclonal rabbit antibody cannot dissociate from the receptor and this causes the receptor to be trapped in the cell. Figs. 10 and 11 show the results of experiments designed to assess the distribution of surface receptors after the internalization of the polyclonal antireceptor antibody. As seen in Fig. 10 B, after its incubation

FIGURE 6 Electron microscopic visualization of the binding of ferritin-labeled monoclonal antireceptor antibody to the surface of formaldehyde-fixed, detergent-treated human fibroblasts. Normal human fibroblasts were grown as described in the legend to Fig. 3. On day 7 of growth, the cells were fixed with 3% formaldehyde in PBS for 15 min at 23°C, treated for 10 min at -10°C with 0.05% Triton X-100, and washed. The cells were then incubated with 5 $\mu\text{g}/\text{ml}$ of IgG-C7- ferritin for 1 h at 23°C. The cells were then fixed with 2% glutaraldehyde for 30 min at 25°C and processed for electron microscopy as described in the legend to Fig. 3. $\times 98,000$.

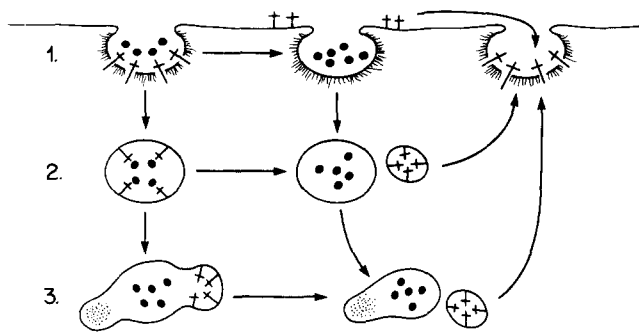


FIGURE 7 Diagram illustrating three possible models for recycling of the LDL receptor. The details and predictions of each model are discussed in the text. The symbols + and ● represent LDL receptors and LDL, respectively.

with cells for 15 min at 37°C, polyclonal antireceptor IgG can be localized by indirect immunofluorescence to numerous vesicles in the perinuclear area of the cell. Therefore, the polyclonal antibody has been internalized and delivered to a lysosomal-like compartment. When these cells were assayed for the presence of surface receptors after antibody internalization, no binding of polyclonal antibody to the cell surface was detected (Fig. 10A). In another experiment cells were pretreated with polyclonal antibody at 4°C and either fixed immediately or allowed to warm to 37°C for 15 min. Cells that had not been warmed displayed numerous clustered receptors (Fig. 11A), whereas cells that had been warmed showed a markedly re-

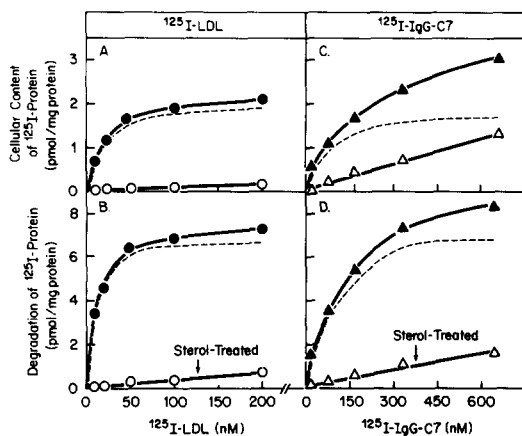


FIGURE 8 Saturation curves for the cellular uptake (upper panels) and degradation (lower panels) of ^{125}I -LDL (left panels) and ^{125}I -labeled monoclonal antireceptor antibody (right panels) by monolayers of normal human fibroblasts that had been treated in the absence (●, ▲) or presence (○, △) of sterols. Fibroblasts were grown in monolayer (15). 2 d before the experiment, each cell monolayer received 2 ml of medium containing 10% lipoprotein-deficient serum in the absence (●, ▲) or presence (○, △) of 1 $\mu\text{g}/\text{ml}$ of 25-hydroxycholesterol plus 12 $\mu\text{g}/\text{ml}$ of cholesterol (delivered in 5 μl of ethanol). On the day of the experiment, each monolayer received 2 ml of medium containing 10% lipoprotein-deficient serum and the indicated concentration of either ^{125}I -LDL (150,000 cpm/pmol) (A, B) or ^{125}I -IgG-C7 (9,000 cpm/pmol) (C, D). After incubation for 5 h at 37°C, the total cellular content of ^{125}I -radioactivity (upper panels) and the amounts of ^{125}I -radioactivity degradation products excreted into the medium (lower panels) were determined. The dashed lines show the receptor-dependent values, which were determined by subtracting the values in sterol-treated cells from those in untreated cells. (Data reprinted from Beisiegel et al. [15]).

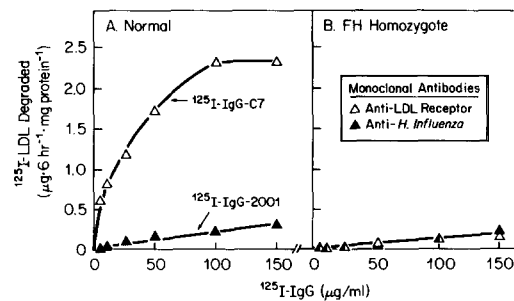


FIGURE 9 Saturation curves for the degradation of ^{125}I -labeled monoclonal anti-receptor IgG-C7 (Δ) and ^{125}I -labeled monoclonal anti-*H. influenza* IgG-2001 (\blacktriangle) by monolayers of human fibroblasts from a normal subject (A) and from a patient with the receptor-negative form of homozygous familial hypercholesterolemia (B). Fibroblasts were grown in monolayer culture and incubated for 48 h in lipoprotein-deficient serum (15). Cells were then incubated with the indicated concentration of either ^{125}I -IgG-C7 (65 cpm/ng) or ^{125}I -IgG-2001 (55 cpm/ng). After incubation for 6 h at 37°C, the total amount of ^{125}I -protein degradation products excreted into the medium was determined (15). Each value represents the average of duplicate incubations.

duced amount of surface antibody binding (Fig. 11B). These morphologic observations were confirmed using ^{125}I -LDL to probe for surface receptors (Fig. 12). After exposure to polyclonal antibody for 2 h at 37°C, virtually no ^{125}I -LDL bound to the cell surface. If the antibody was removed and the cells were incubated for up to 6 h, cell surface ^{125}I -LDL binding returned slowly to 50% of normal. The return of receptors after removal of polyclonal antibody was completely inhibited by cycloheximide (Fig. 12), indicating that new receptors synthesis was required.

Considered together, the results in Figs. 10–12 suggest that the polyclonal antireceptor antibody is internalized like LDL but, unlike LDL, it stays bound to the receptor until the complex reaches the lysosome where the receptor is degraded. Receptors reappear on the surface only after new receptors are synthesized, processed, and transported to the cell surface. Most likely, the ability of the polyclonal antibody to extensively cross-link receptors accounts for its ability to inhibit receptor recycling. Such a cross-linking process undoubtedly would inhibit the receptor-antibody segregation step during recycling. Of interest is that monovalent Fab fragments of the polyclonal antibody are internalized but do not inhibit receptor recycling (unpublished observations). By this formulation the monoclonal antibody does not inhibit recycling because it does not form extensive polymerized networks with the receptor.

Conclusion

The antireceptor antibodies and the ionophore monensin have been useful for studying receptor recycling. But much is still to be learned. While it appears that ligand-receptor dissociation must be a fundamental step in recycling, we have yet to determine the precise cellular compartment where this event takes place. The results of the monensin experiments strongly suggest that separation occurs within the cell rather than on the cell surface. It seems likely that segregation occurs before fusion with lysosomes, but this has not been demonstrated. Hopefully, future experiments with ferritin-labeled antibodies will identify the vesicular compartment in which this step takes place and the mechanism for separation.

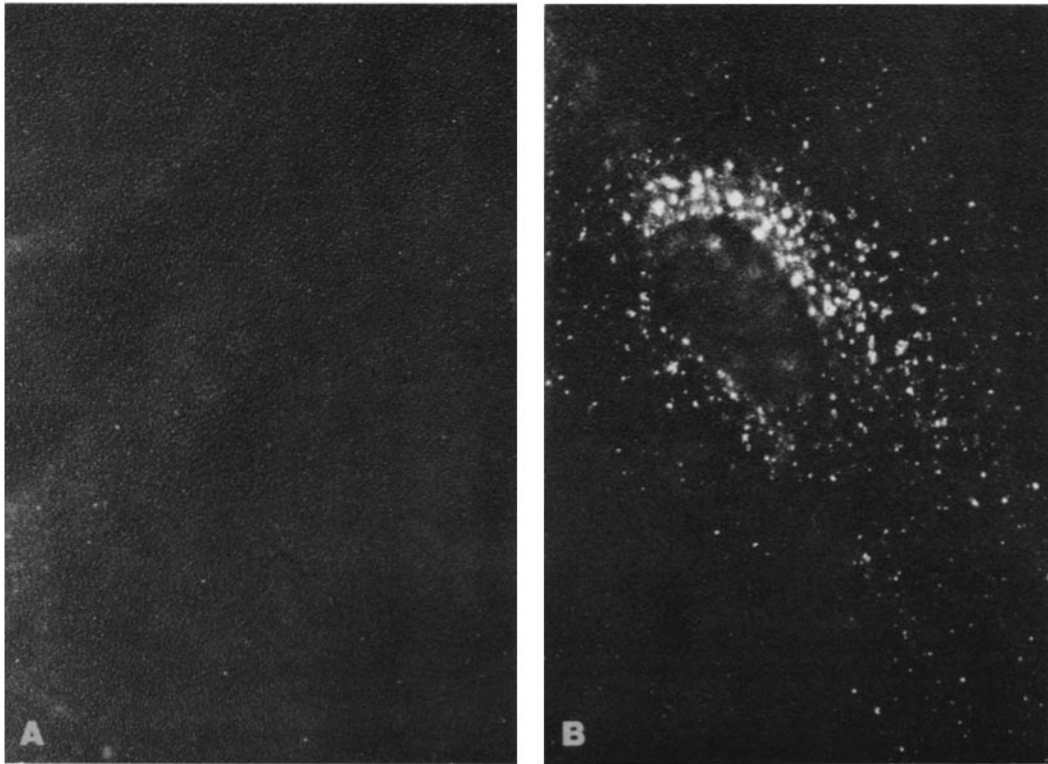


FIGURE 10 Internalization of LDL receptors after incubation for 10 min at 37°C with polyclonal antireceptor antibody. Normal human fibroblasts were grown as described in the legend to Fig. 3 and then incubated with 0.2 mg/ml of rabbit antireceptor IgG for 10 min at 37°C. The cells were then washed and fixed for 15 min at 23°C with 3% formaldehyde in PBS. One set of cells (A) was then incubated at 37°C for 1 h with 0.2 mg/ml of rabbit antireceptor IgG followed by a 1-h incubation at 37°C with 1 mg/ml of FITC-conjugated goat anti-rabbit IgG. The second set of cells (B) was permeabilized by treatment with 0.05% Triton X-100 for 10 min at -10°C and then incubated for 1 h at 37°C with FITC-conjugated goat anti-rabbit IgG. A, $\times 1,000$; B, $\times 1,000$.

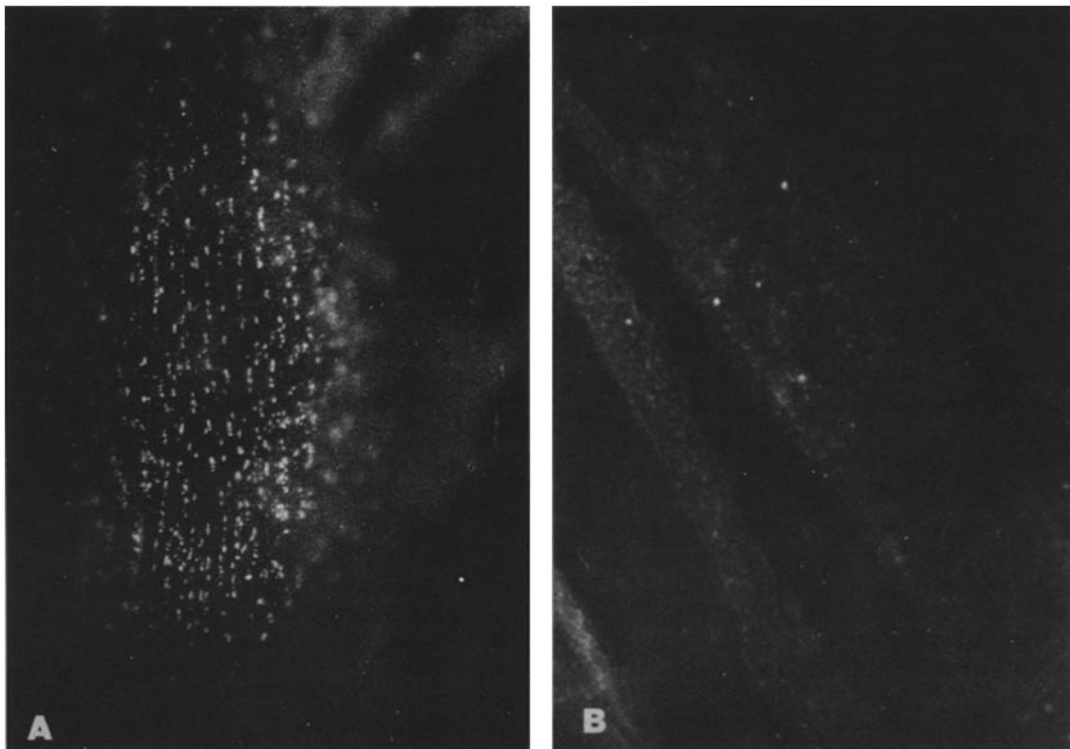


FIGURE 11 Disappearance of LDL receptors from the surface of human fibroblasts after incubation with polyclonal antireceptor antibody at 4°C for 1 h followed by brief warming. Normal human fibroblasts were grown as described in the legend to Fig. 3. Cells were prechilled to 4°C for 30 min and incubated for 1 h at 4°C with 0.2 mg/ml of rabbit antireceptor IgG. Some cells were then fixed immediately with 3% formaldehyde in PBS at 4°C for 5 min, followed by further fixation for 15 min at 23°C (A). Other cells were warmed to 37°C for 15 min before fixation (B). All cells were then treated with 0.2 mg/ml of rabbit antireceptor IgG for 1 h at 37°C followed by 0.5 mg/ml of FITC-conjugated goat anti-rabbit IgG for 1 h at 37°C. A, $\times 900$; B, $\times 900$.

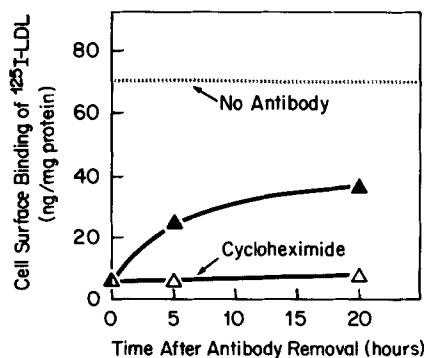


FIGURE 12 Prevention of LDL binding to human fibroblasts by polyclonal antireceptor antibody and recovery of receptor activity after removal of antibody. Fibroblasts from a normal subject were grown in monolayer culture and incubated for 48 h in lipoprotein-deficient serum (15). The cells were then incubated for 2 h at 37°C with medium containing 100 µg/ml of rabbit antireceptor IgG. Each monolayer was then washed and incubated at 37°C with medium containing 10% lipoprotein-deficient serum in the absence (▲) or presence (△) of 30 µg/ml cycloheximide. After the indicated interval, the medium was removed and each monolayer received 2 ml of ice-cold medium containing 10% lipoprotein-deficient serum and 10 µg protein/ml ¹²⁵I-LDL (320 cpm/ng protein) in the absence or presence of 500 µg protein/ml of unlabeled LDL. After incubation for 1 h at 4°C, the amount of high affinity binding of ¹²⁵I-LDL to the cell surface was determined (13). The dashed line indicates the value of ¹²⁵I-LDL binding in cells that were not incubated with the polyclonal antireceptor antibody. Each value represents the average of duplicate incubations.

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