

# Characterization of the estrogen-induced lipoprotein receptor of rat liver

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**Abstract** The ethinyl estradiol-induced lipoprotein receptor of rat liver was purified and characterized. Liver membranes were prepared from ethinyl estradiol-treated rats, solubilized, and subjected to DEAE chromatography. A fraction with a high specific activity for low density lipoprotein (LDL) binding was isolated and used to immunize mice. Hybridomas were prepared from their spleen cells, and a clone that secreted an IgG antibody, which cross-reacted with an ethinyl estradiol-induced protein of the same molecular weight as the bovine adrenal LDL receptor, was expanded. This antibody, designated PIB3, immunoprecipitated the induced lipoprotein receptor. PIB3 was used to purify the receptor, and a polyclonal antibody was raised against the pure protein. This antibody recognized a protein of similar molecular weight in rat liver, adult dog liver, and human skin fibroblasts, thus demonstrating that the induced rat lipoprotein receptor was related to the LDL receptor of other species. This receptor is present in normal rat liver, and its content is reduced by feeding an atherogenic diet, but not by feeding a diet containing 0.5% cholesterol. Moreover, cholestyramine supplementation of the diet did not induce the receptor on liver membranes. The polyclonal antibody could prevent the binding of LDL to liver membranes from control or ethinyl estradiol-treated rats. It decreased chylomicron remnant binding to membranes from ethinyl estradiol-treated membranes, but did not affect chylomicron remnant binding to liver membranes of untreated rats, a result compatible with the existence of a distinct receptor for these latter particles. The amount of LDL receptor-independent, specific remnant binding was the same in both control and ethinyl estradiol-treated rats. This is consistent with the concept that the remnant receptor is not regulated by this treatment. **Based on the above, we conclude that the ethinyl estradiol-induced lipoprotein receptor of rat liver is biochemically and immunologically similar to the LDL receptor of other species. It is present on the liver of normal adult rats and could account for LDL as well as  $\beta$ VLDL and HDL<sub>c</sub> removal. Although it may contribute to chylomicron remnant removal, there appears to be a second unrelated receptor or process which recognizes this lipoprotein. — Cooper, A. D., R. Nutik, and J. Chen. Characterization of the estrogen-induced lipoprotein receptor of rat liver. *J. Lipid Res.* 1987. 28: 59–68.**

**Supplementary key words** lipoprotein • receptor • chylomicron remnant • low density lipoprotein

The liver is now well-established as the organ primarily responsible for removing lipoproteins from the circulation

and subsequently degrading them (1, 2). A variety of mechanisms have been suggested as having roles in this process. First, receptor-mediated endocytosis initiated by the low density lipoprotein (LDL) receptor, as described by Goldstein and Brown (3), has been demonstrated, in studies with isolated hepatocytes (4), liver cell membranes (5) and indirectly, to function in liver in vivo (6). This receptor appears to have a role in LDL and, perhaps, very low density lipoprotein (VLDL) remnant removal (7). Its presence in the liver of adult dogs, however, has been questioned (8). The LDL receptor from bovine adrenal gland has been solubilized (9) and purified (10). This has enabled the structure of the human LDL receptor to be elucidated and studied in detail in normal and abnormal states (11, 12).

In the rat, LDL degradation is normally slow relative to that of triglyceride-rich lipoproteins (13), and it has been difficult to demonstrate high affinity binding of LDL to liver from normal rats (14). Treatment of rats with ethinyl estradiol in pharmacologic doses reduces serum lipoprotein levels (15), markedly accelerates LDL catabolism (15), and induces receptors on the liver which bind LDL (14) and have characteristics similar to the LDL receptor of other species (16). Even in rabbits that lack LDL receptors, LDL accumulates in the liver and is degraded there, establishing the existence of a lower affinity, perhaps less specific, mechanism for LDL removal by liver (17).

The existence of a distinct mechanism for the removal of chylomicron remnants (18, 19), VLDL remnants (20), and cholesterol-rich high density lipoproteins (HDL<sub>c</sub>) (19) has been described in perfused liver and liver membrane binding studies. Genetic evidence for the existence of such a receptor has also been put forth since the livers

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL<sub>c</sub>, cholesterol-rich high density lipoprotein; PMSF, phenylmethylsulfonyl fluoride.

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of Watanabe rabbits, which lack LDL receptors, remove chylomicron remnants efficiently (21). It has been proposed that apoE is the ligand for this receptor (19, 22-24). C apoproteins prevent removal by this pathway (23, 24) and surface phospholipids may also play a role in its function (25). In contrast to the LDL receptor, the remnant removal process is not regulated by the amount of cholesterol in the diet (26-28), by fasting (28), or by the age of the animal (29). Hui et al. (30) recently reported purification of a protein of 56 K dalton from dog liver which bound to HDL<sub>c</sub>. The protein had some immunologic relationship to the LDL receptor, and antibodies to it precipitated the HDL<sub>c</sub> binding activity of solubilized membranes. Others (31) described partial purification of a protein of different molecular weight from rat liver to which remnants bind on ligand blots.

The purpose of the present investigation was to develop and use biochemical tools to allow us to study these processes in rat liver, since the rat has been an important and thoroughly studied model for lipoprotein metabolism. In this study we report the isolation of the receptor and production of mono- and polyclonal antibodies to the estrogen-induced lipoprotein receptor of rat liver. The similarity of this receptor to the human LDL receptor is documented, its regulation by pharmacologic and dietary factors demonstrated, and evidence that chylomicron remnant metabolism proceeds in part by another receptor is also provided.

## METHODS

### Animals

Male Sprague-Dawley rats were housed in a windowless room with a controlled light cycle and fed a standard chow diet. To induce hypercholesterolemia, an atherogenic diet was fed (27). To induce hypolipemia, rats were injected subcutaneously with ethinyl estradiol (10 mg/kg) in propylene glycol on 5 consecutive days (14).

### Lipoprotein preparation

Rat lymph was collected, chylomicrons were isolated, and chylomicron remnants were prepared as described previously (27). Human LDL was prepared from serum of fasted normolipemic donors at d 1.006-1.063 g/ml. Iodination of lipoproteins was by the method of McFarlane (32). The composition and distribution of radioactivity on comparable lipoprotein fractions has been previously reported by this laboratory (27).

### Preparation of rat liver membranes

Livers from four rats (250-300 g) were placed in ice-cold buffer containing 20 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM CaCl<sub>2</sub>, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The tissue was homogenized by two

10-sec pulses from a Polytron (Lucerne, Switzerland) homogenizer. Following centrifugation at 500 g for 5 min, the supernatant was decanted and centrifuged at 100,000 g for 1 hr. This pellet was designated the 500-100,000 g membrane preparation. For certain experiments, the 500 g supernatant was spun at 8,000 g for 20 min, and then this supernatant was spun at 100,000 g for 1 hr. This pellet will be referred to as the 8,000-100,000 g membrane preparation.

For some studies this membrane preparation was solubilized with detergent according to the method of Schneider et al. (9) by resuspending it in buffer containing 250 mM Tris-maleate (pH 6), 2 mM CaCl<sub>2</sub>, and 1 mM PMSF, at a concentration of 9 mg protein/ml. The suspension was sonicated by two 15-sec pulses from a Bronwill (Rochester, NY) Biosonic Sonifier. The preparation was then diluted with an equal volume of water, followed by the addition of octylglucoside and NaCl to final concentrations of 40 mM and 150 mM, respectively. The mixture was stirred on ice for 10 min and then spun at 100,000 g for 1 hr.

### Filter assay of LDL receptor

Solubilized membrane preparations were assayed for LDL binding in a filter assay after precipitation in a phosphatidylcholine-acetone mixture as described by Schneider et al. (9). Egg lecithin vesicles were suspended in a buffer containing 50 mM Tris-maleate (pH 6) and 2 mM CaCl<sub>2</sub>.

Aliquots of solubilized membrane were added to the vesicles with final concentrations of 0.5 mg/ml membrane protein and 0.5 mg/ml phospholipid vesicles in buffer containing 15 mM octylglucoside, 50 mM Tris-maleate (pH 6), 2 mM CaCl<sub>2</sub>, and 0.5 M NaCl. Ice-cold acetone was added to this mixture in the proportion 0.6 volumes of acetone/volume of membrane-vesicle fraction. This mixture was centrifuged at 20,000 g for 20 min at 4°C and the precipitate was resuspended in buffer containing 20 mM Tris-HCl (pH 8), 50 mM NaCl, and 1 mM CaCl<sub>2</sub>. To measure binding, the membrane-vesicles were incubated with <sup>125</sup>I-labeled LDL at room temperature in buffer containing 60 mM Tris-HCl (pH 8), 25 mM NaCl, 1 mM CaCl<sub>2</sub>, and 20 mg/ml BSA with <sup>125</sup>I-labeled LDL. After 1 hr, the mixture was filtered through a cellulose acetate filter N25/45UP (Oxoid, Basingstoke, England). The LDL receptor complex was retained whereas the unbound LDL passed through the filter. Specificity was demonstrated with a 20-fold excess of unlabeled LDL.

### DEAE cellulose chromatography of solubilized membrane preparation

Solubilized membranes (500-100,000 g pellets) from 40 g of liver were diluted 1:4 with a buffer containing 10 mM Tris-maleate (pH 6), 2 mM CaCl<sub>2</sub>, and 40 mM octylglucoside, and applied to a DEAE cellulose column (2.6 × 7.1 cm). The column was then washed with 50 mM Tris-maleate (pH 6), 2 mM CaCl<sub>2</sub>, and 40 mM octyl-

glucoside, and eluted with a 100-ml linear gradient of 0–250 mM NaCl in the washing buffer as described above (10). The fractions demonstrating the greatest LDL binding activity were pooled and designated the DEAE fraction.

### Preparation of monoclonal antibody to the rat liver LDL receptor

Balb/c mice were immunized with the DEAE fraction demonstrating maximal LDL binding prepared from liver membranes of an ethinyl estradiol-treated rat. Each mouse was immunized three times at 3-week intervals with 100  $\mu$ g of protein. Three days after the last injection, the spleen cells of the mice were fused with mouse myeloma cells by a standard procedure (33). Cells were aliquoted into 96-well plates and incubated in RPMI 1640 media containing 20% fetal calf serum and 2% HAT (Sigma, St. Louis, MO) for 2–3 weeks.

### Screening hybridomas for antibodies to the LDL receptor

A two-step screening procedure was used. For the first screen, approximately 30  $\mu$ g of partially purified receptor (DEAE fraction) was applied to each well of a 96-well polyvinyl plate. After drying overnight at 37°C, the receptor was fixed to the plate with 50% methanol. Media from each growing colony of hybridomas was transferred into one of the pre-coated wells and incubated at room temperature for 2 hr. After washing,  $^{125}$ I-labeled goat anti-mouse IgG (6:25  $\mu$ g IgG/well) was added. The plates were washed, the wells were cut from the plate, and radioactivity was measured. All wells containing at least twice as many counts as background were designated positive, and their media were subjected to a second screening step. For the second screen, each positive sample from screen 1 was compared for its ability to bind to normal and ethinyl estradiol-treated membranes. The procedure was identical to that used in the first screen, except that pairs of wells were pre-coated with solubilized membranes from normal or ethinyl estradiol-treated rats. Hybridoma cultures that were positive for both screens were cloned by limiting dilution according to the method of Kennett, McKearn, and Bechtol (34). Cells from each positive clone were grown and injected into pristane primed retired breeder Balb/c mice. After 1–2 weeks, the ascites fluid was withdrawn and IgG was prepared.

### Ligand blotting and immunoblotting

The solubilized 8,000–100,000 *g* liver membranes from normal and ethinyl estradiol-treated rats, the DEAE fractions from these membranes, or the purified LDL receptor were electrophoresed on 6% polyacrylamide gels prepared by the method of Laemmli (35). Samples contained no reducing agents and were not heated. Unless otherwise noted, between 150–200  $\mu$ g of protein was loaded on the

gels. Proteins were transferred to nitrocellulose paper with a Bio-Rad (Richmond, CA) blotting apparatus in a buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol at 175 mA for 16 hr according to the method of Daniel et al. (36). Strips for immunoblotting were incubated with 3–12  $\mu$ g/ml of purified IgG or 10  $\mu$ l/ml of monoclonal media or 1  $\mu$ l/ml of polyclonal rabbit serum for 2 hr at room temperature and were then washed and incubated with  $^{125}$ I or horseradish peroxidase-conjugated goat anti-mouse or  $^{125}$ I-labeled goat anti-rabbit IgG. After washing, autoradiograms were obtained, when appropriate, by exposing the dried nitrocellulose paper to Kodak (Rochester, NY) XAR-2 film. Horseradish peroxidase-conjugated IgG was developed according to instructions in the Bio-Rad (Richmond, CA) Immuno-Blot™ (GAR-HRP) assay kit. Those strips that were to be used in ligand blotting were incubated with human LDL at a concentration of 20  $\mu$ g/ml for 1 hr. Following four washes, the strips were incubated with rabbit anti-human LDL IgG at a concentration of 3  $\mu$ g/ml for 2 hr. Development was as described above, using either autoradiography or horseradish peroxidase localization.

### Immunoprecipitation of the LDL receptor from solubilized membranes

Octylglucoside-solubilized membranes from ethinyl estradiol-treated rats were incubated at 4°C for 1 hr with specific monoclonal antibody or non-immune monoclonal antibody in the proportions of 2.5 mg of membrane protein to 40  $\mu$ g of IgG in a volume of 1 ml in a buffer containing 40 mM octylglucoside, 0.125 M Tris-maleate (pH 6) and 2 mM CaCl<sub>2</sub>. After 4 hr, 800  $\mu$ g of goat anti-mouse IgG was added, and the incubation was continued overnight at 4°C. Pansorbin was added to the incubation mixture in the proportion of 500  $\mu$ l of Pansorbin/mg of goat anti-mouse IgG and the mixture was incubated for 1.5 hr. The total incubation volume was 890  $\mu$ l. The immunoprecipitate was isolated by centrifugation at 8,000 *g* for 10 min. An aliquot (160  $\mu$ l) of the supernatant was run on a 6% polyacrylamide gel and used in blotting experiments with LDL as the ligand.

### P1B3 immunoaffinity column for purification of the rat liver LDL receptor

Monoclonal P1B3 IgG (12 mg) was coupled to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) by a standard method. The 3-ml column was equilibrated with buffer A containing 50 mM Tris-HCl (pH 8), 2 mM CaCl<sub>2</sub>, and then washed with the same buffer containing 40 mM octylglucoside and 0.1% Nonidet P-40 (buffer B), and finally rewashed with buffer A. The DEAE fraction from ethinyl estradiol-treated rat liver membranes (from 40 *g* of tissue) was dialyzed against 10 liters of buffer containing 50 mM Tris-maleate (pH 6) and 2 mM CaCl<sub>2</sub> to remove the octylglucoside, and the dialyzed solution was

adjusted to pH 8 by titration with 250 mM Tris-HCl, 2 mM CaCl<sub>2</sub> (pH 7.5), and centrifuged at 100,000 *g* for 1 hr at 4°C. The sample was slowly applied to the affinity column at 4°C, and the column was washed with 30 ml of buffer A, 60 ml of buffer B, and finally 250 ml of buffer A. Elution of the LDL receptor was accomplished with 50 mM triethylamine chloride (pH 11) and was monitored with a UV Spectrophotometer at A<sub>280</sub>. The peak fractions were immediately frozen in liquid nitrogen.

#### <sup>125</sup>I-labeled P1B3 binding to rat liver membranes

Monoclonal P1B3 IgG was iodinated by the Iodogen method (Bio-Rad, Richmond, CA) to a specific activity of 400 cpm/ng. Liver membranes from control and treated animals were incubated with P1B3 for 1 hr at 37°C in buffer containing 50 mM Tris (pH 7.4), 100 mM NaCl, 0.5 mM CaCl<sub>2</sub>, and 20 mg/ml BSA. Each tube contained 1.3 mg/ml membrane protein and the designated <sup>125</sup>I-labeled P1B3 concentration. Specific binding was measured by incubating a duplicate tube containing a 40-fold excess of cold IgG for each concentration of <sup>125</sup>I-labeled P1B3. The number of counts bound in this tube was subtracted from the value obtained in the corresponding tube, and the difference was assumed to be specific binding. The incubation was terminated by spinning the tubes in a Microfuge for 5 min and washing the pellets twice with 10% sucrose. The tips of the tubes were cut and counted in a gamma counter.

#### Preparation of polyclonal antibody to the LDL receptor

Purified LDL receptor obtained from the monoclonal P1B3 affinity column was injected into a female New Zealand White rabbit in three doses at intervals of 2 weeks. The presence of antibody was shown by the Ouchterlony Double Diffusion Technique as well as by using the rabbit serum in blotting experiments against rat liver membranes. The antibodies were purified by chromatography on Protein A Sepharose CL-4B (Pharmacia, Piscataway, NJ).

#### Other methods

Protein was determined by the method of Lowry et al. (37).

## RESULTS

#### Production of monoclonal antibody to the estrogen-induced lipoprotein receptor

Monoclonal antibodies were produced to the estrogen-induced lipoprotein receptor of the rat liver, using a strategy similar to that employed by Beisiegel et al. (38), for the LDL receptors of bovine adrenal gland. A partially purified receptor was obtained by DEAE cellulose chro-

matography of solubilized liver membranes. The greatest LDL binding was obtained in the fraction that eluted from the column when the conductivity of the NaCl gradient was 4 μmhos. Aliquots of the most active fractions were injected into mice, and the spleen from one mouse, whose serum could distinguish normal liver membranes from those of an estrogen-treated rat, was fused with myeloma cells. The media from successful fusions were screened for production of antibodies to the DEAE fraction and then for the ability to distinguish between membranes from liver of normal and estrogen-treated animals. These were subcloned by limiting dilution and again screened. There were eight positive clones.

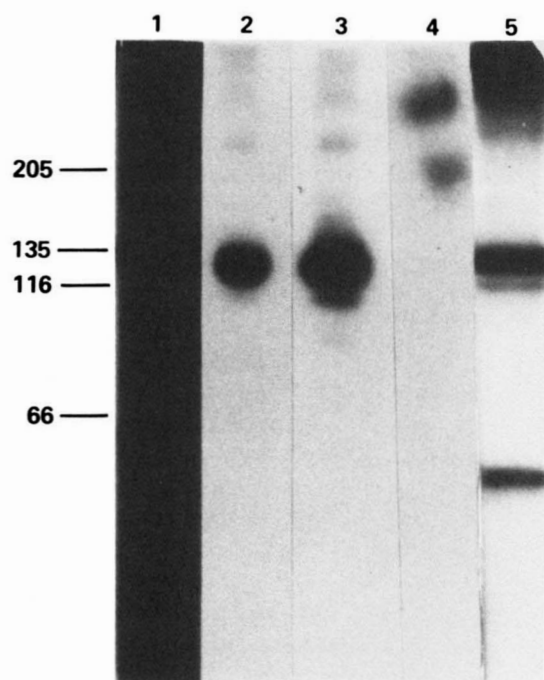
Membranes from estrogen-treated animals were subjected to gel electrophoresis and then blotted to nitrocellulose. The location of the LDL binding site was identified by ligand blotting with human LDL. The specificity of this site was confirmed by the fact that LDL blotting required Ca<sup>2+</sup>, and methyl LDL did not bind (not shown). Immunoblots with media from the hybridomas were then prepared. Six reacted primarily with a protein of the molecular weight (135,000) identical to that with which LDL reacted (Fig. 1). In all of these instances there was also a small amount of reactivity with a protein with a molecular weight of about 115,000. It has been demonstrated that this is the molecular weight relationship of the precursor of the LDL receptor and the mature receptor (10). One of these clones produced an antibody that recognized a second protein of much lower molecular weight as well. Two recognized exclusively proteins of different molecular weights. One of the positive clones, designated P1B3, was expanded and used in further studies. It was found to be an IgG of the γ<sub>1</sub> isotype.

#### Effect of P1B3 on ligand blotting

P1B3 did not compete with <sup>125</sup>I-labeled LDL binding to whole membranes nor did pre-incubation with P1B3 prevent <sup>125</sup>I-labeled LDL binding in the ligand blotting assay (not shown). However, when solubilized liver membranes were incubated with P1B3, followed by anti-mouse IgG and then protein A, and the supernatant was electrophoresed, transferred to nitrocellulose, and incubated with LDL, the amount of LDL binding at 135 Kdalton decreased as a function of the concentration of P1B3 (see Methods for the details of this protocol). At high antibody concentrations, there was virtually no residual LDL blotting. Identical treatment of membranes with non-immune monoclonal IgG did not affect ligand blotting of LDL. This result supports the contention that the antibody was to the receptor protein.

#### Production of a monospecific polyclonal antibody to the LDL receptor of rat liver

An immunoaffinity column was prepared using P1B3. This removed primarily a single protein of 135 Kdaltons



**Fig. 1.** Immunoblots of hybridoma media. The DEAE fraction of rat liver membranes which demonstrated the maximal LDL binding in Fig. 2 was used to immunize Balb/c mice. In addition, portions of this fraction were electrophoresed on 6.0% polyacrylamide and then transferred to nitrocellulose as described by Daniel (36). Non-reducing conditions were used. Lane 1, a strip of the nitrocellulose was incubated with human LDL followed by rabbit anti-human apoB, followed by  $^{125}\text{I}$ -labeled goat anti-rabbit IgG; lanes 2-5, strips were incubated with the supernatant of hybridoma colonies that passed the various screens described in the text and then with  $^{125}\text{I}$ -labeled goat anti-mouse IgG; autoradiographs of the strips were then prepared. Molecular weight standards were run and used to determine the molecular weights of the proteins indicated.

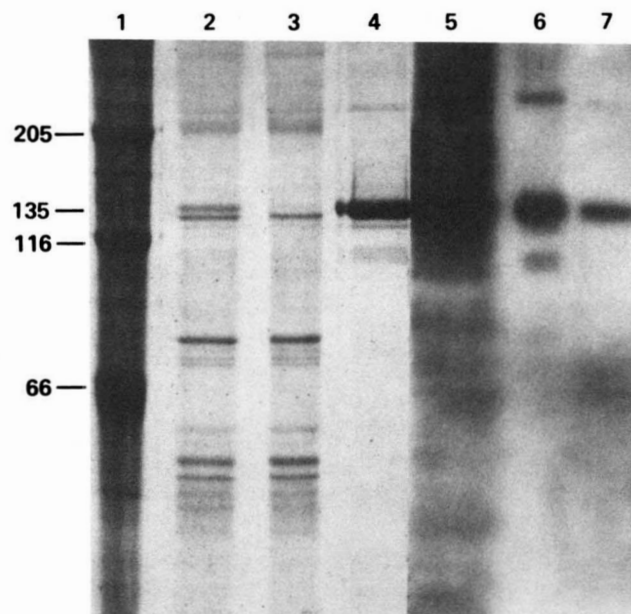
with only traces of other protein (**Fig. 2**). LDL bound to this protein on Western blots. A rabbit was immunized with this protein and IgG was prepared from its serum. This IgG reacted primarily with a 135 Kdalton protein (**Fig. 3**). It was concluded that the other proteins with which the antiserum cross-reacts are related to the receptor based on several lines of evidence. First, the proteins were also recognized by the monoclonal antibody when it was present at a high concentration (**Fig. 3**, lane 3). Second, the bands were all induced by estrogen therapy (**Fig. 3**, lanes 1 and 2). Third, when the antiserum was incubated with down-regulated rat hepatoma cells (not shown) or liver membranes from normal rats (**Fig. 3**, lanes 4-7), the bands were not reduced as would have been expected if they were those of liver proteins unrelated to the LDL receptor. This polyclonal antibody also cross-reacts on immunoblots with protein of the appropriate molecular weight from cultured rat hepatoma cells (39), cultured murine macrophage-derived cells

(J774) (40), as well as liver membranes of adult dog and membranes from cultured human skin fibroblasts (**Fig. 4**).

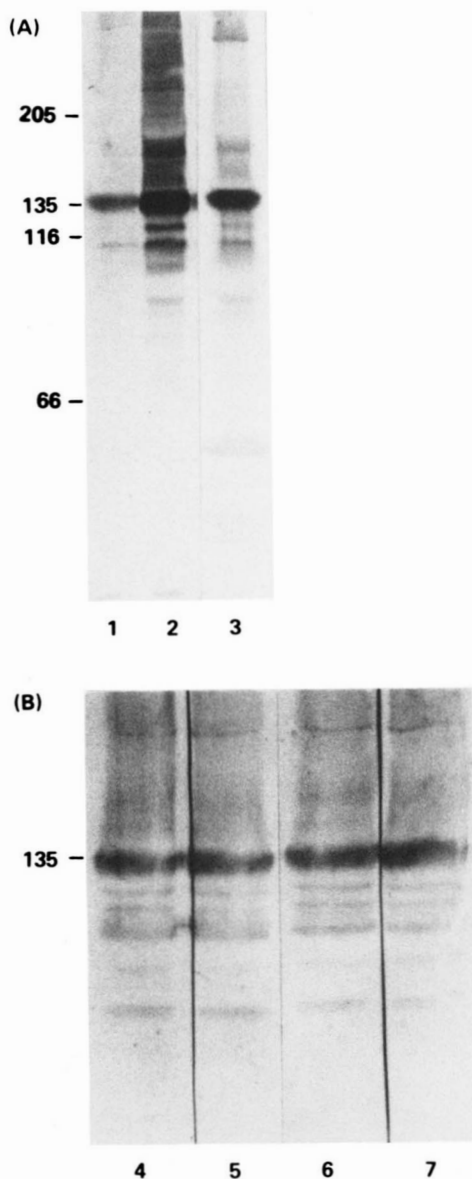
### Regulation of P1B3 binding

In order to study the regulation of the number of LDL receptors on liver membranes, the amount of P1B3 binding to tissue was studied. Antibody binding was complete within 1 hr, was linear with respect to membrane protein, and was saturated at  $4 \mu\text{g}$  of antibody/ml incubation buffer. Treatment of rats with ethinyl estradiol significantly increased LDL binding and comparably increased P1B3 binding to whole membrane (**Fig. 5A**). At saturation, the fold difference was not as great (not shown), possibly because nonspecific binding is more difficult to eliminate. The same phenomena occurs with LDL binding to membranes (14). P1B3 binding to nitrocellulose blots of these membranes revealed a similar increase (**Fig. 5B**).

Feeding a diet that rendered the animals hypercholesterolemic consistently decreased the amount of specific P1B3 binding to liver cell membrane by about 20% and reduced the amount of antibody binding to Western blots (**Fig. 5**). This is in contrast to remnant binding which is not affected by this feeding regimen (not shown). Feeding animals a smaller amount of cholesterol (0.5%) did not



**Fig. 2.** Purification of the ethinyl estradiol-induced lipoprotein receptor. The DEAE fraction that demonstrated maximal LDL binding was passed through a P1B3 immunoaffinity column prepared as described in Methods. Lanes 1-4 are Coomassie Blue-stained 6.0% polyacrylamide gels. Lanes 5-7 are ligand blots prepared as described in the legend to Fig. 1, lane 1. Lane 1, molecular weight markers; lane 2, maximal LDL binding DEAE fraction; lane 3, immunoaffinity column run-through; lane 4, immunoaffinity-retained fraction; lane 5, LDL blot to whole cell membrane; lane 6, LDL blot to DEAE fraction; lane 7, LDL blot to immunoaffinity-retained fraction.



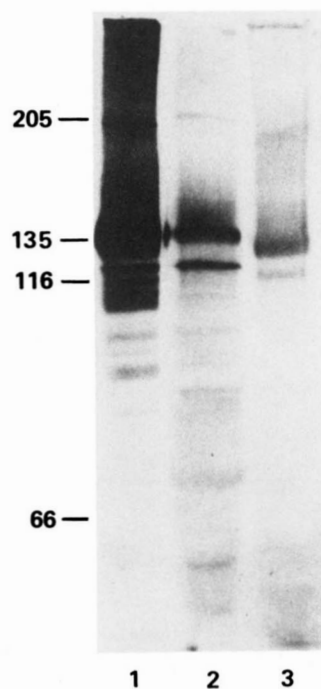
**Fig. 3.** Characterization of the polyclonal anti-receptor antibody. Liver membranes from control or estrogen-treated rats were prepared, and immunoblotting was carried out as described in the legend to Fig. 2, except that bound antibody was visualized with horseradish peroxidase-labeled goat anti-rabbit IgG. A, Lane 1, polyclonal antibody versus liver membranes from control rat; lane 2, polyclonal antibody versus liver membranes from ethinyl estradiol-treated rat; lane 3, PIB3 ( $10 \times$  the usual concentration) versus membranes from ethinyl estradiol-treated rats. B, Lanes 4-7, the polyclonal antibody was incubated with an increasing concentration of liver cell membranes from a control rat. After 4 hr of incubation, the membranes were removed by centrifugation, and the supernate was used to immunoblot membranes from an ethinyl estradiol-treated rat; lane 4, no membrane; lane 5, 0.5 mg of membrane protein/ml; lane 6, 1 mg of protein/ml; lane 7, 2 mg of membrane protein/ml.

affect PIB3 binding nor did feeding the animal cholestyramine (not shown). These results are consistent with the effects of these agents on LDL turnover in the whole

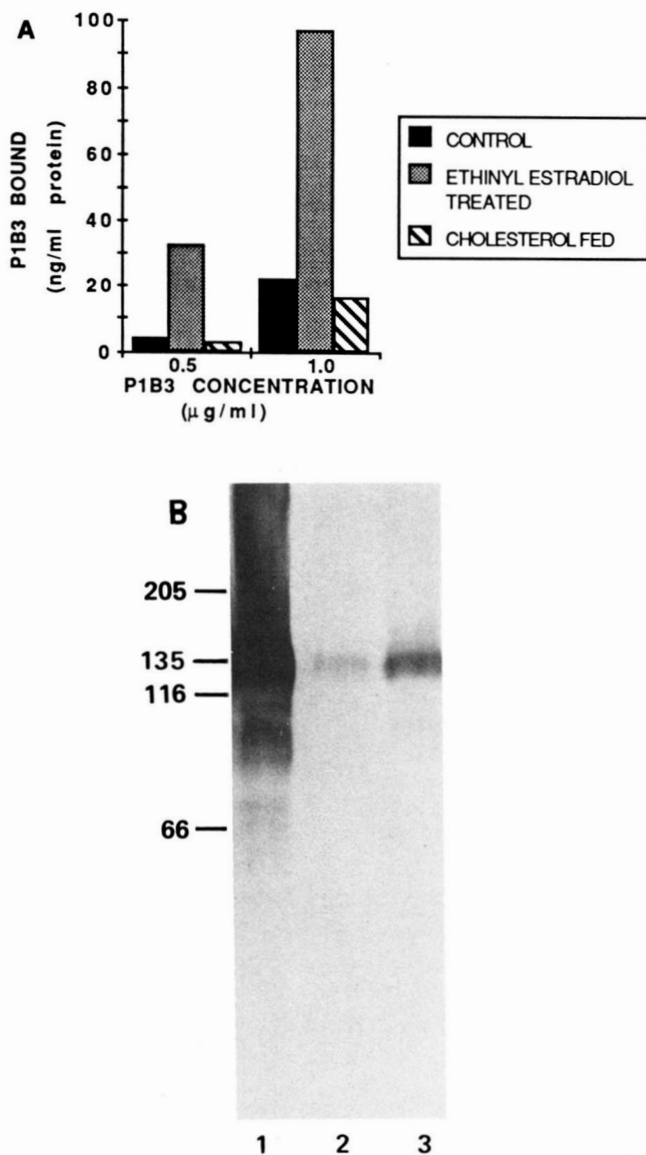
animal, where it has been demonstrated that LDL removal by rat liver can be both up- and down-regulated (41), albeit with somewhat less responsiveness than in other species.

### Competition between the polyclonal antibody and lipoprotein

The polyclonal antibody competed with LDL for binding to liver membranes from normal and estrogen-treated rats. It was as effective as unlabeled LDL as a competitor with both types of membranes (**Fig. 6A,B**). The antibody also competes for HDL<sub>c</sub> and  $\beta$ VLDL binding (not shown). In contrast, this antibody was able to compete with chylomicron remnant binding to membranes from estrogen-treated animals (**Fig. 6D**), but did not compete significantly with remnant binding to membranes from control (untreated) rats (**Fig. 6C**). A similar result, but with less nonspecific binding, was obtained when the membranes were first solubilized and the binding of remnants to the solubilized proteins was measured by a filter assay. Once again, in membranes from normal animals, the polyclonal antibody did not affect remnant binding, although it diminished LDL binding (**Fig. 6E,F**). In contrast, in membranes from estrogen-treated animals, the antibody did compete for both remnant and LDL binding.



**Fig. 4.** Immunoblotting of dog and human cell membranes with the polyclonal antibody. Immunoblotting was carried out as described in the legend to Fig. 5. Lane 1, membranes from ethinyl estradiol-treated rat; lane 2, membranes from control-fed adult dog; lane 3, membranes from human skin fibroblasts.



**Fig. 5.** Regulation of rat liver LDL receptors. Animals were fed a control diet or an atherogenic (27) diet; a separate group of control-fed animals was injected with ethinyl estradiol as described in Methods. A, Membranes were prepared from their livers and incubated with  $^{125}\text{I}$ -labeled PIB3 or  $^{125}\text{I}$ -labeled PIB3 and a 40-fold excess of unlabeled PIB3. The difference in cpm recovered in the pellets between these is the specific binding. Two different antibody concentrations were used; (■) control; (▨) ethinyl estradiol-treated; (▩) cholesterol-fed. Duplicate determinations were made, and a representative experiment is shown. B. Identical amounts of the membranes described above were immunoblotted with the polyclonal antibody after polyacrylamide gel electrophoresis as described in the legend to Fig. 2. They were developed with horseradish peroxidase as described in Methods. Lane 1, ethinyl estradiol; lane 2, cholesterol-fed; lane 3, control. A representative experiment is shown.

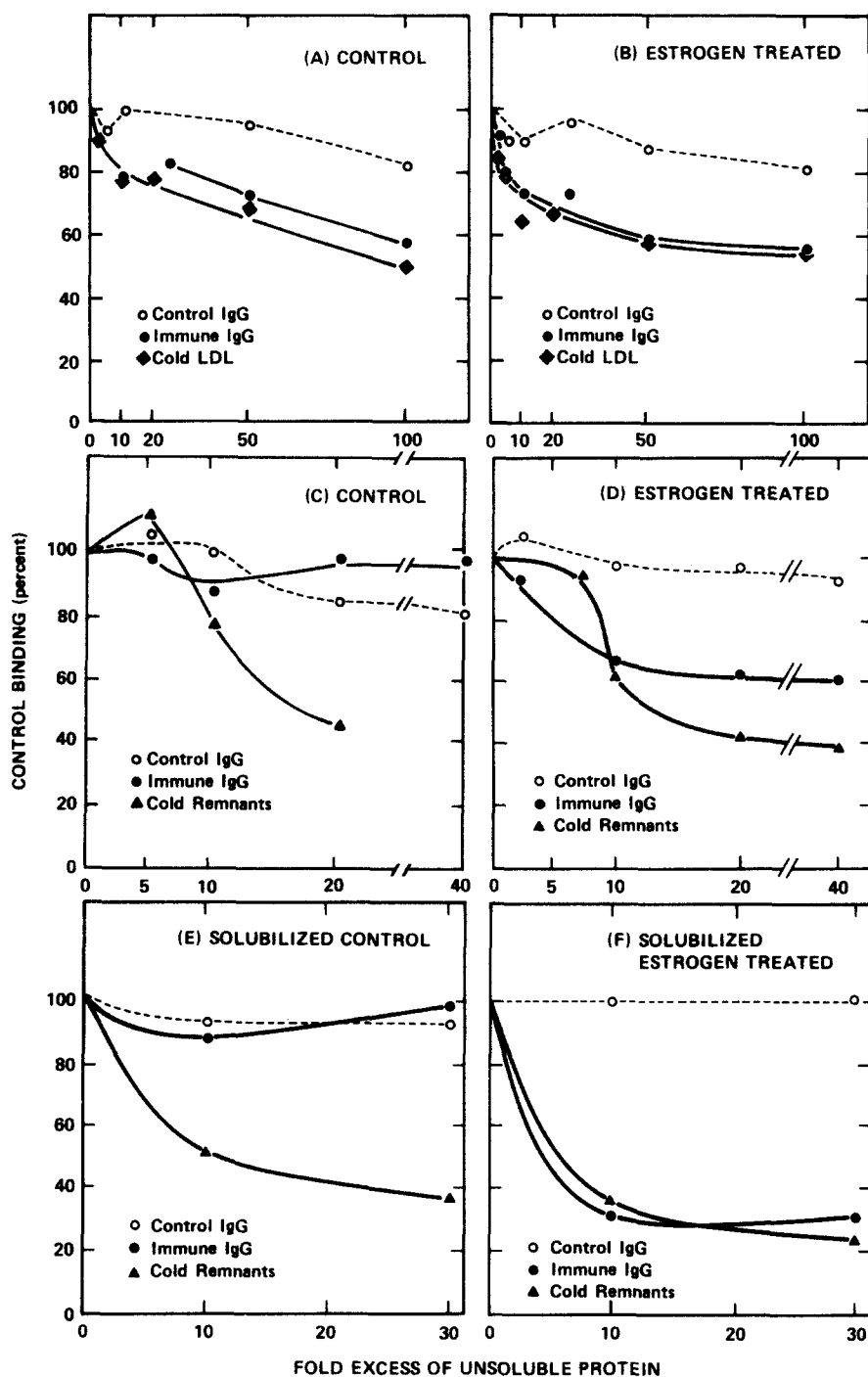
This result supports the concept that there is a remnant binding site on normal liver that is not related to the LDL receptor site.

In principle, one can use the data of Fig. 6 to calculate the absolute value of LDL receptor-independent, specific remnant binding by subtracting the amount of remnant binding in the presence of excess cold remnants from that in the presence of excess anti-LDL receptor antibody. When this is done with the data of Fig. 6C and D, the amount of remnant specific binding to control membranes (18 ng/mg of membrane protein) is comparable to the amount of remnant specific binding to membranes from ethyl estradiol-treated rats (17 ng/mg membrane protein). This is consistent with the idea that ethinyl estradiol specifically induced LDL receptors. This assay should prove useful for future studies of remnant receptor activity in a variety of physiologically perturbed states.

## DISCUSSION

Two general conclusions can be drawn from the present study. First, rat liver possesses a receptor that is very similar to the LDL receptor of human tissue. The receptor is of a similar molecular weight and is immunologically related to the LDL receptor of mouse, dog, and human tissue. In previous reports, evidence for LDL binding in rat liver was observed only after treatment of the animal with ethinyl estradiol (14). Roach and Noël (16) recently reported ligand blotting of LDL to a partially purified protein of this molecular weight, but again this was in the estrogen-treated animal, as was the report of cross-reactivity of antibody to LDL receptor from bovine adrenal gland with membrane of liver from estrogen-treated rats (42). Since the liver is the principal site of LDL degradation in the rat, as in the rabbit, and since more than one process can lead to LDL removal and degradation, the biochemical demonstration of the receptor in this organ provides a useful new probe for studies of receptor physiology. The current report documents the presence of the LDL receptor in the normal animal, thus providing a biochemical basis for the observation that the disappearance of LDL is delayed by alterations that affect the ability of the LDL particle to bind to the receptor (6).

In vivo the expression of the receptor is reduced by a cholesterol-rich atherogenic diet, suggesting that the number of receptors expressed, even in the normal animal, is under physiologic control. In concert with the results of Spady, Turley, and Dietschy (41), who found that cholesterol feeding at a more modest level did not reduce, and bile acid sequestration did not increase, receptor-mediated removal of LDL, these perturbations did not affect LDL receptor expression on liver membranes. Thus, the precise threshold at which LDL receptor activity in rat liver is down- or up-regulated remains to be determined. These results, along with the physiologic studies of Carew, Pittman, and Steinberg (6) and Spady et al. (41), make it highly likely that this receptor is responsible for LDL



**Fig. 6.** Competition of the polyclonal antibody for lipoprotein binding. Liver cell membranes from control and ethinyl estradiol-treated animals were prepared as described in Methods. The membranes were then incubated with a trace of  $^{125}\text{I}$ -labeled lipoprotein and varying concentrations of unlabeled lipoprotein or unlabeled immune or non-immune rabbit IgG. The amount of lipoprotein bound at 100% is indicated in parentheses. A,  $^{125}\text{I}$ -labeled LDL binding to membranes from control rats (79 ng/mg protein); B,  $^{125}\text{I}$ -labeled LDL binding to membranes from ethinyl estradiol-treated rats (228 ng/mg protein); C,  $^{125}\text{I}$ -labeled remnant binding to membrane from control rats (292 ng/mg protein); D,  $^{125}\text{I}$ -labeled remnant binding to membranes from ethinyl estradiol-treated rats (676 ng/mg protein); E,  $^{125}\text{I}$ -labeled remnant binding to solubilized membranes from control rats (120 ng/mg protein); F,  $^{125}\text{I}$ -labeled remnant binding to solubilized membranes from ethinyl estradiol-treated rats (513 ng/mg protein). Each point is the mean of duplicate determinations of a representative experiment.



degradation in the normal adult animal. Since HDL<sub>c</sub> and  $\beta$ VLDL are also very good ligands for this receptor, it seems likely that this receptor plays a role in their metabolism.

A minor issue resolved by these studies relates to the activity of human LDL as a ligand for the rat receptor. Although Innerarity, Pitas, and Mahley (43) have suggested that human LDL is not a ligand for the rat receptor, we found previously in rat hepatomas that it was a ligand (44). In the present study we have unequivocally demonstrated that human LDL binds to the rat LDL receptor. Whether the low affinity of human LDL is due to differences between rat and human apoB, with equivalent differences between the rat and human LDL receptors, or whether it is due to contamination with or acquisition by rat LDL of apoE is not yet clear. However, it suggests that the use of human LDL as a marker for the low affinity or nonspecific pathway is not entirely appropriate.

Secondly, these studies provide further, albeit indirect, evidence for the existence of an LDL receptor-independent mechanism for chylomicron remnant removal. Remnant binding to liver membranes from normal animals was not blocked by the polyclonal antibody to the LDL receptor. This antibody could prevent the binding of other lipoproteins to the LDL receptor and could reduce remnant binding to liver membranes from estrogen-treated rats. Although one might question the physiologic relevance of the membrane binding assay, we have recently reported data demonstrating that binding of remnants, remnant removal, and hepatic remnant accumulation are all regulated in concert when rats are treated with ethionine (Barnard, G. F., E. Daniels, S. K. Erickson, and A. D. Cooper. Manuscript submitted.). Why there is so little remnant binding to LDL receptors under normal conditions is not clear. The relative contribution of the two receptors to removal of various lipoprotein classes is of considerable importance and will be facilitated by the availability of these antibodies for studies in rats and mice.

A second finding supporting the existence of a separate remnant removal mechanism was the observation that the number of LDL receptors in liver membrane was down-regulated by feeding the animals an atherogenic diet. Remnant binding and metabolism have been found not to be affected by this diet. These observations, along with physiologic evidence demonstrating that dietary lipid removal is normal in animals and patients who lack LDL receptors, provide strong support for the concept of an LDL receptor-independent remnant removal process.

The biochemical nature of the remnant receptor remains unresolved by this study. Hui et al. (30) reported the purification of a protein that bound to HDL<sub>c</sub> and remnants and was immunologically cross-reactive with anti-LDL receptor antibodies. The molecular weight of

this protein was about 66,000. Based on ligand blotting, Kinoshita et al. (31) have suggested that the receptor has a molecular weight of 36,000. In this study we found no evidence of any proteins that were cross-reactive to the LDL receptor and were not increased with ethinyl estradiol treatment. Furthermore, polyclonal anti-LDL receptor antibody did not interfere with remnant binding to normal liver, suggesting that the two receptors are immunologically dissimilar in the rat. Whether any of these proteins are the remnant receptor remains to be elucidated.

In summary, we have purified and prepared antibodies to the rat liver lipoprotein receptor which is induced by ethinyl estradiol treatment. The receptor is biochemically and immunologically similar to the LDL receptor of man and several other species. It is present in normal adult rat liver and can account for LDL as well as  $\beta$ VLDL and HDL<sub>c</sub> removal. Although it may contribute to chylomicron remnant removal as well, there seems to be a second unrelated receptor or process which recognizes this lipoprotein. ■

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