papers and notes on methodology

ELISA measurement of LDL receptors

Karen May,*,† Fredric B. Kraemer,† Jean Chen,* and Allen D. Cooper[1,*,†]

Research Institute* Palo Alto Medical Foundation, Palo Alto, CA 94301, and Department of Medicine,† Stanford University School of Medicine, Stanford, CA 94305

Abstract An enzyme-linked immunosorbent assay was developed for measurement of low density lipoprotein (LDL) receptors. A monospecific polyclonal antibody to LDL receptor purified from rat liver that reacted with rat, mouse, canine, and human LDL receptor was used. With this assay, LDL receptors could be measured on 2–4 × 10^5 adherent cells and 1.0 × 10^5 cells in suspension, although results were more variable with cell suspensions. Membranes from a variety of receptor-rich and receptor-poor tissues could be assayed directly after adherence of the membranes to the ELISA plate by an overnight incubation. In some instances, the quality of the assay was improved by first solubilizing the membranes. The sensitivity of the assay is such that between 0.15 and 2 μg of membrane protein is required. This could be obtained from leukocytes in a modest (20–30 ml) quantity of human blood. The assay was used to demonstrate the rapid down-regulation of LDL receptors in human mononuclear leukocytes in response to a cholesterol-containing meal. Overall, the results support the use of ELISA technology to measure LDL receptors, particularly for physiologic studies. —**May, K., F. B. Kraemer, J. Chen, and A. D. Cooper.** ELISA measurement of LDL receptors. *J. Lipid Res.* 1990. **31:** 1683–1691.

Supplementary key word cholesterol

The serum concentration of low density lipoprotein (LDL) is a well-established risk factor for susceptibility to atherosclerosis (1). In most individuals, the rate of LDL catabolism is determined by the number of LDL receptors present on the liver and, perhaps, at extrahepatic sites (2). In the past, determination of the number of LDL receptors has been accomplished primarily by measurement of the binding and/or degradation of iodinated lipoprotein to cells and cell membranes (3, 4). This approach suffers from a number of disadvantages: *1)* the ligand (LDL) is heterogeneous and not very stable; *2)* it is difficult to obtain fresh unperturbed cells from an organ such as the liver; and *3)* lipoprotein binding to cell membranes is difficult to interpret because of the existence of non-LDL receptor-mediated "specific" binding (5). Thus, more convenient, specific, and reproducible methods are necessary, particularly for physiologic studies in humans. Immunoassays have been used to measure many proteins, particularly if they are soluble. It has been more difficult to establish such assays for membrane-bound proteins (6). In the present report, we describe an enzyme-linked immunosorbent assay (ELISA) for LDL receptors that allows accurate determination of relative receptor content in cells and membranes of a variety of species. The assay was initially validated with adherent cells, was then adapted to cells in suspension, and finally to cell membranes. The latter allowed the measurement of LDL receptors on hepatic and extrahepatic membranes from several species as well as membranes from freshly isolated human mononuclear leukocytes.

MATERIALS AND METHODS

Male Sprague-Dawley rats were purchased form Simonsen Laboratories (Gilroy, CA). Rats, weighing 180–220 g, were used to prepare liver membranes. All were maintained on a standard chow diet with a controlled light cycle. Some rats were injected subcutaneously with ethinyl estradiol (5 mg/kg body weight) in propylene glycol on 5 consecutive days to increase LDL receptor number (7). Phenylmethylsulfonyl fluoride (PMSF), 3-[3-cholamidopropyl)dimethyl-amino]-1-propanesulfonate (CHAPS), and phosphatase substrate (*p*-nitrophenyl phosphate disodium) tablets were purchased from Sigma (St. Louis, MO). Tissue culture media were purchased from GIBCO (Grand Island, NY). Goat anti-rabbit IgG was purchased from Cappel (Malvern, PA). Bovine serum albumin (BSA) was purchased from Armour Pharma-

Abbreviations: LDL, low density lipoproteins; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[3-cholamidopropyl]-dimethylammoniol-propane-sulfonate; MEM, minimum essential medium; BSA, bovine serum albumin; HBSS, Hank's buffered salt solution; LPDS, lipoprotein-deficient serum; HSF, human skin fibroblasts; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; LRP, LDL receptor-like protein.

[1]To whom correspondence should be addressed at: Research Institute, Palo Alto Medical Foundation, 860 Bryant Street, Palo Alto, CA 94301.

ceuticals (Kankakee, IL). Alkaline phosphatase-conjugated goat anti-rabbit IgG (heavy and light chain-specific) was purchased from Caltag Laboratories (San Francisco, CA). Immulon 2 "U" plates were purchased from Dynatech Laboratories, Inc. (Chantilly, VA). All other chemicals were obtained from Sigma or J. T. Baker Chemical Co. (Phillipsburg, NJ).

Cell culture

Human skin fibroblasts (HSF) were maintained in modified Eagle's medium (MEM) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), glutamine (2 mM), and 10% fetal calf serum under a humidified atmosphere of 95% air, 5% CO_2 at 37°C. For ELISA, cells were subcultured in 24-well cell culture plates (day 0). Cells were grown to confluence; on day 3, the growth medium was replaced with fresh medium supplemented with lipoprotein-deficient serum to up-regulate LDL receptor expression (3). The cells were used on day 4, and at that time the cell density was 2–4 × 10^5 cells/cm^2 and 60 μg cell protein/cm^2. Rat hepatoma (HTC 7288C) cells were grown in suspension in Swimm's S-77 medium supplemented with 10% fetal calf serum as previously described (8). For ELISA binding studies, cells were subcultured at a density of 1 × 10^5 cells/ml media (day 0). On day 3, the growth medium was replaced with one of the following: fresh medium, fresh medium supplemented with 10% lipoprotein-deficient serum, or fresh medium supplemented with 25-hydroxycholesterol. Cells were used in experiments on day 4. At the time of assay, cell density was 6–8 × 10^5 cells/ml, and 1 × 10^5 cells were aliquoted to each silanized glass tube for assay.

Preparation of membranes

Liver, adrenal, and intestinal membranes were prepared from normal and ethinyl estradiol-treated rats by the method of Kovanan et al. (4) as modified by this laboratory previously (8). For some experiments, the liver membrane preparations were solubilized with either 30 mM CHAPS by the method of Hui et al. (9) or with octylglucoside as described by Cooper, Nutik, and Chen, (10). All protien assays were by the method of Lowry et al. (11) or by the Bio-Rad (Richmond, CA) microprotein assay.

Enzyme-linked immunosorbent (ELISA) assay—standard curve

Polyclonal rabbit anti-rat LDL receptor IgG was prepared as described previously (10). Polystyrene (Immulon 2 "U") 96-well microtiter plates were used as a solid phase. A curve of anti-rabbit IgG binding to known amounts of polyclonal anti-LDL receptor antibody was constructed. To do this, 100 μl goat anti-rabbit IgG (1–5 μg/ml in phosphate buffer) was added to each well and incubated overnight at 37°C. The wells were washed three times (all washes had twice the incubation volume, e.g., 200 μl, and were performed at 4°C, e.g., on ice) with 0.3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (buffer A). A 10-fold higher concentration of BSA (3.0%) in PBS (200 μl) was then added and the plates were incubated at 37°C for 45–60 min to cover nonspecific binding sites. After washing three times with buffer A (200 μl), polyclonal anti-LDL receptor IgG was added to duplicate wells (0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5.0 ng/100 μl in buffer B; 0.3% BSA and 1 mM $CaCl_2$ in PBS) and incubated for 1.5 h at 37°C. The wells were washed five times with 200 μl of buffer B, and alkaline phosphatase-conjugated goat anti-rabbit IgG in buffer B (100 μl) was added and incubated at 37°C for 45 min to detect bound anti-LDL receptor antibody. For each preparation, to achieve an optimal signal expression in the absorbance range measured by the microelisa autoreader, 1:5000, 1:2500, or 1:1250 dilutions of alkaline phosphatase-conjugated antibody in buffer B were used. The wells were washed with buffer B five times. Then, 100 μl substrate solution (0.1% *p*-nitrophenyl phosphate, disodium in 0.5 M bicarbonate buffer containing 1 mM $MgCl_2$, pH 9.8) was added and incubated at room temperature for 45–60 min. Alkaline phosphatase was quantified colorimetrically with a Dynatech MR580 microelisa reader. Absorbance was plotted as a function of anti-LDL receptor antibody, using the Statview (Brain Power, Inc., Calabasas, CA) linear regression program for the Macintosh computer. It was assumed that all of the added anti-receptor antibody was bound, allowing conversion of the absorbance units of unknown samples to the amount of antibody bound.

Assaying adherent cell lines by ELISA

Cell lines were grown as monolayers in 24-well tissue culture plates at 37°C. After growing to confluence, cells were washed twice with buffer A (600 μl), and then incubated at 37°C for 45 min with appropriate media plus 3.0% BSA (300 μl). Cells were then chilled at 4°C for 15 min. All incubations and buffers used beyond this step were performed at 4°C to prevent LDL receptor internalization and recycling. In all washes, supernatants were carefully removed by aspiration to minimize cell stripping from wells. Cells were washed twice with buffer A (600 μl) and then an excess amount of anti-LDL receptor antibody in cell media supplemented with 0.3% BSA and 1 mM $CaCl_2$ (buffer C) was added (300 μl), and incubated for 1.5 h. Excess amount of antibody was determined by titration (0, 25, 50, 100, 200, 400 μg/ml). Cells were washed five times with buffer B (600 μl each), and then alkaline phosphatase-conjugated goat anti-rabbit antibody in buffer C (300 μl) was added and incubated for 45 min. (The amount of alkaline phosphatase-conjugated goat anti-rabbit antibody for optimal binding in the stan-

dard curve was predetermined in separate experiments.) Cells were washed five times with buffer B (600 μl) and substrate solution was added (300 μl) and incubated at room temperature for 45–60 min. When the substrate incubation was complete, 100-μl aliquots of the supernatants were transferred to 96-well microtiter plates and absorbance values were measured by microelisa reader. This transfer prevented cells from interfering with absorbance measurement.

Assaying cell suspensions by ELISA

The assay of LDL receptors, expressed in cell lines grown in suspension or in freshly isolated primary cells, was performed as described for adherent cell lines with the following exceptions. Cells were assayed in silanized glass tubes instead of plates. The incubation buffer used was Swimm's S-77 supplemented with appropriate concentration of $CaCl_2$ and bovine serum as outlined for adherent cell lines. Cells were pelleted at 500 *g* for 5 min in a Sorvall RC-5B centrifuge prior to changing wash buffer, resuspending in incubation buffer, and aliquoting.

Assaying membrane proteins for LDL receptor by ELISA

The ELISA assay was also used to measure LDL receptors on either solubilized or nonsolubilized membrane proteins from dog as well as rat tissue. When measuring LDL receptors from a new tissue type or species, the amount of membrane protein added to the microtiter wells for LDL receptor quantitation was titered. For liver membranes, we have found that the optimal amount of membrane protein is between 0.125 and 1.000 μg per well. With membranes from nonhepatic tissues, up to 2 μg of protein/well may be required. Membrane proteins were incubated overnight at 37°C in 96-well plates. Membrane proteins were assayed using the same protocol outlined above for standards, with the following exceptions. An antibody titration was performed for each new type of membrane protein. Polyclonal anti-LDL receptor antibody was added in excess to get a complete quantitation of LDL receptors present in membrane protein. Usually conditions were optimal with 100 μg/ml antibody. Parallel samples were assayed using normal rabbit IgG instead of anti-LDL receptor IgG to detect nonspecific binding. The nonspecific binding was subtracted from the anti-LDL receptor binding to determine specific binding. The assay was read in the plates in which the reactions were carried out.

Human studies

Subjects and protocol. Three normal, healthy male subjects (age 22.7 ± 1.5 yr) volunteered for the study. After an overnight fast, 50 ml of heparinized blood was obtained for mononuclear leukocyte isolation. The subjects then consumed 750 mg of cholesterol in the form of three hard-boiled eggs and 80 g of triglyceride in the form of cheesecake within 20 min. After 2 h, blood was again obtained for mononuclear leukocyte isolation. Fasting triglyceride and cholesterol values averaged 42.7 ± 3.3 and 128.0 ± 13.0 mg/dl, respectively, while 2 h postprandial values were 152.3 ± 16.4 and 128.0 ± 14.0, respectively (mean ± SE).

Mononuclear leukocyte membrane preparation. Mononuclear leukocytes were isolated as described by Böyum (12). Each 50-ml sample of blood was divided in half and diluted with 15 ml of Hank's buffered salt solution (HBSS) without Ca^{2+} and Mg^{2+}. Each sample was underlaid with 10 ml of Hypaque-Ficoll and centrifuged at room temperature for 30 min at 500 *g*. The interface containing the mononuclear leukocytes from the two gradients was removed with a pipette, combined, diluted with 50 ml of HBSS without Ca^{2+} and Mg^{2+} and centrifuged at 4°C for 10 min at 600 *g*. The cell pellets were then washed two times with HBSS without Ca^{2+} and Mg^{2+} and resuspended in 6 ml of 150 mM NaCl, 1 mM $CaCl_2$, and 10 mM Tris-HCl (pH 7.4). Membranes were prepared from the mononuclear cells as previously described for macrophages (10). The cell suspension was homogenized with a Brinkman Polytron at setting 6 for 20 sec × 3 at 4°C. The homogenate was centrifuged at 4°C for 4 min at 800 *g*, and the supernatant was then centrifuged at 4°C for 60 min at 100,000 *g*. The resulting pellet was resuspended in 0.25 M sucrose, 1 mM EDTA, 100 μM leupeptin, and 20 mM Tris-HCl (pH 7.4) and used for measurement of LDL receptors as described in the assay for membranes.

RESULTS

Test of antibody specificity

The polyclonal antibody to the rat LDL receptor has been characterized in a number of previous studies. It does not recognize the LDL receptor-related protein (LRP) described by Herz et al. (13) (**Fig. 1, left**). To further test its specificity, immunoreactivity was compared to that of a monoclonal anti-LDL receptor antibody which purifies only the LDL receptor. On PAGE gels, which were overloaded with solubilized liver membranes from ethinyl estradiol-treated rats, a number of bands are identified. These are all induced with the estrogen treatment (10). The same bands are recognized by the mono- and polyclonal antibodies (Fig. 1, right), suggesting that all are from the LDL receptor and that the polyclonal antibody does not recognize any proteins that are not derived from the LDL receptor. To test its specificity in monocytes, comparable protein was loaded on the gel. There was very little immunoreactivity and that present was at the appropriate molecular weight region of

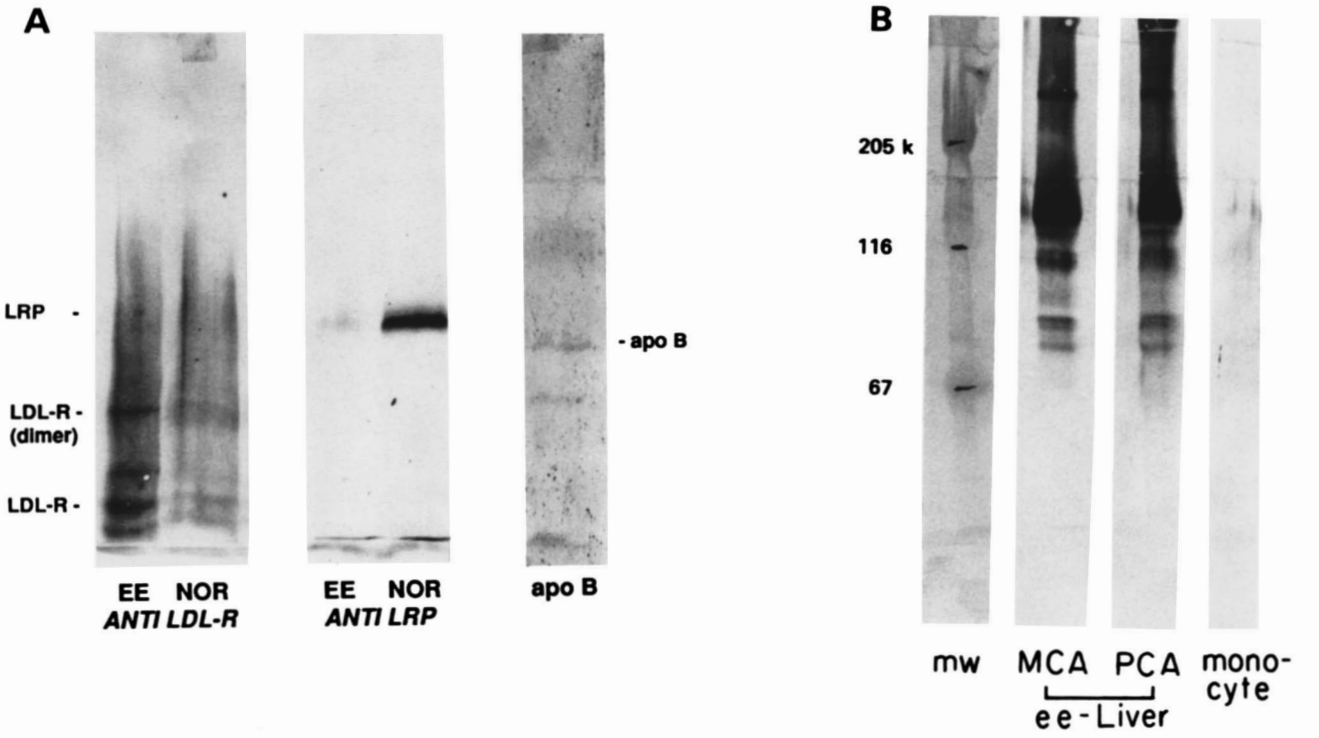

Fig. 1. Immunoreactivity of anti-LDL receptor antibody. Left: The antibody does not recognize the LDL receptor-like protein (LRP). A 2% polyacrylamide gel, containing 0.1% SDS reinforced with agarose (23), was run on liver membranes from normal (nor) or ethinyl estradiol-treated (EE) rats and the proteins were transferred to nitrocellulose. The nitrocellulose was incubated with either polyclonal anti-LDL receptor antibody or the anti-LRP antibody supplied by Herz et al. (13). The blot was developed with a horseradish peroxidase-conjugated second antibody. VLDL was also run on the gel and the protein stain showing apoB is included as a marker. The location of the LDL receptor (LDL-R), its dimer, and the LRP are noted. Right: The antibody recognized only LDL receptor material in rat liver and human monocytes. Membranes were prepared from the liver of ethinyl estradiol-treated rats and from human monocytes as described in Methods. Fifty μg protein dissolved in 30 mM CHAPS was applied to 6% polyacrylamide gels containing 0.1% SDS, and no reducing agents were added. After electrophoresis, the proteins were transferred to nitrocellulose and incubated with either a monoclonal (E) or the polyclonal anti-rat LDL receptor antibody and developed with a second antibody conjugated with horseradish peroxidase. A molecular weight standard (MW) is included.

LDL receptor (Fig. 1, right). This confirms the specificity and suggests that the ELISA is very sensitive since, despite the faintness of the immunoblot, monocyte membranes gave a clear reaction in the ELISA (see below). In previous reports, we have demonstrated its monospecificity to J774 cells (14), dog liver and adrenal (15), and rat adrenal, intestine, skeletal muscle, and brain (16).

Standard curve, internal standard, and optimization of the ELISA method

To construct a standard curve, ELISA plates were coated with excess goat anti-rabbit IgG, and varying amounts of rabbit anti-rat LDL receptor were added. After incubation and appropriate washing, alkaline phosphatase-conjugated goat anti-rabbit IgG was added to the wells, washed, and substrate was added. The reaction was quantified by absorbance at A405/A490. A linear range was identified, and a line was constructed by least squares linear regression. The R^2 was always greater than 0.95. Such a standard line was constructed on each ELISA plate. In the linear range, it was assumed that the amount of anti-LDL receptor antibody added was completely bound. The absorbance of unknown samples was expressed in ng anti-LDL receptor antibody bound, derived from the standard curve.

Because different preparations of anti-LDL receptor antibodies that had varying affinities were used, it was not always possible to compare results directly among assays. In order to compare the results of assays done with different antibody preparations, an aliquot of a control membrane protein was assayed on each plate. The control membrane protein was derived from the same tissue and species as the samples being studied, if possible, and was titered at the same concentrations as the unknown sample. The correction was rarely more than 10%.

The conditions for the assay described in Methods were maximized with respect to time and temperature. Since the anti-LDL receptor antibody was found to bind with much higher affinity to cell surfaces and membrane proteins in the presence of 1.0 mM $CaCl_2$, calcium was included during incubation with anti-LDL receptor antibody. Ca^{2+} did not affect the reactivity between the goat

anti-rabbit antibodies and the anti-LDL receptor antibody. However, when $CaCl_2$ was present throughout the assay, a decrease in signal was observed. Thus, two buffers were used for both standards and unknown membranes or cells: a calcium-free buffer before the anti-LDL receptor antibody was added, and a buffer supplemented with $CaCl_2$ in the presence of anti-LDL receptor antibody and during subsequent washes. The use of 3.0% BSA to block nonspecific binding sites prior to antibody incubation was critical in attaining sensitivity, with as much as an 8-fold increase in signal obtained.

Quantitating surface LDL receptors by ELISA in adherent cell cultures

The majority of studies of LDL receptor regulation have been done in adherent cells in culture; thus, the ELISA was first applied to such cells. To minimize LDL receptor internalization and recycling, assays were performed at 4°C. The ELISA assay quantitated human LDL receptors on HSF cells. LDL receptor concentration on lipoprotein-deficient serum (LPDS)-treated HSF cells was compared with LDL receptor concentration on untreated control HSF cells. The ELISA detected the increase in LDL receptors that is induced by culture in LPDS (**Fig. 2**). Four experiments were performed in parallel with ^{125}I-labeled apoE-rich lipoprotein binding studies for comparison purposes. The mean ratio of LDL receptor concentration in LPDS treated/untreated cells for these experiments was 3.95 (± 1.03 SE) by the ELISA method and 3.37 (± 0.65 SE) by the lipoprotein binding method. Thus, there was good agreement between the two approaches. Similar results were obtained with the adherent murine cell line J774 grown in control or LPDS (2.7-fold induction with ELISA, 2.5-fold induction of ^{125}I-labeled chylomicron binding, respectively).

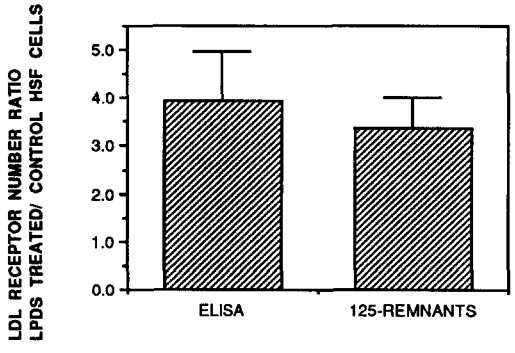

Fig. 2. Comparison of ELISA measurement of LDL receptors on human skin fibroblasts with ^{125}I-labeled chylomicron remnant binding. Human skin fibroblasts were grown to confluence in 24-well tissue culture plates (2–4 × 10^5 cells/well). During the 24 h before the determination, they were grown in either complete or delipidated media. After cooling to 4°C and washing, rabbit anti-LDL receptor antibody was added (100 μg/ml) and incubated for 1.5 h. After further washing, alkaline phosphatase-conjugated goat anti-rabbit IgG was added and incubated for 45 min. After washing, substrate solution was added, and after color development was complete, 100-μl aliquots were transferred to ELISA plates and absorbance was measured. Anti-LDL receptor IgG bound was determined from a standard curve. The mean and standard error of four experiments is given. ^{125}I-labeled chylomicron remnant binding was measured as previously described (14). The ratio of serum-free to control receptors by each method of determination is shown. Mean ± SE of four separate experiments.

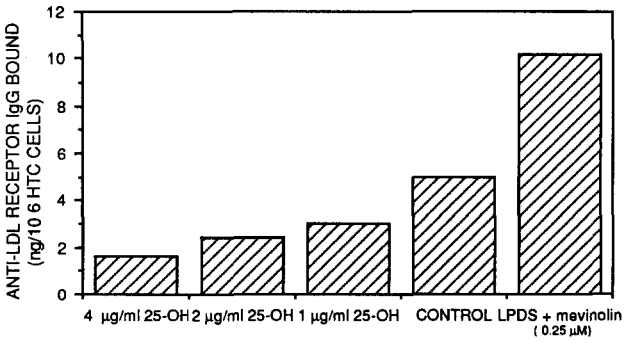

Fig. 3. ELISA measurement of LDL receptors on rat HTC (7288C) cells. HTC cells were maintained in suspension culture. During 14 h before use, the media was supplemented with the indicated concentration of 25-hydroxycholesterol or replaced with delipidated media containing 0.25 μM mevinolin. The ELISA was carried out as described in the legend to Fig. 2, except that the cells were pelleted between washes and incubations. Duplicate determinations were made.

Measuring LDL receptor concentrations in cell suspensions

For physiologic studies, adherent cells cannot be used, but circulating cells or cell suspensions may be available. To explore further the sensitivity of the method and assess its use with cell suspensions, rat HTC (7288C) cells were studied. A representative experiment comparing four treatment groups to a control is shown (**Fig. 3**). Cells treated with 25-hydroxycholesterol had a dose-dependent decrease in the number of LDL receptors compared to control cells. Conversely, cell LDL receptor expression was up-regulated with 0.25 μM mevinolin supplemented with 10% LPDS. In this experiment, replicas were within 10% of each other, and there was no overlap between replicates of the experimental groups, although there was some overlap between individual control samples and the lowest concentration of 25-hydroxycholesterol. These findings are consistent with previous observations using ligand binding (8). In a separate experiment, a comparison to ^{125}I-labeled chylomicron remnant binding was carried out. With HTC 7288C cells grown in complete media or LPDS plus mevinolin, the induction of ^{125}I-labeled chylomicron remnant binding was 5.6-fold and of ELISA activity was 6.2-fold. We have previously reported (8) that in this cell line, the induction of remnant binding is due to an increase in LDL receptors. Freshly isolated rat adipocytes were also successfully assayed (17). Thus, the assay can be adapted to cell suspensions, and the results of metabolic perturbations can be assessed. However, because of the need for a larger volume and repeated cen-

trifugation, the assay was less sensitive, more laborious, and less precise with these types of cells than with adherent cells.

Quantitating LDL receptor density in rat membrane proteins by ELISA

Because of the difficulties encountered with cells in suspension, and the fact that suspensions cannot always be prepared from tissues, the assay was adapted to membranes. Immunoassay of membrane protein has certain limitations (6) so the assay was carefully validated for membranes. The concentrations of LDL receptors in normal rat liver membranes were compared to liver membranes taken from rats treated with ethinyl estradiol for 5 days. Membranes were bound directly to the solid phase (0.25 μg/well), and increasing concentrations of anti-LDL receptor antibody were added to microtiter wells, containing the liver membrane protein. At 100 μg/ml anti-LDL receptor antibody, the binding plateaued, indicating sufficient antibody had been added to recognize almost all of the exposed LDL receptors in the liver membrane preparations (**Fig. 4A**), and this concentration was used in subsequent experiments. The two types of membranes were then compared, while varying the concentration of membrane protein with constant anti-LDL receptor antibody concentration (100 μg/ml) (Fig. 4B). A linear response was obtained with <1.0 μg membrane protein. Thus, the assay is zero order with respect to antibody at a modest antibody concentration, and first order with respect to liver membrane receptors at a level that requires a very small amount of tissue. With membranes, the background is considerably lower than with cells and never exceeded 20% of the total signal under optimal assay conditions.

Quantitating LDL receptor density in membranes derived from extrahepatic rat tissues and tissues of other species

The ability of the assay to determine receptor density in an extrahepatic tissue of the rat was studied by using membranes prepared from rat intestine. Using the same conditions as with rat liver, a linear response was obtained (**Fig. 5A**). Intestinal membranes have about one-fourth the amount of LDL receptors that liver has. Even in a tissue with a low content of LDL receptor, nonspecific binding is not a problem (Fig. 5A). The background is a greater percentage of total binding than with liver membranes, but, even in this worst case, was still only 20% of the original.

Rabbit polyclonal anti-rat LDL receptor antibody cross-reacts with the LDL receptor from a variety of species, including mouse, dog, and human. The utility of antibody in quantifying receptors from some of these species was studied. With dog liver, linear response as a function of membrane was obtained over the range of protein up to 2 μg protein. In a number of experiments, the ELISA results were compared to those obtained by immunoblots and densitometric scanning with good correlation found. However, immunoblotting required up to 1,000-fold more membrane and considerably more antibody (data not shown).

In order to quantify LDL receptors in the adrenal gland of the dog, a different protocol was necessary because intact membranes gave variable results, presumably because they could not be homogenized well

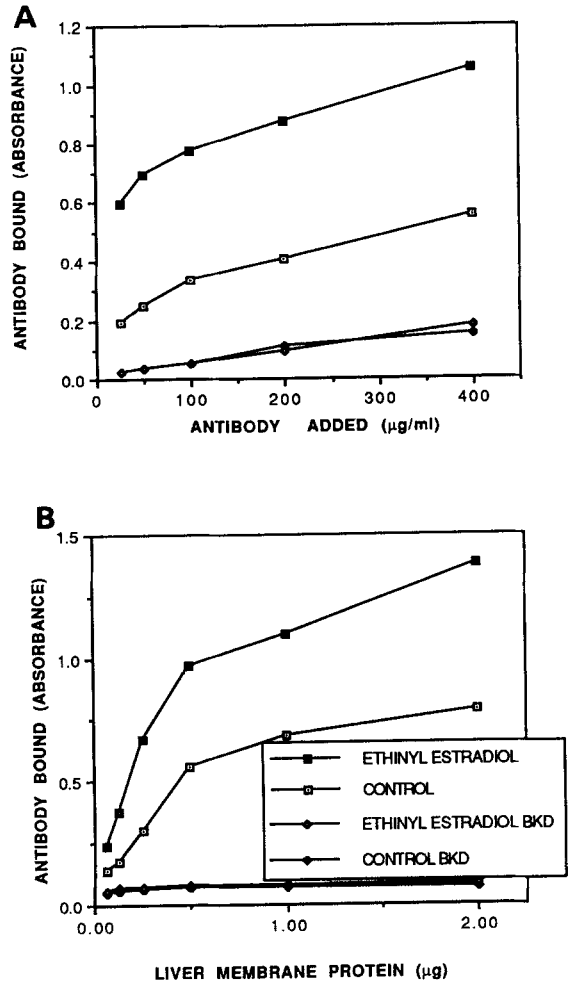

Fig. 4. ELISA measurement of LDL receptors on rat liver membranes. Membranes were prepared by the method of Kovanen, Brown, and Goldstein (7) from livers of control rats or rats that had been pretreated with ethinyl estradiol for 5 days. Membranes adhered to the ELISA plates by incubating overnight at 37°C. The remainder of the procedure was as described in the legend to Fig.2, except that the absorbance was determined in the original plate. A) The amount of anti-LDL receptor antibody bound as a function of antibody concentration. Each well contained 0.25 μg membrane protein. B) The amount of anti-LDL receptor antibody bound as a function of membrane protein. The antibody concentration was 100 μg/ml. In order to display the background, absorbance units are shown. In A), the peak binding was 9 ng IgG; in B), it was 14 ng IgG.

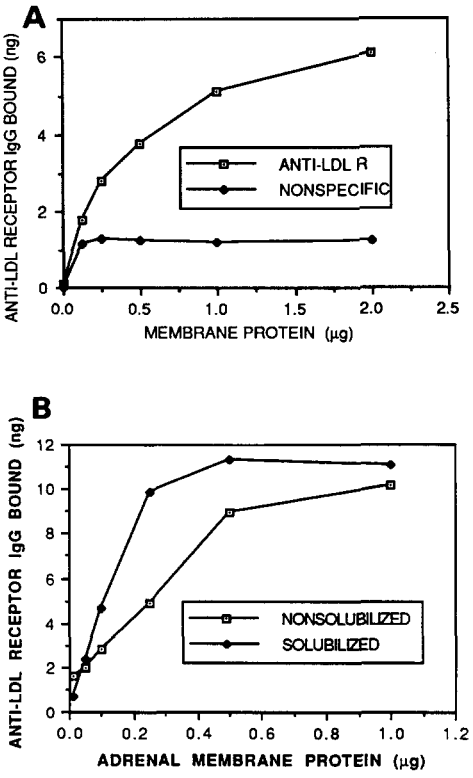

Fig. 5. ELISA measurement of LDL receptors on rat intestinal and adrenal membranes. The same protocol as in Fig. 4B. The adrenal membranes were solubilized in CHAPS before incubation with the microtiter plates. A) rat intestinal cells; B) dog adrenal gland.

enough to give a uniform particle size in the small volume needed for the assay. However, when the membranes were solubilized first, a linear assay was obtained (Fig. 5B). Thus, the assay can be adapted to quantify LDL receptors in a variety of tissues from a number of species.

Measurement of LDL receptors on human mononuclear leukocytes and the response to a cholesterol-rich meal

It has been suggested that the cholesterol metabolic responses of human mononuclear leukocytes reflect those in the liver (18–20). Mononuclear leukocytes were isolated, membranes were prepared and assayed for LDL receptor content by ELISA. A linear response was obtained with 0.25 to 1.0 μg of protein (not shown). To test the ability of the assay to detect changes in LDL receptor number, blood was obtained from three individuals before and 2 h after they were fed a high fat, high cholesterol meal. Consistent with a previous report (19), the number of LDL receptors decreased in each individual (**Fig. 6**) ($P<0.05$ by paired t test). Thus, this assay can be used on human mononuclear leukocytes and should prove useful in a variety of clinical studies.

DISCUSSION

The LDL receptor, by virtue of its ability to bind apoB and apoE with high affinity, initiates the removal of lipoproteins containing these apoproteins and, thus, plays a central role in determining serum cholesterol concentration (2). Although the bulk of LDL receptors are in the liver, most, if not all, cells are capable of expressing LDL receptors under appropriate conditions. Thus, measuring LDL receptors has proved to be a useful tool for studying cholesterol homeostasis in many cells and tissues both in vitro and in vivo. In general, receptors are measured by determining the amount of binding of radiolabeled LDL to the relevant cell or to membranes prepared from a tissue homogenate. This approach has a number of drawbacks, including poor sensitivity, the presence of substantial nonspecific binding (especially in circumstances where the number of receptors is low), and the presence of LDL receptor-independent specific binding in some tissues (5). Furthermore, the ligand, LDL, cannot be stored for more than a few weeks and may be heterogeneous even in the same donor. Thus, it is difficult to compare results from day to day, particularly in terms of absolute values. This is particularly a problem in devising assays that will be useful for conducting clinical studies of patients over time. Accordingly, we have applied ELISA technology to the problem and now report the establishment of such a procedure and document its utility.

The success of the assay depends on the quality and specificity of the antibody. The antibody used in these studies was prepared against LDL receptor purified from a rat liver. It has previously been shown to recognize only

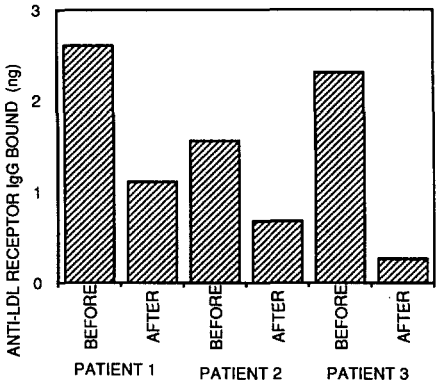

Fig. 6. ELISA measurement of LDL receptors on membranes from mononuclear leukocytes before and after a cholesterol-rich fatty meal. Thirty ml of blood was drawn from three normal volunteers before they consumed a cholesterol-rich fatty meal. Two hours after the meal, an additional 30 ml of blood was drawn. Mononuclear leukocytes were isolated, and their membranes were prepared as described in Methods. An ELISA was done as described in the legend to Fig. 4. Each well was incubated with 1 μg membrane and 100 μg/ml antireceptor antibody. Pre versus post $P<0.05$ by Student's T test.

the LDL receptor and closely related peptides (10, 16), which probably represent the receptor precursor, a dimer of the receptor, and degradation fragments of the receptor. It does not appear to cross-react to any extent with the LRP, a protein with significant homology to the LDL receptor (13). Although prepared against a rat antigen, such an antibody has considerable ability to recognize the LDL receptor of may other species including dog, mouse, and humans. This is not surprising since the receptor is well conserved with about 75% homology between the human and rat proteins (21). Although monoclonal antibodies might have advantages compared to polyclonal antibodies in terms of the availability of a continuous supply of a homogeneous reagent, in our experience, they tend to have lower affinities and greater species specificities, characteristics which limit their usefulness for many purposes.

The assay described has several major advantages. These include its simplicity and the avoidance of radioactivity, as well as the fact that samples can be stored and assayed in large numbers simultaneously. This can be a particular advantage in large complex studies involving feeding regimens. Perhaps most notable is the sensitivity of the assay; less than 1 μg of liver membranes is needed per determination. Thus, the approach should facilitate studies of biopsy material. Similarly, white cell membranes prepared from only 15–30 ml of blood can be assayed, making it possible to assess the response of individuals to drugs and dietary manipulations. For example, although most individuals respond to a cholesterol-containing meal with a decrease in LDL receptors, this is not always the case nor is the magnitude of the decrease predictable (F. Kraemer, K. May, and A. D. Cooper, unpublished observations). By studying this variability, we may gain insight into the basis for hypo- and hyperresponsiveness to dietary cholesterol and its role in the susceptibility to atherosclerosis.

The assay was applicable to all of the tissues we studied. This included tissues where background binding makes the data obtained with radioligand assays difficult to interpret. Even in monocytes, where Western blotting reveals only a small amount of antigen, a clear signal was obtained. However, the ratio of background-to-specific binding (signal-to-noise) varies appreciably, depending on the strength of the signal. With membranes, background varied from 4% of the total signal with membranes from liver of ethinyl estradiol-treated rats to a worst case of 20% of the total signal in intestine. For white cell membranes, background was an average of about 10%. As noted, the background was generally higher with cells ranging from 22% in up-regulated cells to 55% in maximally down-regulated cells. Thus, as long as an adequate number of replicates are carried out, valid estimates can be readily made.

Although solid phase technology has been applied successfully to membrane proteins, there are several specific caveats that must be recognized when measuring LDL receptors (6). First, when bound to solid phase, the immunoreactivity of some epitopes may be altered. This is more problematic with monoclonal antibodies, but may account for different reactivities among batches of polyclonal antibodies. Second, to get absolute values for antibody binding, the complete titration curves should be constructed and analyzed mathematically. However, for relative studies, such as those described here, the approach of seeking a near-zero order for antibody and near-first order for membranes should suffice. Third, it is necessary to monitor the antibody for monospecificity following each boost of the donor and to titer each bleed carefully. Fourth, when first assaying a new type of membrane by ELISA, whether varying tissue type or animal species, a membrane titer at constant antibody concentration must be performed to establish binding conditions. Further, when a new antibody preparation is used in the assay, it is necessary to re-establish binding conditions because antibody affinities vary somewhat with different antibody preparations. Fifth, when used with cultured cells, the assay will measure only the number of surface receptors. Conversely, when used with cell membranes, the assay will measure total cell receptors, which will obscure any differences in the subcellular distribution of receptors induced by a treatment. Similarly, differences in rates of internalization or degradation must be assessed with the appropriate ligand in intact cells. Lastly, genetic defects that affect receptor function, but not receptor synthesis or localization to the plasma membrane, may not be apparent with an immunologic assay as compared to a functional assay (18). However, when used with appropriate care, ELISA measurement of LDL receptors should facilitate studies of the physiology of the LDL receptor.

After the completion of this work, a report of a solid phase radioassay for LDL receptors, using domain-specific antibodies, was reported (22). Since purified receptors were used in that report, it is difficult to compare the two assays, but their general similarities suggest that a variety of approaches to solid phase assay of the LDL receptor are possible and should become standard technology in the future.

This work was supported by grants DK38318, DK36659, HL29589, and HL42865 from the National Institutes of Health and the Research Service of the Department of Veterans Affairs. We would like to thank Dr. Y. Nagata for assistance with some of the studies and Ms. J. Gill for preparing the manuscript.

Manuscript received 20 July 1989, in revised form 27 March 1990, and in re-revised form 24 May 1990.

REFERENCES

1. Kannel, W. B., W. P. Castelli, T. Gordon, and P. M. McNamara. 1971. Serum cholesterol, lipoproteins, and the risk of coronary heart disease. The Framingham study. *Ann. Intern. Med.* **74:** 1–12.
2. Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science.* **232:** 34–47.
3. Brown, M. S., and J. L. Goldstein. 1975. Regulation of the activity of the low density lipoprotein receptor in human fibroblasts. *Cell.* **6:** 307–316.
4. Kovanen, P. T., J. R. Faust, M. S. Brown, and J. L. Goldstein. 1979. Low density lipoprotein receptors in bovine adrenal cortex. I. Receptor-mediated uptake of low density lipoprotein and utilization of its cholesterol for steroid synthesis in cultured adrenocortical cells. *Endocrinology.* **104:** 599–609.
5. Nagata, Y., J. Chen, and A. D. Cooper. 1988. Role of low density lipoprotein receptor-dependent and -independent sites in binding and uptake of chylomicron remnants in rat liver. *J. Biol. Chem.* **263:** 15151–15158.
6. Butler, J. E., J. H. Peterman, M. Suter, and S. E. Dierks. 1987. The immunochemistry of solid-phase sandwich enzyme-linked immunosorbent assays. *Fed. Proc.* **46:** 2548–2556.
7. Kovanen, P., M. S. Brown, and J. L. Goldstein. 1979. Increased binding of low density lipoprotein to liver membranes from rats treated with 17-alpha-ethinyl estradiol. *J. Biol. Chem.* **254:** 11367–11373.
8. Barnard, G., S. K. Erickson, and A. D. Cooper. 1984. Lipoprotein metabolism by rat hepatomas: studies on the etiology of defective dietary feedback inhibition of cholesterol synthesis. *J. Clin. Invest.* **74:** 173–184.
9. Hui, D. Y., W. J. Brecht, E. A. Hall, G. Friedman, T. L. Innerarity, and R. W. Mahley. 1986. Isolation and characterization of the apolipoprotein E receptor from canine and human liver. *J. Biol. Chem.* **261:** 4256–4267.
10. Cooper, A. D., R. Nutik, and J. Chen. 1987. Characterization of the estrogen-induced lipoprotein receptor of rat liver. *J. Lipid Res.* **28:** 59–68.
11. Lowry, O. H., N. J Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
12. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* **21:** 77–89.
13. Herz, J., U. Hamann, S. Rogne, O. Myklebost, H. Gausepohl, and K. K. Stanley. 1989. Surface location and high affinity for calcium of a 500 kDa liver membrane protein closely related to the LDL receptor suggests a physiological role as a lipoprotein receptor. *EMBO J.* **7:** 4119–4128.
14. Ellsworth, J. L., A. D. Cooper, and F. B. Kraemer. 1986. Evidence that chylomicron remnants and β-VLDL are transported by the same receptor pathway in J774 murine macrophage-derived cells. *J. Lipid Res.* **27:** 1062–1072.
15. Cahill, P. D., G. E. Sarris, A. D. Cooper, P. D. Wood, J. C. Kosek, R. S. Mitchell, and D. C. Miller. 1988. Inhibition of vein graft intimal thickening by eicosapentaenoic acid: reduced thromboxane production without change in lipoprotein levels or low-density lipoprotein receptor density. *J. Vasc. Surg.* **7:** 108–118.
16. Fong, L. G., E. Bonney, J. C. Kosek, and A. D. Cooper. 1989. Immunohistochemical localization of low density lipoprotein receptors in adrenal gland, liver and intestine. *J. Clin. Invest.* **84:** 847–856.
17. Sather, S., K. A. May, A. D. Cooper, S. W. Cushman, and F. B. Kraemer. 1988. Distribution of LDL receptor in the isolated adipocyte. *Clin. Res.* **36:** 159.
18. Cuthbert, J. A., C. A. East, D. W. Bilheimer, and P. E. Lipsky. 1986. Detection of familial hypercholesterolemia by assaying functional low-density-lipoprotein receptors on lymphocytes. *N. Engl. J. Med.* **314:** 879–883.
19. Applebaum-Bowden D., S. M. Haffner, E. Hartsook, K. H. Luk, J. J. Albers, and W. R. Hazzard. 1984. Down-regulation of the low density lipoprotein receptor by dietary cholesterol. *Am. J. Clin. Nutr.* **39:** 360–367.
20. Daga, A., M. Fabbi, T. Mattioni, S. Bertolini, and G. Corte. 1988. Pvu II polymorphism of low density lipoprotein receptor gene and familial hypercholesterolemia. Study of Italians. *Arteriosclerosis.* **8:** 845–850.
21. Lee, L. Y., W. A. Mohler, B. L. Schafer, J. S. Freudenberger, N. Byrne-Connolly, K. B. Eager, S. T. Mosley, J. K. Leighton, R. N. Thrift, R. A. Davis, and R. D. Tanaka. 1989. Nucleotide sequence of the rat low density lipoprotein receptor cDNA. *Nucleic Acids Res.* **17:** 1259–1260.
22. van Driel, I., M. Brown, and J. Goldstein. 1989. Stoichiometric binding of low density lipoprotein and monoclonal antibodies to LDL receptors in a solid phase assay. *J. Biol. Chem.* **264:** 9533–9538.
23. Gabelli, C., D. G. Stark, R. E. Gregg, and H. B. Brewer, Jr. 1986. Separation of apolipoprotein B species by agarose-acrylamide gel electrophoresis. *J. Lipid Res.* **27:** 457–460.