JCI The Journal of Clinical Investigation

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J Clin Invest. 1978;61(5):1298-1308. https://doi.org/10.1172/JCI109047.

Research Article

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Identification of a Lymphocyte Surface Receptor for Low Density Lipoprotein Inhibitor, an Immunoregulatory Species of Normal Human Serum Low Density Lipoprotein

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ABSTRACT The present study demonstrates the existence on human peripheral blood lymphocytes of a saturable cell surface receptor for low density lipoprotein inhibitor (LDL-In), a subset of normal human serum low density lipoprotein (LDL) that has been previously demonstrated to suppress selected lymphocyte functions in vivo and in vitro. The binding of radioiodinated LDL-In of demonstrable biological activity occurs rapidly and is quantitatively augmented by prior cultivation of the lymphocytes in lipoprotein-depleted serum, suggesting regulation of receptor density by lipoproteins in vivo. Binding is temperature dependent, facilitated by calcium ions, saturable at 4°C within 40–60 min, and blocked by prior exposure to unlabeled LDL-In. The lymphocyte receptor is trypsin sensitive and regenerates in vitro with a $t_{1/2}$ of 3.6 h. LDL-In receptors are calculated to have a maximum density of 4,860±460 per cell if uniformly distributed on all lymphocyte subsets. These receptors have an estimated average association constant of 1.47×10^7 liters/mol. When considered in context of the estimated concentration of LDL-In in blood, the receptors should be partially occupied in vivo by endogenous plasma LDL-In. Prior site occupancy inhibition experiments designed to analyze the specificity of LDL-In binding demonstrate that (*a*) LDL-In is 13.7-fold more effective than whole LDL in blocking the subsequent binding of ^{125}I-LDL-In to cells; and that (*b*) LDL is 11-fold more effective than LDL-In in blocking the binding of ^{125}I-LDL. This is consistent with the degree of contamination of each lipoprotein with the other lipoprotein. An independent identity of the LDL-In receptor is also supported by observations that in contrast to the previously described LDL receptor, synthesis and expression of the LDL-In receptor on lymphocytes are not suppressed by cultivation of the cells in the presence of 25-hydroxycholesterol and cholesterol. These findings suggest the existence of a previously undescribed and discrete receptor on lymphocytes for LDL-In, and that the modulation of lymphocyte function by LDL-In may be mediated by a specific cell surface receptor pathway.

INTRODUCTION

Cell surface membrane receptors serve as specific recognition sites for the regulation of cellular metabolism by certain exogenous signals. Although a variety of molecules interact with surface receptors, serum lipoproteins have only recently been shown to participate in surface receptor-dependent bioregulatory phenomena. Low density lipoprotein (LDL),[1] the first described bioregulatory lipoprotein, has been shown to regulate cholesterol biosynthesis in vitro (1, 2). LDL, which binds with high affinity to specific cell surface membrane receptors, appears to be internalized by a process resembling endocytosis and subsequently

This is publication no. 1294 of the Scripps Clinic and Research Foundation.

Dr. Curtiss is a recipient of Fellowship 1 F32 CA-05804 from the U. S. Public Health Service.

Received for publication 3 May 1977 and in revised form 30 December 1977.

[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; DS, Dulbecco's phosphate-buffered saline; E receptor, nonimmune lymphocyte receptors for SRBC; FCS, fetal calf serum; HDL, high density lipoprotein; LDL, low density lipoprotein; LDL-In, low density lipoprotein inhibitor; LPDS, lipoprotein-depleted serum; PBL, human peripheral blood lymphocytes; PBM, human peripheral blood mononuclear cells; PHA, phytohemagglutinin; RIF, rosette inhibitory factor; SRBC, sheep red blood cells.

transported to lysosomes where the cholesterol ester component of LDL is hydrolyzed and the resultant unesterified cholesterol released. This cholesterol then reduces the cellular biosynthesis of unesterified cholesterol by regulating the activity of a rate-controlling enzyme.

Independent studies of humoral factors that influence lymphocyte function have led to the recognition of two subsets of LDL that also possess bioregulatory properties, namely: (*a*) rosette inhibitory factor (RIF), and (*b*) low density lipoprotein inhibitor (LDL-In). RIF, first discovered in some sera from individuals with hepatitis virus infection, is an inducible minor species of serum LDL capable of metabolically regulating the functional capacity of human thymus-derived lymphocytes to bind to sheep red blood cells (SRBC) via the E receptor (3, 4). RIF, which appears to possess an unusual apolipopeptide composition, binds with very high affinity to a limited number of lymphocyte surface receptors (RIF receptors) that are independent of the E receptor (3, 4).

LDL-In was subsequently identified by us in normal human serum by its capacity to suppress human lymphocyte responses to mitogen and allogeneic cells in vitro (5). We have suggested that LDL-In may represent a normal physiologic repressor of lymphocyte activation (6). LDL-In that has been partially purified from whole LDL appears to represent a relatively minor species of serum LDL (5). The biological effect of human LDL-In on lymphocytes is not species specific because isolated LDL-In suppresses both murine mixed lymphocyte reactions (6) and the primary generation of murine cytotoxic T cells in vitro (7). Administration of less than 10 pmol of human LDL-In to mice induces a significant suppression of the primary cytotoxic T-cell response (7). The bioregulatory effects of LDL-In are temporally dependent and only a transient exposure of lymphocytes to LDL-In is required to suppress their subsequent stimulation by lectins or allogeneic cells (5, 6). Furthermore, the regulatory effects of LDL-In are selective in that LDL-In appears to influence only the primary inductive events required for lymphocyte stimulation (5–7).

Recent evidence has identified the lymphocyte rather than the macrophage as the primary target of LDL-In-mediated immunoregulation (8), and it is now appropriate to ask whether or not a cellular receptor is involved in the initial steps of LDL-In-mediated regulation. This study is addressed to the interaction of LDL-In with human lymphocytes and the identification of a discrete saturable cell surface receptor for this immunoregulatory molecule.

METHODS

Cells. Peripheral blood mononuclear cells (PBM) from normal donors were isolated from heparinized venous blood by a modification of the method of Boyum (9). Blood was diluted with an equal volume of RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.), and 10-ml aliquots layered onto 3 ml of Ficoll-Hypaque (1.074 g/cm^3; Pharmacia Fine Chemicals, Piscataway, N. J.). After centrifugation at 2,260 *g* (at the interface) for 6 min at 20°C, the mononuclear cells were harvested from the interface, mixed with 20 μl of 1% washed homologous carrier erythrocytes, washed three times with RPMI-1640, and recovered by centrifugation at 1,200 *g* for 5 min at 20°C. Cells were resuspended in complete medium consisting of RPMI-1640 supplemented with 100 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, and 10% (vol/vol) fetal calf serum (FCS). Yields of >75% that contained 83–90% lymphocytes, 10–15% monocytes, and <2% polymorphonuclear leukocytes were obtained. Viability was >98% in all cases.

Peripheral blood lymphocytes (PBL) to be used in the binding assays were prepared from PBM resuspended to a final concentration of 2×10^6/ml in complete medium containing 10% (vol/vol) lipoprotein-depleted serum (LPDS). 20-ml aliquots were then transferred to plastic tissue culture flasks with a 75-cm^2 growth area (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and maintained at 37°C in humidified 5% CO_2 in air. When present, the sterols, cholesterol and 25-hydroxycholesterol (Steraloids, Inc., Pawling, N. Y.) were added in absolute ethanol (final volume 0.5% or less). Lymphocytes were recovered after 48–72 h of culture. Viability was >96% with more than 98% of the cells identified as lymphocytes by cytologic criteria and the peroxidase (10) reaction.

Lipoprotein isolation. Serum LDL (*d* 1.006–1.063 gm/cm^3), high density lipoproteins (HDL; *d* 1.063–1.215 g/cm^3), and LPDS ($d > 1.215$ g/cm^3) were isolated from pooled normal human sera according to standard ultracentrifugation flotation techniques in a Beckman 45-Ti rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) using solid KBr for density adjustment (11). LDL-In, purified >70,000-fold relative to serum proteins, was purified from pooled normal human serum by dextran sulfate precipitation followed by density gradient ultracentrifugation in NaCl and molecular exclusion chromatography on a 2.5 × 85-cm column of Biogel A-15 (200–400 mesh; BioRad Laboratories, Richmond, Calif.) (5). As described previously, LDL-In is characterized by a mean buoyant density lower than the peak density of total LDL and as currently isolated represents a subset of serum whole LDL (5). The absolute degree of purity relative to whole LDL cannot be assessed in the absence of a quantitative means of analysis for LDL that is not LDL-In; however, preparations are purified 25- to 80-fold relative to whole LDL using immunosuppressive activity as the criterion. Ouchterlony analysis in gel demonstrated the presence of apolipoprotein chains B and D but not of A_I, A_{II}, C_I, C_{II}, or C_{III} by precipitation formation. Other major serum proteins were not demonstrable with antisera to albumin and IgG. Negatively stained preparations, examined in a Hitachi 11-E electron microscope (Hitachi Ltd., Tokyo, Japan) at 75 keV on a carbon-coated Forminar film demonstrated spherical particles of 246±26 Å diameter ($n = 50$) that are not morphologically distinguishable from whole LDL (160–260 Å) other than the greater mean diameter.

Isolated preparations of LDL, HDL, LPDS, and LDL-In were dialyzed against lipoprotein buffer (0.15 M NaCl, 0.05 mM EDTA, 0.0005% alpha-tocopherol, pH 7.4). Preparations were analyzed by double diffusion in gel with monospecific rabbit antisera to human albumin, IgG, beta lipoprotein, and alpha lipoprotein as indicators of the presence of appropriate and of contaminating serum proteins. Preparations devoid of detectable contaminants were stored at

4°C in lipoprotein buffer. Protein concentrations were determined by the method of Lowry et al. (12) using an albumin standard, and lipoprotein concentrations are expressed as micrograms protein per milliliter. The LPDS contained <10 μg/ml total cholesterol as determined by Abell's modification (13) of the method of Liebermann and Burchard.

Iodination. 100 μg of LDL or LDL-In were labeled with 2 mCi of ^{125}I (17 Ci/mg; New England Nuclear, Boston, Mass.) using critically limited oxidation with 5 μg of chloramine T (14). About 4% (3.0–4.8%) of ^{125}I was incorporated. After extensive dialysis against lipoprotein buffer and determination of protein concentration, the radiolabeled lipoproteins were chromatographed on a 1.5 × 30-cm column of BioGel A-5, 200–400 mesh (BioRad Laboratories) in lipoprotein buffer containing 1% bovine serum albumin (BSA); and the radiolabeled lipoproteins recovered in the void peak. Greater than 94% of the radioactivity was precipitated by incubation for 30 min in 15% trichloroacetic acid at 90°C, 7% or less was extracted into 20 vol of 3:1 ethanol-ether (15). More than 95% of the trichloroacetic acid-soluble radioactivity was identified as free iodide by chloroform extraction according to Bierman et al. (16). Mock-iodinated LDL-In, prepared using the same number of moles of iodide ions containing trace ^{125}I (sp act 0.17 Ci ^{125}I/mg), was similarly characterized.

Lymphocyte-suppressive activity. Lipoprotein preparations were assayed for biological activity by measuring the suppression of phytohemagglutinin (PHA)-induced lymphocyte responses in vitro as described previously (5, 8). 24 h after incubating serial dilutions of LDL-In or LDL with 1×10^5 PBM in 250-μl microcultures in complete medium, 10 μg PHA-M (Difco Laboratories, Detroit, Mich.) was added. 1 μCi [^{3}H]Methylthymidine (2 Ci/mM; ICN Pharmaceuticals, Inc., Cleveland, Ohio) was added 72 h after initiation of the cultures, and the cells were harvested with a MASH I unit (Microbiological Associates, Walkersville, Md.) 18 h later. Washed cells were transferred on filter disks to 3 ml of toluene-based Omnifluor (New England Nuclear) and the beta emission measured in a liquid scintillation counter (65% efficiency for ^3H). Cultures containing both ^{125}I-LDL-In and [^{3}H]thymidine were counted in both gamma and beta counters. Experimentally obtained ^3H counts from these cultures were corrected to true ^3H counts by subtraction of the ^{125}I contribution as estimated from a standard curve obtained by counting ^{125}I standards in the ^3H window of the beta counter. Thymidine uptake, expressed as observed cpm, was used as the index of lymphocyte response; and percent inhibition was calculated from percent uptake relative to control cultures containing no LDL-In. In all experiments, unstimulated cultures incorporated <400 cpm. Preparations of LDL-In used in these experiments had specific biological activities of 3–8 μg/ml. Biological activity is defined as the concentration of lipoprotein protein required for 50% suppression of [^{3}H]thymidine uptake (5).

Lipoprotein binding assays. The binding of LDL-In to PBL was analyzed by a modification of the techniques of Ho et al. (2). After cultivation for 48 h in complete RPMI-1640 medium containing 10% (vol/vol) LPDS, the lymphocytes were harvested by centrifugation at 1,200 *g* for 5 min at 20°C. Carrier erythrocytes were lysed by incubation for 30 min at 37°C in 0.14 M NH_4Cl, 0.015 M tris, pH 7.2; and the lymphocytes washed twice in ice-cold Dulbecco's phosphate-buffered saline (DS) containing 100 μM $CaCl_2$ and 20 mg/ml bovine serum albumin (DS-BSA). Subsequent steps were carried out at 4°C unless otherwise noted. Portions of the cell suspension ($4–5 \times 10^6$ cells) were added to 1.2-ml silicone-coated polycarbonate tubes, centrifuged for 2 min at 1,000 *g* and the supernate discarded. Assays were conducted in duplicate at a final reaction volume of 100 μl, which included the cells, 50 μl DS-BSA, ^{125}I-LDL, or ^{125}I-LDL-In in DS, and competing unlabeled lipoprotein in DS when present. Binding assays performed to assess the effects on the lymphocytes of prior incubation in the presence of 24-hydroxycholesterol and cholesterol were carried out in plastic tissue culture flasks with a 25-cm^2 growth area (Falcon Plastics) for 5 h at 37°C. These assays were conducted in duplicate at a final reaction volume of 2.0 ml, which included the cells at 2×10^6/ml in complete medium containing 10% (vol/vol) LPDS, and 50 μl of the labeled ligand in DS. Labeled and unlabeled lipoprotein preparations were centrifuged at 7,000 *g* for 5 min immediately before use. After incubation, the cells were collected by the addition of 1.0 ml of DS-BSA and centrifugation at 7,000 *g* for 5 min. The cell pellet was washed with 1.0 ml of DS-BSA, immediately centrifuged at 7,000 *g* for 3 min, resuspended in 100 μl of DS-BSA, layered onto a second tube containing 1.0 ml of 100% FCS, and recentrifuged at 7,000 *g* for 5 min. The radioactivity of the recovered cell pellets was determined in a gamma counter, and the pellets hydrolyzed by incubation overnight at 37°C with 0.5 ml 0.1 N NaOH. The protein content was determined by the method of Lowry et al. (12) using BSA standards, and results are expressed as nanograms of ^{125}I-LDL-In or ^{125}I-LDL protein bound per milligram cell protein.

Proteolysis and regeneration of surface receptors. After cultivation in LPDS for 48 h, 8×10^7 PBL were washed four times with Puck's saline G (Grand Island Biological Co.) at 20°C, and 40×10^6 cells (4.0 ml) treated with 100 μg recrystallized trypsin (190 U/mg; Worthington Biochemical Corp., Freehold, N. J.). After 10 min of incubation at 37°C, trypsin-treated and control cells were washed twice with 40 ml of cold DS-BSA and resuspended to a concentration of 2×10^6/ml in complete media containing 10% LPDS. The cells were incubated at 37°C in 5% CO_2 in air. Aliquots of control and trypsin-treated cells were removed at the times indicated and assayed for their capacity to bind ^{125}I-LDL-In and to form E rosettes. Viability was >94%, and 87% of the cells were recovered.

E receptor assay. Human T lymphocytes positive for E receptors were assayed by adherence to neuraminidase-treated SRBC (4). The PBL (1×10^6 in 0.5 ml) were washed twice in Puck's saline G by centrifugation at 1,000 *g* for 2 min. The washed pellet was mixed with 0.1 ml of a 1% suspension of neuraminidase-treated SRBC, immediately centrifuged at 400 *g* for 5 min, and maintained at room temperature for 15 min. Wet mounts were prepared by adding 5 μl of 0.02% trypan blue, gently resuspending the cells and transferring them to a microslide. 400 viable lymphocytes were counted and scored as positive if three or more SRBC adhered to each lymphocyte.

Immunofluorescent assays. The presence of LDL or LDL-In on the surface of viable PBL grown in LPDS after incubation with up to 280 μg/ml LDL-In for 4 and 24 h at 4° or 37°C was assayed: (*a*) by direct immunofluorescent antibody assays using fluorescein isothiocyanate-coupled IgG from high titer antiserum produced by immunizing rabbits with LDL-In, and (*b*) by indirect assays using either rabbit antisera to LDL or LDL-In, or fluorescein isothiocyanate-coupled rabbit antisera to LDL-In followed by fluorescein isothiocyanate-coupled IgG fraction of goat anti-rabbit IgG. Direct assays were performed at 4°C, and reagents prepared as described for surface immunoglobulin (4). Indirect assays employed 30-min reaction periods at 4°C for both the first and the second antibody. The sensitivity of the assays was determined by coupling LDL-In to Affi-Gel 10 beads (BioRad Laboratories) which

were substituted for the cells in preliminary experiments. Beads conjugated with as few as 86 molecules of LDL-In/μm^2 area of bead surface were positive using the indirect assays. This provides an estimated sensitivity of <13,000 molecules per lymphocyte based on an observed mean diameter of 7.1 μm and a relatively smooth surface at 4°C to yield a mean surface area of 160 μm^2/lymphocyte.

Calculations and statistical analyses. The number of cells present in binding assays was calculated from parallel analyses of cellular protein, typically 1.92×10^7 cells/mg cell protein. The molar content of LDL or LDL-In was calculated from an estimated molecular weight of 2.5×10^6 and an estimated composition of 22% protein, 78% lipid (5, 17). Inhibition slopes, calculated by best fit/least square linear regression analysis of the observed data, were performed according to Goldstein (18) with a HP-2000F computer and represent ^{125}I-lipoprotein bound per milligram cell protein vs. $\log_{10}$ of competing lipoprotein protein. Points on the Scatchard plots were analyzed for error variance. Significance of linear regression slopes was determined in both directions from the error variance using double-tailed Student's t tests (18). Mean binding affinity (K) of the ligand-receptor system at half saturation of receptor sites was calculated from $K = (L_b \cdot R_b)/(L_f)(R_f)$, where $(L_b \cdot R_b)$ is the molar concentration of bound ligand-receptor complex, (L_f) is the molar concentration of free ligand, and (R_f) is the molar concentration of free unoccupied receptor.

RESULTS

Biological activity of iodinated LDL-IN. ^{125}I-LDL-In was used as a receptor probe in identifying and characterizing a lymphocyte surface receptor for LDL-In. Because this method may be valid only if the presence of an iodine atom on LDL-In does not interfere with biological activity, we compared I LDL-In prepared with sodium iodide containing trace (1% mol/mol) ^{125}I to noniodinated LDL-In for its capacity to suppress the response of PBM to PHA stimulation (Fig. 1). The specific suppressive activity of I LDL-In containing 0.98±0.05 atoms of iodine per molecule was 6 μg/ml, which was equal to that of noniodinated LDL-In. The specific suppressive activity of total LDL isolated by sequential ultracentrifugation is also shown in Fig. 1, and gave 50% suppression at a 24-fold lower specific activity (150 μg/ml).

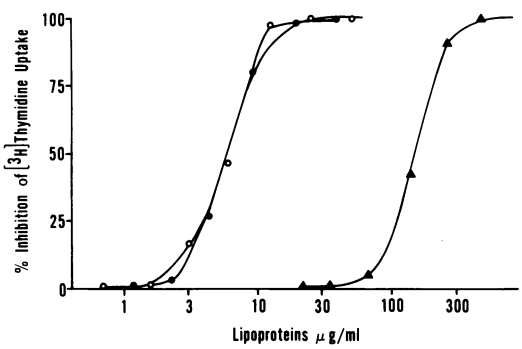

FIGURE 1 Suppression of PHA-stimulated PBM cultures by normal serum lipoproteins. The biological activity of LDL-In (○ —— ○) containing 0.98 mol of I per mole of LDL-In is compared with noniodinated LDL-In (● —— ●) and noniodinated whole LDL isolated by sequential ultracentrifugation (▲ —— ▲). Mean [^{3}H]thymidine uptake of control cultures = 57,303 cpm. Lipoprotein concentrations expressed as micrograms protein per milliliter.

Characterization of the binding reaction. When LDL-In containing 0.98 atoms of ^{125}I per molecule was incubated with human PBL, initial rapid binding occurred within approximately 5 min. This was followed by a second and slower binding phase. For example, when 14.5 μg/ml of ^{125}I-LDL-In was incubated with $4-5 \times 10^6$ PBL/ml, at 4°C, equilibrium conditions were achieved within 40–50 min (Fig. 2). In contrast, at 37°C equilibrium conditions were not observed within an equivalent time period. Instead, binding progressed linearly for more than 24 h (not shown). The probability that the ^{125}I-LDL-In was internalized after binding to the cell, rather than accumulated at the surface, was suggested by direct and indirect immunofluorescent assays. After incubating the cells with 280 μg/ml LDL-In at 37° or 4°C for 4 h, neither LDL nor LDL-In could be detected on the lymphocyte surface, although by reference to standard beads with covalently attached LDL-In, an estimated 86 molecules/μm^2 of surface area were detectable by fluorescence. From these data it appears unlikely that cell-associated LDL-In progressively accumulates on the cell surface.

Binding of ^{125}I-LDL-In by lymphocytes was enhanced threefold when cells had been cultivated for 48 h in the absence of exogenous lipoproteins, as shown by comparing PBL precultured in LPDS to whole FCS (Fig. 2). Surface-bound LDL-In was also not demonstrable by immunofluorescent assays. The approximate number of LDL-In molecules bound per lymphocyte at 4°C was derived from the saturation levels at 60 min. When PBL were cultured for 48 h in medium containing FCS, approximately 876 molecules of LDL-In were bound per cell, whereas after the same time in medium containing LPDS approximately 2,410 molecules of LDL-In were bound per cell. This suggests that the number of detectable LDL-In receptors may be influenced by lipoproteins present as a serum constituent of the medium.

The binding of ^{125}I-LDL-In to lymphocyte is influenced by calcium concentrations. Minimal levels of binding were observed in the presence of <30 μM Ca^{++}, whereas binding of ^{125}I-LDL-In increased 10-fold when Ca^{++} was increased to 100 μM (Table I).

Effect of trypsin. Evidence that the saturable binding of LDL-In by lymphocytes is mediated by a cell surface receptor was provided by demonstration of the decreased binding of ^{125}I-LDL-In by lymphocytes after limited proteolysis of the cells with trypsin. Chisari et al. (19) have demonstrated that incubation of lymphocytes at 37°C for 10 min with 25 μg trypsin/ml completely abrogates E rosette formation. The trypsin-

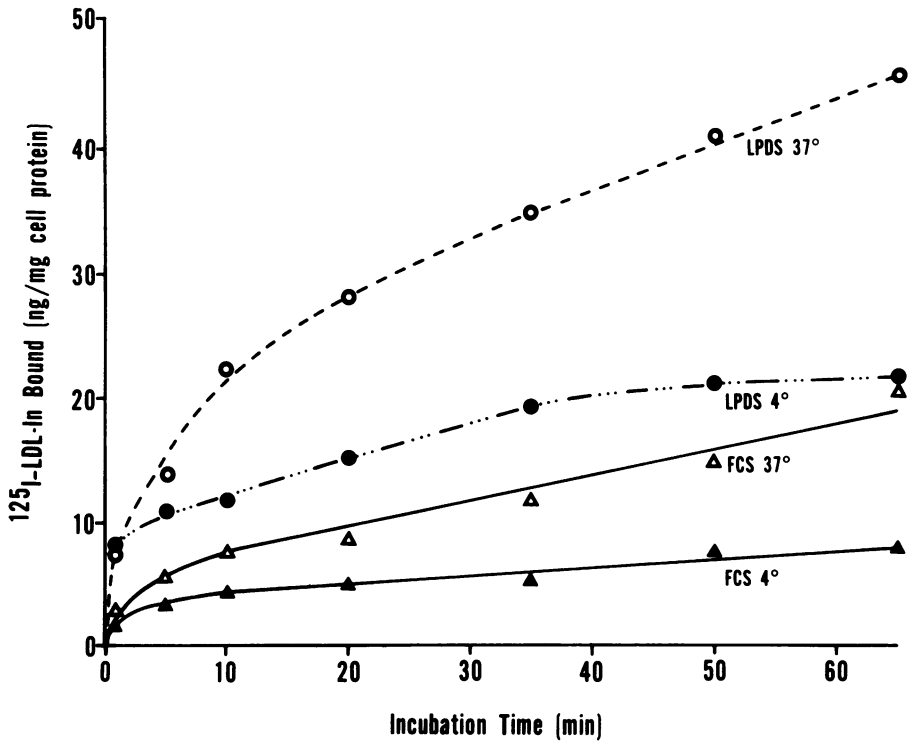

FIGURE 2 Binding of ^{125}I-LDL-In to PBL. The effect of time, temperature, and preculture. All PBL were cultured for 48 h in either LPDS or unfractionated FCS before assay of ^{125}I-LDL-In binding. All points represent the mean of duplicate assay tubes containing approximately 4 × 10^6 cells (194–292 μg total cell protein) in DS-BSA. 14.5 μg/ml of ^{125}I-LDL-In (317 cpm/ng) was added and the tubes incubated at either 4° or 37°C for the times indicated after which the total amount of ^{125}I-LDL-In bound to the cells was determined as described in Methods.

treated cells regenerated the ability to form E rosettes upon subsequent culture. We used the same conditions to demonstrate removal and regeneration of the capacity to bind ^{125}I-LDL-In. After trypsin treatment, E receptor-positive lymphocytes decreased 100% from 53.4±3.5% to 0, and the binding of ^{125}I-LDL-In decreased 76% from 34.05±1.15 to 8.18±0.59 ng bound/mg cell protein. When cultured in complete media containing 10% LPDS, these same lymphocytes regenerated both receptors; however, the rate of regeneration differed significantly (Fig. 3). An estimated half regeneration time of 3.6 h was calculated for LDL-In receptor in contrast to 13.6 h for E receptor.

TABLE I
Influence of Calcium Ions on the Binding of LDL-In by Lymphocytes‡*

$CaCl_2$	^{125}I-LDL-In bound
μM	*ng/ml cell protein*
10	4.3
20	4.7
30	6.3
50	11.9
70	22.0
100	48.0
130	45.2

* The binding assay was carried out at 4°C for 60 min with 10.5 μg/ml of ^{125}I-LDL-In (453 cpm/ng).
‡ Cells cultured in 10% LPDS for 48 h before assay.

Binding affinity. To estimate the number of LDL-In receptors per lymphocyte, the binding of ^{125}I-LDL-In by lymphocyte receptors at 4°C was permitted to reach equilibrium at a series of ^{125}I-LDL-In concentrations between 5.3 and 26.6 μg/ml. The number of receptors per lymphocyte was then calculated from a modified Scatchard plot (Fig. 4). Extrapolation to saturation (bound/free = 0), gave an estimate of 22.12 ng LDL-In protein bound per 5 × 10^6 lymphocytes. Assuming that the receptor is a single molecular complex or molecule capable of interacting with LDL-In in an equimolar fashion, the number of saturable receptor sites per lymphocyte was estimated at 4,860±456. Half saturation of this calculated number of receptors occurred at a free LDL-In concentration of 68.2 nM. From these values the mean binding affinity (K) was estimated to be approximately 1.47 × 10^7 liters/mol.

Binding specificity. The specificity of binding was evaluated in sequential competitive inhibition or blocking experiments we refer to as prior site occupancy studies. An unlabeled ligand was incubated with the lymphocytes at 4°C for 60 min and without washing, then incubated for an additional 60 min after the same or the alternate radiolabeled ligand had been added. The validity of these experiments in determining binding site specificity is based upon the assumption that the first ligand, if bound to the cell, will remain associated and will not be released during incubation with the radiolabeled second ligand. Stability of the association of cell-bound ^{125}I-LDL-In at 4°C, and the rate of its exchange with free LDL-In was therefore determined. As shown in Table II, the amount of ^{125}I-LDL-In bound to the cell during the first incubation was not decreased after a second incubation of up to 90 min in the presence of 23.3 μg/ml of LDL-In.

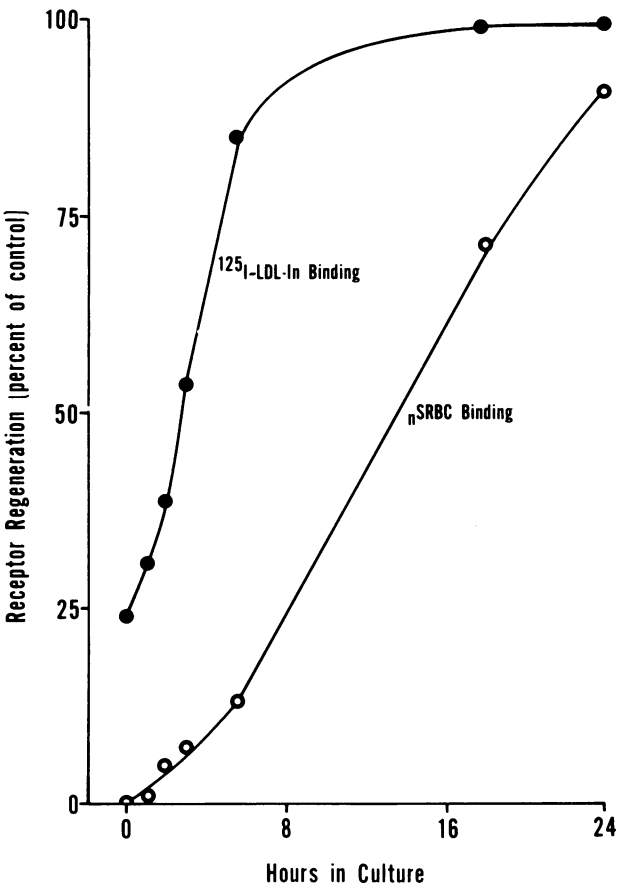

FIGURE 3 Regeneration of trypsin-sensitive PBL surface membrane receptors. The binding of ^{125}I-LDL-In and percent of E-receptor positive PBL were assayed as described after limited trypsin treatment and incubation at 37°C in complete media containing 10% LPDS. Mean untreated E rosette-positive PBL, 53.4±3.5%; mean untreated ^{125}I-LDL-In binding, 34.05±1.15 ng/mg cell protein.

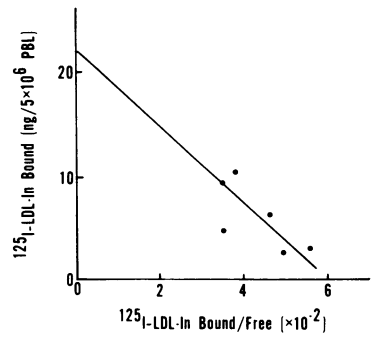

FIGURE 4 Estimation of number of molecules bound by PBL at saturation from Scatchard plot of ^{125}I-LDL-In binding. Cells precultured 48 h in LPDS before assay. Between 5.3 and 26.6 μg/ml of ^{125}I-LDL-In (658 cpm/ng) was added to 5×10^6 PBL, allowed to incubate for 60 min at 4°C, and the cells harvested as described. Extrapolation of the linear regression line to bound/free of zero results in 22.12±2.08 ng ^{125}I-LDL-In bound to 5×10^6 PBL or 4,860±456 receptors per cell.

Because the rate of exchange between bound and free ligand was insignificant, the dissociation constant is extremely high, and the use of prior site occupancy appears to represent a valid approach to determining receptor specificity. When PBL were incubated with LPDS or purified HDL (5.8 mg/ml) for 60 min at 4°C, the subsequent binding of ^{125}I-LDL-In was not diminished (Fig. 5a). Homologous LDL-In inhibited the subsequent binding of ^{125}I-LDL-In, and 50% inhibition was observed at 67 μg/ml. An equal concentration of total LDL was without effect (Fig. 5a). In fact, a 13.4-fold greater concentration of total serum LDL (900 μg/ml) was required to occupy 50% of the ^{125}I-LDL-In binding sites. The inhibition slopes for LDL (−17.4) and LDL-In (−19.7) (Fig. 5a) were not statistically different ($P = 0.4$). This is consistent with the concept of prior site occupancy by molecules of similar affinity for the same binding site, but by molecules that are present at different concentrations.

TABLE II

*Stability of Association of Lymphocyte-Bound ^{125}I-LDL-In and its Rate of Exchange with Free LDL-In at 4°C**

Time of reaction sequence, *min*		
^{125}I-LDL-In‡, 100 μg/ml	LDL-In, 23.3 μg/ml	ng ^{125}I-LDL-In bound/mg cell protein
60	0	38.3±3.2
60	30	46.1±4.1
60	60	44.2±6.5
60	90	37.7±5.8

* Cells cultured in 10% LPDS for 48 h before assay.
‡ 715 cpm/ng.

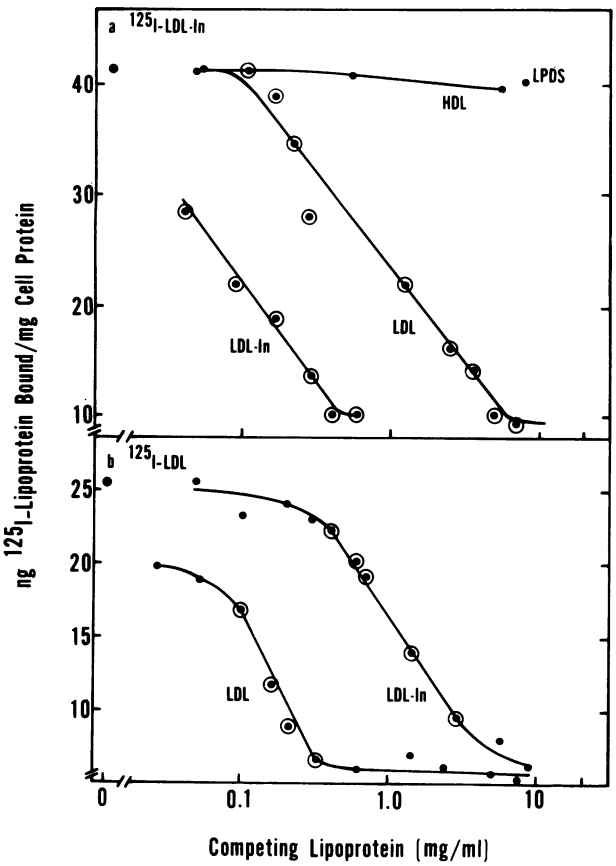

FIGURE 5 The ability of lipoproteins to inhibit the subsequent binding of ^{125}I-labeled lipoproteins. All PBL were cultured for 48 h in LPDS before assay. Points represent the mean of duplicate assays containing approximately 5×10^6 PBL (187–302 μg total cell protein) in DS-BSA. Tubes were incubated for 60 min at 4°C with competing lipoprotein before the addition of labeled ligand which in Fig. 5a is ^{125}I-LDL-In at 23.3 μg/ml (658 cpm/ng) and in Fig. 5b is ^{125}I-LDL at 23.3 μg/ml (757 cpm/ng). After an additional 60 min at 4°C the total amount of ^{125}I bound was determined as described in Methods. Circled dots indicate points used for calculation of linear regression slopes.

A receptor for LDL has been identified on human lymphocyte lines (2) as well as PBL (20). To determine whether the receptor for LDL and LDL-In is identical, whole LDL isolated by classic sequential ultracentrifugation was radiolabeled under conditions identical to those used for LDL-In. The capacity of unlabeled LDL and LDL-In to occupy receptor sites and inhibit the subsequent binding of ^{125}I-LDL was then analyzed. Representative results, illustrated in Fig. 5b, indicate that LDL-In does not preferentially occupy the LDL receptor. In the homologous reaction, LDL inhibited the subsequent binding of ^{125}I-LDL by 50% at 120 μg/ml, whereas an equal concentration of LDL-In was without effect (Fig. 5b). A 10-fold greater concentration of LDL-In was required to occupy 50% of the ^{125}I-LDL binding sites. In contrast to the inhibition slopes shown in Fig. 5a, the inhibition slope for LDL-In (−13.6) is significantly less than that observed for LDL (−21.3), and the two slopes are nonidentical ($P < 0.005$) by analysis of variance.

Inasmuch as the prior site occupancy experiments suggest that the LDL-In and the LDL receptors are not identical, additional evidence to support this conclusion was sought. The inclusion of 25-hydroxycholesterol (2 μg/ml) and cholesterol (16 μg/ml) in the lipoprotein-deficient preincubation medium has been demonstrated (20) to prevent the appearance of enhanced receptor-mediated degradation of ^{125}I-LDL by PBL presumably by suppression of synthesis of the LDL receptor. By using this experimental maneuver, we compared the amount of ^{125}I-LDL and ^{125}I-LDL-In bound in 5 h at 37°C by PBL cultured for 48–72 h before assay in complete medium containing 10% LPDS and these sterols. Three representative experiments are summarized in Table III. As predicted, incubation of the PBL for 48 h in LPDS in the presence of 25-hydroxycholesterol (2–10 μg/ml) and cholesterol (16 μg/ml) resulted in marked attenuation of the binding of ^{125}I-LDL. However, these same conditions had no discernible effect on the quantity of ^{125}I-LDL-In bound by these lymphocytes.

TABLE III
*Effect of Sterols on the Enhancement of Binding Observed with Prior Culture of the PBL in LPDS**

Experiment number	25-hydroxycholesterol‡	Prior culture period	Input	Total ng bound/mg cell protein	
				LDL	LDL-In
	μg/ml	h	μg/ml		
A	0	48	10§	146±30	ND‖
	2	48	10	85±1	ND
	5	48	10	72±1	ND
	10	48	10	57±4	ND
B	0	48	10§	152±5	219±16
	10	48	10	45±11	188±31
C	0	72	4.7¶	ND	40±8
	2	72	4.7	ND	33±3
	10	72	4.7	ND	42±4
	20	72	4.7	ND	60±8

* The binding assay was carried out at 37°C for 5 h.
‡ Control cultures contained 10% LPDS, plus 0.5% vol/vol ethanol; all cultures that contained 25-hydroxycholesterol also contained 16 μg/ml cholesterol.
§ Sp act 200 cpm/ng.
¶ Sp act 568 cpm/ng.
‖ ND, not done.

DISCUSSION

Demonstration of the existence of a limited number of saturable lymphocyte receptors with a moderately high binding affinity for LDL-In, an immunoregulatory species of serum LDL (5–8), lends credence to a potential physiologic role for this lipoprotein and suggests that a cell surface receptor might be implicated in the cellular pathways responsible for the phenotypic expression of its effect on lymphocytes. The evidence presented in this study supports the existence of a receptor for LDL-In on the surface of lymphocytes. As suggested by Cuatrecasas and Hollenberg (21), a number of criteria must be fulfilled to convincingly support the existence of a cellular receptor. In this study, we have provided four lines of evidence that imply a receptor-mediated association between LDL-In and lymphocytes. First, binding is temperature dependent, calcium ion dependent, and occurs with reasonably high affinity ($K = 1.47 \times 10^7$ liters/mol) at reasonably low molar concentrations (e.g. 50% binding occurs at 0.2 nM), all of which favor specific rather than nonspecific interactions. Second, the binding can be inhibited by proteolysis of the lymphocyte surface with trypsin and then regenerated to control levels, which implies that binding involves a trypsin-sensitive surface protein. Third, the kinetics of binding are applicable over the range in which LDL-In is capable of eliciting a biological response both in vivo and in vitro. Fourth, unlike HDL or other serum protein, LDL-In specifically competes with ^{125}I-LDL-In for binding to the lymphocyte.

In an attempt to define the optimal conditions for binding of LDL-In, it was found that cells grown in LPDS bind more LDL-In than cells grown in whole serum. There may be two reasons. First, whole serum contains LDL-In in equilibrium with cell surface-bound LDL-In that may partially saturate available LDL-In receptors. The ^{125}I-LDL-In ligand would be required to compete for receptors, thus diminishing the binding of ^{125}I-LDL-In. In contrast, cells grown in LPDS for 48–72 h should have little if any residual cell-bound LDL-In occupying available cellular receptors. Second, information regarding the binding of LDL by fibroblasts indicates that bound and internalized LDL not only reduces the biosynthesis of cholesterol, but also regulates the surface density of the LDL receptor (22). Analogously, LDL-In present in serum may regulate the cell surface density of its own receptor. The contribution of each of these mechanisms remains to be determined.

The binding of LDL-In is facilitated by Ca^{++}, which implies a degree of specificity. Adding up to 100 μM Ca^{++} significantly augments the binding of LDL-In, whereas further amounts do not (Table I). These findings are particularly relevant in light of the recent observations by Dana et al. (23) that the specific binding of ^{125}I-LDL to nonsiliconized glass is Ca^{++} independent. The calcium requirement is also dependent upon the concentration of added ^{125}I-LDL-In, and is typified by the observation that at higher LDL-In concentrations the calcium dependence is less evident (data not shown).

Lymphocytes readily bind LDL-In at concentrations of 2–10 μg/ml even though albumin is present in excess (400-fold by mass or 136,000-fold by molar concentration) in the reaction medium, and the cells are subsequently sedimented through FCS. This suggests that binding is specific and not simply a nonspecific adsorption phenomenon.

Finally, binding of LDL-In is saturable under conditions that arrest the metabolic activities of the cell, i.e., low temperature. The observation that saturable binding could not be demonstrated at 37°C and that there is a progressive linear association of LDL-In with the lymphocytes suggests internalization similar to that described for LDL (2, 24). This interpretation is further supported by our failure to detect LDL-In accumulation at the surface of viable cells. Although at least 10,000 molecules can be anticipated to bind per cell within the 1st h (estimated from data in Fig. 2), neither LDL nor LDL-In could be visualized by immunofluorescence. 24 h later, when more than 100,000 molecules ought to be detectable per cell, LDL-In could still not be visualized at the surface of viable cells. Cell-associated LDL-In remained at all times below the limits of visualization which we estimate at $<13{,}000$ molecules/cell. Metabolic-dependent internalization of receptor bound ^{125}I-LDL-In such as described for LDL (2, 24) could reasonably account for these observations, and is the subject of a current study of the cellular catabolism of LDL-In.

More than 75% of the binding of LDL-In to lymphocytes at 4°C was abrogated by limited stripping of the cell surface proteins with trypsin. This does not appear to be artifactual because in culture the same cells regenerate LDL-In receptors and E receptors de novo. These data suggest that lymphocytes are capable of synthesizing and inserting a specific LDL-In binding protein in their cell membrane.

Demonstration that certain structurally related molecules can compete with the binding of LDL-In whereas others cannot, further serves to identify a specific receptor binding process. HDL at 5.8 mg/ml (a 500-M excess), which is not immunoregulatory at physiologically significant concentrations (5, 6), does not block the binding of ^{125}I-LDL-In to lymphocytes, but 500 μg/ml of whole LDL does (Fig. 5a). Although less efficient than LDL-In, the limited capacity of whole LDL to compete for the binding of ^{125}I-LDL-In can be explained in two ways. First, LDL-In is a minor subset of LDL and is present in preparations of LDL.

Second, all LDL molecules or a subset may be bound less efficiently by the LDL-In binding site. Because LDL contains LDL-In (5), a comparison of the binding curves (Fig. 5a) suggests that the concentration required for inhibition of binding of the ^{125}I-LDL-In ligand would occur if 7.3% of the LDL preparation were LDL-In. This estimated percentage does not differ greatly from the 4% LDL-In content of LDL required to account for the immunosuppressive properties of this preparation of LDL (Fig. 1). Further evidence that the LDL-In present in total LDL may be responsible for the observed binding of LDL to the ^{125}I-LDL-In receptor is the statistically significant identity ($P < 0.005$) of the inhibition slopes of LDL and LDL-In (Fig. 5a).

If LDL-In is a discrete subset of LDL, then is the receptor responsible for the binding of LDL-In identical to the LDL receptor or are there two distinct and independent receptors on PBL? If only one receptor exists, then according to the results of the prior site occupancy experiments (Fig. 5a), LDL-In represents a selected subset of LDL molecules with an affinity greater than whole LDL for the LDL receptor. In this case, LDL-In should also occupy this single receptor more efficiently when the radiolabeled ligand is ^{125}I-LDL. If there are two independent receptors, one for LDL and another for LDL-In, then LDL-In would either not compete for the LDL receptor or, at the very least, compete less efficiently for the LDL receptor. A distinction between a one- vs. a two-receptor hypothesis is possible from analysis of the binding of LDL and LDL-In to the ^{125}I-LDL receptor (Fig. 5b). LDL-In does not inhibit the subsequent binding of ^{125}I-LDL to the LDL receptor as efficiently as LDL. These results support a two-receptor hypothesis. However, LDL-In is capable of saturating LDL receptor sites (Fig. 5b), and complete saturation is achieved at very high concentrations (10 mg/ml). If the LDL-In preparation used in these experiments contained only 4% LDL, and this LDL contaminant were responsible for occupancy of the LDL receptor, the slopes would be identical. The lack of identity suggests that LDL-In might also be bound at lower affinity by the LDL receptor.

The net binding and the blocking by each of the lipoproteins are tabulated for comparison in Table IV. This supports the hypothesis that just as the two ligands, LDL and LDL-In, do not appear to be identical, their respective binding sites on the lymphocyte surface are also not strictly identical. The hypothesis that one receptor binds both ligands is inconsistent with the observation that at a concentration of 400 μg/ml, LDL-In completely blocks the binding of ^{125}I-LDL-In; but at the same concentration permits 84% occupancy of the ^{125}I-LDL binding sites (Table IV). Similarly, LDL at 400 μg/ml has completely blocked the binding of ^{125}I-LDL, but still permits 64% occupancy of the ^{125}I-LDL-In binding sites (Table IV). We conclude from this that at least two discernible lipoprotein receptors exist on the lymphocyte, each of which is capable of binding a different subset of normal human serum LDL.

This initial conclusion is supported by two additional observations which serve to further distinguish between LDL and LDL-In receptors. First, the enhanced binding of LDL-In that results from preincubation of

TABLE IV

*Evidence for Differential Specificity of Binding of Lipoproteins by Lymphocytes**

Blocking lipoprotein		Lipoprotein binding capacity			
		LDL-In		LDL	
		net ng ^{125}I-LDL-In bound		net ^{125}I-LDL bound	
Type	Concentration	mg cell protein	Percent maximum	mg cell protein	Percent maximum
	μg/ml				
Buffer	—	31.5	100	19.3	100
LDL-In	50	17.7	56	19.3	100
	400	0.4	1	16.3	84
LDL	100	31.5	100	11.1	58
	400	20.2	64	0.3	2
HDL	5,800	29.5	94	—	—
LPDS	7,800	31.0	98	—	—

* Net nanograms lipoprotein bound was corrected for noninhibitable counts. ^{125}I-lipoprotein present at 23.3 μg/ml in assays. Data obtained from Fig. 5.

the cells in LPDS is not abrogated by the addition of 25-hydroxycholesterol and cholesterol to the preincubation medium as is the LDL binding (Table III). Second, LDL-In is capable of suppressing the in vitro PHA response of PBLs isolated from a homozygous familial type II hypercholesterolemia patient with demonstrated deletion of surface LDL receptor.[2] Taken together, these observations suggest that the receptors and, furthermore, the mode of action of LDL and LDL-In are probably distinct. Further proof of this hypothesis must await purification of LDL-In that is completely devoid of LDL other than LDL-In and vice versa, a task not currently possible.

Although this study was not designed to permit simultaneous measurements of binding and biological activity, estimates of receptor number and binding affinity derived from the data illustrated in Fig. 4 suggest that the binding of ^{125}I-LDL-In could be associated with the observed suppression of lymphocyte proliferation by LDL-In. First, the calculated affinity of LDL-In for its receptor is sufficiently high to permit binding under physiological conditions. If it is assumed from current data that at least 1% of LDL normally present in serum at 0.7–0.9 mg LDL protein/ml (25) is LDL-In, then based on the calculated association constant, 16–20% of the LDL-In receptors should be occupied in vivo at any given time. If the percentage of LDL-In is greater, e.g., 5%, then LDL-In should occupy 50–55% of available receptor sites in vivo. The actual degree of saturation may be even greater because of the very high dissociation constant. Second, the calculated affinity of LDL-In binding is sufficient to permit occupancy of the LDL-In receptor on the lymphocyte during suppression of PHA or allogeneic stimulation in vitro. At LDL-In concentrations sufficient to produce 90% suppression of the PHA response in complete medium containing 10% FCS, approximately 40% of LDL-In receptors should be occupied; whereas at LDL-In concentrations that result in <2% suppression of the PHA response, fewer than 10% of the receptors should contain LDL-In.

It is of interest that the biological activity of RIF at 20 ng/ml, the immunoregulatory species of serum LDL induced by hepatitis virus infections (3, 4), is the same whether it is assayed in the presence of serum or not. Although normal serum contains a 10^{5}–10^{6} M excess of LDL, and a 10^{3}–10^{5} M excess of LDL-In, it does not diminish the binding of RIF. This leads us to suggest that neither LDL nor LDL-In interact with the previously described RIF receptor. We therefore propose that at least three independent surface membrane receptors for LDLs may exist on lymphocytes: (*a*) RIF receptors, (*b*) LDL receptors, and (*c*) LDL-In receptors.

[2] Cells kindly provided by Dr. J. Goldstein, University of Texas, Dallas.

ACKNOWLEDGMENTS

We wish to thank Dr. P. Alaupovic for the gift of antisera to apolipoprotein chains, and Dr. F. V. Chisari for his helpful suggestions. The excellent technical assistance of Diana Peterson and the assistance of Mary Gortmaker in preparation of the manuscript are readily acknowledged.

This investigation was supported by U. S. Public Health Service research grants CA-14346 and CA-16600.

REFERENCES

1. Brown, M. S., and J. L. Goldstein. 1976. Receptor mediated control of cholesterol metabolism. *Science (Wash. D. C.).* **191:** 149–154.
2. Ho, Y. K., M. S. Brown, H. J. Kayden, and J. L. Goldstein. 1976. Binding, internalization, and hydrolysis of low density lipoprotein in long term lymphoid cell lines from a normal subject and a patient with homozygous familial hypercholesterolemia. *J. Exp. Med.* **144:** 444–455.
3. Chisari, F. V., and T. S. Edgington. 1975. Lymphocyte E rosette inhibitory factor: a regulatory serum lipoprotein. *J. Exp. Med.* **142:** 1092–1107.
4. Chisari, F. V., J. A. Routenberg, and T. S. Edgington. 1976. Mechanisms responsible for defective human T lymphocyte sheep erythrocyte rosette function associated with hepatitis B virus infections. *J. Clin. Invest.* **57:** 1227–1238.
5. Curtiss, L. K., and T. S. Edgington. 1976. Regulatory serum lipoproteins: regulation of lymphocyte stimulation by a species of low density lipoproteins. *J. Immunol.* **116:** 1452–1458.
6. Curtiss, L. K., D. H. DeHeer, and T. S. Edgington. 1977. *In vivo* suppression of the primary immune response by a species of low density lipoprotein. *J. Immunol.* **118:** 648–652.
7. Edgington, T. S., C. S. Henney, and L. K. Curtiss. 1977. The bioregulatory properties of serum low density lipoprotein inhibitor (LDL-In) on the generation of killer T cells. *In* Regulatory Mechanisms in Lymphocyte Activation. D. O. Lucas, editor. Academic Press, Inc., New York. 736.
8. Curtiss, L. K., and T. S. Edgington. 1977. Effect of LDL-In, a normal immunoregulatory human serum low density lipoprotein, on the interaction of macrophages with lymphocytes proliferating in response to mitogen and allogeneic stimulation. *J. Immunol.* **118:** 1966–1970.
9. Boyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest. Suppl.* **21:** 77–89.
10. Kaplow, L. S. 1965. Simplified myeloperoxidase stain using benzidine dihydrochloride. *Blood.* **26:** 215–219.
11. Havel, R. J., H. S. Eder, and J. H. Bvagdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
12. 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.
13. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. 1958. Cholesterol in serum. *Stand. Methods Clin. Chem.* **2:** 26–33.

14. Chisari, F. V., J. L. Gerin, and T. S. Edgington. 1974. Immunochemistry of the hepatitis B virus: ^{125}I HB Ag ligand. *J. Immunol.* **113:** 543–553.
15. Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
16. Bierman, E. L., O. Stein, and Y. Stein. 1974. Lipoprotein uptake and metabolism by rat aortic smooth muscle cells in tissue culture. *Circ. Res.* **35:** 136–150.
17. Scanu, A. M., C. Edelstein, and P. Keim. 1977. Serum lipoproteins. *In* The Plasma Proteins. Vol. I. Academic Press, Inc. New York. 318–391.
18. Goldstein, A. 1971. Quantitative data. *In* Biostatistics: An Introductory Text. The Macmillan Company, New York. 34–92.
19. Chisari, F. V., W. J. Gealy, and T. S. Edgington. 1977. Recovery of soluble sheep erythrocyte receptor from the T lymphocyte surface by proteolytic cleavage. *J. Immunol.* **118:** 1138–1142.
20. Ho, Y. K., M. S. Brown, D. W. Bilheimer, and J. L. Goldstein. 1976. Regulation of low density lipoprotein receptor activity in freshly isolated human lymphocytes. *J. Clin. Invest.* **58:** 1465–1474.
21. Cuatrecasas, P., and M. M. Hollenberg. 1976. Membrane receptors in hormone action. *Adv. Protein Chem.* **30:** 251–451.
22. 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.
23. Dana, S. E., M. S. Brown, and J. L. Goldstein. 1977. Specific, saturable and high affinity binding of ^{125}I-low density lipoprotein to glass beads. *Biochem. Biophys. Res. Commun.* **74:** 1369–1376.
24. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts. *J. Biol. Chem.* **249:** 5153–5162.
25. Fredrickson, D. S., and R. I. Levy. 1972. Familial hyperlipoproteinemia. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, editor. McGraw-Hill Book Company. 3rd edition. 545–614.