Improved detection of familial hypercholesterolemia by determining low density lipoprotein receptor expression in mitogen-induced proliferating lymphocytes

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Abstract **In view of the presence of some 190 mutations in the low density lipoprotein receptor (LDL-R) gene and a lack of simple detection methods, we have developed an improved assay system for detecting familial hypercholesterolemia (FH) using mitogen-induced proliferating lymphocytes. Freshly isolated mononuclear cells were cultured for 3 days in RPMI 1640 supplemented with 10% human lipoprotein-deficient serum (LPDS) and 1% phytohemagglutinin (PHA). LDL-R expression was measured by flow cytometry using a monoclonal anti-LDL-R antibody or DiI-LDL. Mitogenic responses were monitored by cell size (FSC), interleukin-2 receptor (IL2-R) expression, and stimulation index (SI). The LDL-R expression in PHA-stimulated lymphocytes was significantly higher than lymphocytes or monocytes cultured without PHA (15.2- and 3.6-fold, respectively). The gradation of the LDL-R expression was highly correlated to FSC, IL2-R expression, and SI ($r > 0.9$ in each case). However, no difference in FSC, IL2-R expression, or SI existed between 30 clinically diagnosed FH and 42 normolipemic control subjects. The significantly lower LDL-R expression in the FH group (45.2 ± 15.3% versus 100 ± 14.1%; unpaired *t* test, $P < 0.0001$) indicated the presence of genetic defects. Normocholesterolemic first degree relatives and non-FH hypercholesterolemic subjects demonstrated normal LDL-R expression as did the controls. The assay carries an efficiency of 97% and both sensitivity and specificity of 98.5%.—Measurement of low density lipoprotein receptor expression in phytohemagglutinin- and lipoprotein-deficient serum-stimulated lymphocytes offers a simple method for detecting familial hypercholesterolemia with improved sensitivity.—**Chan, P-c., A. Edwards, R. Lafrenière, and H. G. Parsons. **Improved detection of familial hypercholesterolemia by determining low density lipoprotein receptor expression in mitogen-induced proliferating lymphocytes. *J. Lipid Res.* 1998.** 39: **2261–2270.**

Supplementary key words familial hypercholesterolemia • low density lipoprotein receptor • proliferating lymphocytes • mitogen stimulation • flow cytometry

Familial hypercholesterolemia (FH) is a monogenic lipid disorder caused by mutations in the low density lipoprotein receptor (LDL-R) gene (1). Affected individuals develop elevated LDL and total cholesterol levels, tendinous xanthomas and premature coronary heart disease (CHD) (2, 3). Fifty to 80% of male FH individuals develop symptoms or signs of CHD by age 50 and females manifest about 10 years later (4, 5). The mortality due to CHD is increased in FH patients (6–8), up to 30-fold higher than age- and gender-matched control subjects (6). When compared to unaffected individuals with the same elevated plasma cholesterol level, the mortality due to CHD in FH is not similar as one may expect but is eight times higher (4). Hence, the presence of an LDL-R abnormality represents an independent risk factor for CHD. Early and accurate diagnosis of the condition is, therefore, important to ensure timely treatment and appropriate counselling.

Given the importance of diagnosing the condition, a number of methods have been proposed including a variety of biochemical and genetic/molecular techniques. DNA-based methods are more specific and often definitive but have limited value in detecting LDL-R anomalies in the general populations (9–11) due to the presence of a large number of mutant alleles (more than 190) (12, 13). One alternative is to directly demonstrate a deficiency in the LDL-R function. Receptor assays that have been described include measurement of ^{125}I-, fluorescent-, or colloidal gold-labeled LDL binding and/or uptake in skin fibroblasts (14, 15), lymphocytes (16–18), or monocytes (19, 20, 21). Measurement of LDL-mediated suppression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-Co-A) reductase activity, LDL-mediated stimulation of acyl-

Abbreviations: FH, familial hypercholesterolemia; LDL, low density lipoprotein; LDL-R, low density lipoprotein receptor; PHA, phytohemagglutinin; LPDS, lipoprotein-deficient serum; IL2-R/CD25, interleukin 2 receptor; SI, stimulation index; CHD, coronary heart disease; PI, propidium iodide; DiI, 3,3′-dioctadecylindocarbocyanin iodide; FL1/FL2, green/red fluorescence; CDA, cumulative distribution analysis; FITC, fluorescein isothiocyanate; SE, standard error; *r*, correlation coefficient.

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CoA:cholesteryl acyltransferase (ACAT) activity or rate of proteolytic degradation of ^{125}I-labeled LDL in skin fibroblasts (15) as well as indirect evaluation of the LDL-R function using tritiated-thymidine incorporation (22–24) have also been reported. The latter method is based on the fact that cholesterol is required for cell division and inhibition of HMG-CoA reductase arrests proliferation in PHA-stimulated lymphocytes cultured in lipoprotein depleted medium (LPDS). The LDL-R status is reflected by the minimum amount of LDL required to reverse the inhibition of proliferation.

Evaluation of LDL-R expression on peripheral blood mononuclear cells is of interest. It offers easy accessibility and processing of cell cultures. Recently, it has been reported that 22–32% of clinically diagnosed FH patients do not have deficiencies in LDL-R function based on a lymphocyte binding assay (25, 26). Whether this finding represents a true deficiency in classifying FH patients based on clinical criteria or a reflection of the insensitivity of the binding assay itself is not clear. One of the major factors limiting the sensitivity of most binding assays for LDL-R activity is the extent of receptor up-regulation within the assay system as the LDL-R gene is normally repressed in vivo due to circulating LDL. Most LDL-R assays utilize the sterol-mediated receptor up-regulation such as depleting cells of cholesterol and/or including HDL_3 to promote reverse cholesterol transport (14, 27). However, the LDL-R can also be greatly up-regulated through growth-related mechanisms (28, 29). As the normal allele does not compensate for the defective allele in heterozygous FHs (30), we propose that up-regulating LDL-R activity through both sterol- and growth-mediated mechanisms will maximize the LDL-R expression and thereby improve its discrimination between FHs and unaffected individuals. In the current study, we *1*) compared the LDL-R expression in lymphocytes cultured in LPDS and in LPDS plus PHA; *2*) examined its relationship with the mitogenic response; and *3*) compared both the LDL-R expression and the mitogenic response in PHA-stimulated lymphocytes between a cohort of 30 clinically diagnosed FH and 42 normolipemic control subjects. Our results indicate superior sensitivity in the detection of FH using proliferating lymphocytes whose LDL-R expression has been synergistically up-regulated by LPDS and mitogen.

MATERIALS AND METHODS

Materials

EDTA Vacutainer tubes, tissue culture flasks (Falcon), fluorescein (FITC)-conjugated antibodies to cell surface markers (CD14 and IL2-R) were from Becton-Dickinson, Mountain View, CA. Monoclonal anti-LDL-R antibody (C7) was from Amersham and phycoerythrin-conjugated goat anti-mouse IgG $F(ab')_2$ (PE-GAM) was from Bio/Can, Mississauga, Canada. Phytohaemagglutinin M form (PHA-M), RPMI 1640, and penicillin/streptomycin were purchased from Gibco, Burlington, Canada. PHA of the same lot was aliquoted in RPMI and frozen at −20°C until use. Propidium iodide (PI) and enzymatic cholesterol analysis kit were from Sigma, St. Louis, MO. 3,3′-Dioctadecylindocarbocyanin iodide (DiI) was obtained from Molecular Probe Inc., Eugene, OR and was dissolved in dimethylsulfoxide (DMSO) for labeling of LDL. Lovastatin was obtained from Merck Frosst Canada Inc., Quebec, Canada, and was converted to active form by dissolving in 50% ethanol and 0.5 mol/L NaOH. Human lipoprotein-deficient serum (LPDS) was prepared from pooled human plasma from more than 40 healthy donors by flotation ultracentrifugation ($d > 1.215$ g/mL) as described (31). The plasma was kindly provided by the Canadian Red Cross, Calgary Centre. The absence of cholesterol in the LPDS was confirmed enzymatically using a commercial cholesterol analysis kit (Sigma).

Study design

Index cases of familial hypercholesterolemia were obtained from local lipid clinics. The selection of these cases was made based on the presence of primary hypercholesterolemia with plasma LDL greater than the 99th percentile for the same gender and age population, presence of tendinous xanthoma, a family history of coronary heart disease, and the absence of the apoB-3500 mutation. Twenty to 40 mL of EDTA blood was then collected from these individuals, their first degree family members (affected or not), unrelated non-FH hypercholesterolemic and normolipemic subjects in a local lipid clinic. Aliquots of the blood sample were then coded and sent for the LDL-R assay to be performed in a research laboratory. Previous medical histories including lipid levels were subsequently collected from respective family or primary care physicians. Lipid profiles were performed at an independent clinical laboratory on those whose lipid levels had never been measured or unavailable. No clinical history nor laboratory data was made available to the analyst at the time of LDL-R assay.

Subjects

A total of 31 subjects (17 male and 14 female) including 15 index cases and one genetically characterized homozygote was studied and a positive diagnosis of FH was made based on a history of persistent primary hypercholesterolemia (LDL cholesterol level greater than 95th percentile for age and sex in the absence of secondary causes for hypercholesterolemia) and either the presence of tendon xanthoma in patient or a family history of hypercholesterolemia, tendon xanthoma, or CHD. The age of these subjects ranges from 8 to 65 years (40 ± 15.6, mean ± SD). Seven normocholesterolemic first degree relative and six non-FH hypercholesterolemic (plasma cholesterol greater than 6.2 mmol/L) subjects were also included in the study. Approximately 70% of all hypercholesterolemic subjects were on lipid-lowering drugs for 2 weeks or longer. Control subjects included 20 male and 22 female healthy normocholesterolemic individuals with age ranging from 8 to 65 years (31 ± 13.4, mean ± SD). None was on medication of any kind. The study protocol was approved by the Ethics Committee of the University of Calgary and informed consent was obtained from all participating subjects.

Mononuclear cell isolation and culture

Mononuclear cells were isolated using a modified procedure of Boyum (32). Briefly, 20–40 mL of EDTA blood collected from each subject was diluted 1 in 2 with a balanced salt solution or phosphate-buffered saline (PBS), pH 7.4, and then layered over ficoll-paque (Pharmacia) in a height ratio of 1 to 0.8 (blood:ficoll-paque). After centrifugation at 400 *g* for 30 min at 18–20°C, mononuclear cells were recovered at the interface. The cells were then washed three times in PBS and cultured at 37°C in 5% CO_2 at a concentration of 1×10^6 cells/mL (0.4×10^6/cm^2) in RPMI 1640 supplemented with 10% LPDS, 100 U/mL penicillin, and 100 μg/mL streptomycin with and without 1% PHA. In some experiments, 20 or 103 μg/ml of LDL was added

at zero hour to the media to abrogate the effects of LPDS up-regulation of the LDL-R expression. Rather than using whole serum with variable LDL concentrations, LDL was added to the LPDS to control for serum growth factors and LDL apolipoprotein composition. Previous authors had identified modulation of LDL binding to the LDL-R by LDL apolipoproteins C-I, C-II, C-III, and E in addition to apoB (33).

Two approaches were taken to investigate the relationship between the cell mitogen response and LDL-R expression. First, changes to mitogen response (measured as FSC, surface IL-2R, and SI, further defined below) and LDL-R surface expression were measured at various times over 4 days of cell culture in 10% LPDS and 1% PHA. Second, to further clarify the role of cell proliferation and cell cycle on LDL-R expression, in some cultures on day 0, in addition to LPDS and PHA, we added lovastatin (0.1–5.0 μmol/L) and the mitogen response and LDL-R expression were measured at 72 h of culture. Lovastatin, an inhibitor of mevalonate biosynthesis (34), inhibits growth by arresting, in a dose-dependent manner, cells in the G_1 phase of the cell cycle and this effect is not LDL dependent, but can be prevented by the addition of mevalonate to the media (35–38).

Preparation of DiI-labeled LDL (DiI-LDL)

Human LDL ($1.019 < d < 1.063$ g/mL) was isolated by density gradient ultracentrifugation as described (39). LDL protein concentration was determined by a modified Lowry method (40). To label LDL with DiI, LDL, 0.5 mg protein/mL in 2 mL of LPDS, was incubated at 37°C overnight with 50 μL of DiI (3 mg/mL) in DMSO as described (41). Labeled LDL was recovered by density gradient ultracentrifugation and dialyzed extensively against PBS containing 5 mmol/L EDTA. DiI-LDL was then passed through a 0.22-μm filter and stored at 4°C in the dark until use, normally within 2 weeks of preparation.

Dual color flow cytometry for surface expression of LDL-R and IL2-R or CD14

Harvested cells were washed once with cold PBS, pH 7.4, supplemented with 0.5% of bovine serum albumin (BSA). The cell pellet was then resuspended in PBS/0.5%BSA/0.5 mmol/L $CaCl_2$ (PBC) and incubated on ice for 30 min with a final concentration of 2 μg/mL of the anti-LDL-R antibody (42). After incubation, the cells were washed twice with PBC and then incubated on ice with PE-GAM in the dark for another 30 min. The labeled cells were washed twice in PBC. Unoccupied binding sites on PE-GAM were then blocked by incubation with diluted mouse whole serum (1:20) for 20 min before the addition of fluorescein isothiocyanate (FITC)-conjugated anti-IL2-R (CD25) or -CD14 antibody, the latter being a surface marker for monocytes. After another 30 minutes of incubation, the double-labeled cells were washed twice with PBC, fixed in 1% buffered formaldehyde in PBS (pH 7.4), and stored at 4°C in the dark until flow cytometric analysis.

Labeled samples were analyzed on an FACScan flow cytometer (Becton-Dickinson, Mountainview, CA) equipped with an argon laser emitting at 488 nm. The laser was used to measure light-scattering properties and to excite fluorochromes on the cells. Forward-scatter, FSC (a reflection of cell size) and side-scatter, SSC (cell granularity) readings were captured from each single cell and were used to exclude cell debris or aggregates as well as to delineate lymphocyte and monocyte populations (see below). From these selected or gated cell populations, the green fluorescence (FL1) from FITC-stained cells was measured using a 530 nm bandpass filter and the orange-red fluorescence (FL2) from PE and DiI was measured using a 585 nm bandpass filter. Fluorescence signals from 10,000 cells were routinely collected from each sample and analyzed using the LYSYS II program (Becton-Dickinson) to give a mean fluorescence intensity (per cell) in arbitrary units. The instrument was calibrated daily using fluorescent-labeled beads (CaliBRITE, Becton-Dickinson). Cell viability was assessed by propidium iodide (PI) exclusion in unfixed samples and was always maintained at greater than 95%. Background fluorescence due to cell autofluorescence and nonspecific binding of isotype-matched control antibodies was subtracted to give a net mean fluorescence (MF) which is a reflection of the average quantity of receptor/protein present on a single cell in the population. To allow direct comparison of the LDL-R measurements among experiments, the MF for each sample was expressed as a percentage of the average MF of the controls (usually 3 or more) within the same experiment (%LDL-R).

Monocyte and lymphocyte populations from cultures without PHA were separated by "electronic gating" based on their FSC and SCC readings (**Fig. 1A**). The purity of the monocyte population as assessed by CD14 expression was always greater than 97% while that of the lymphocyte population was less than 3% (data not shown). In the PHA-stimulated samples, gating for monocytes was impossible due to the presence of blast cells (Fig. 1B). However, the population contained only an average of 5% of cells positive for CD14.

Flow cytometric analysis of DiI-LDL uptake

The specific DiI-LDL uptake provides a measure of the LDL-R activity (15, 43) and was determined as described (21, 41). Briefly, harvested cells were washed once in PBC and then incubated with 30 μg/mL protein of DiI-LDL for 1 h at 37°C. Nonspecific uptake was assessed by including 20-fold excess of unlabeled LDL in the reaction mixture. After the incubation, cells were washed twice with PBC and fixed in 1% formaldehyde buffered with PBS (pH 7.4). Cell-associated fluorescence due to DiI (FL2) was analyzed on an FACScan flow cytometer as described above. Specific uptake was calculated as the difference between total and nonspecific uptake.

Cell cycle analyses by propidium iodide

Harvested cells were washed once with PBS, fixed in 67% ethanol in PBS (2:1, v/v), and stored at 4°C until analysis. On the day of analysis, $1–1.5 \times 10^6$ ethanol-fixed cells in a 12 × 75 mm round-bottom tube were washed once with PBS and treated with RNAase to remove any double-stranded RNAs before final suspension in 50 μg/mL of propidium iodide in PBS, pH 7.4. Cellular fluorescence (FL2) from 15,000 cells was routinely collected in a flow cytometer. The distribution of DNA was analyzed by the CELLFit program (Becton-Dickinson) to calculate, based on their respective DNA content, the percentage of cells in G_0G_1, S, and G_2M phases. Stimulation index (SI), calculated as $(S + G_2M)/G_0G_1$, was taken as a measure of the degree of cell activation and proliferation (44).

Statistical analysis

Sample means were tested for differences using the unpaired Student's *t* test. Correlation between LDL-R expression and mitogenic response was analyzed by simple linear regression and the significance of the correlation was tested by the *t* test ($t = r\,[(n-2)/(1-r^2)]^{1/2}$). A *P* value of less than 0.05 was considered significant. The Kolmogorov-Smirnov two-sample test was used to test whether the two sample populations could have come from the same parent population. Indicators of diagnostic efficiency were evaluated in terms of sensitivity, specificity, and efficiency as described (45). The cutoff value for distinguishing FH patients and unaffected individuals was determined graphically as the intersection point of the sensitivity and specificity curves on the cumulative distribution analysis (CDA) chart (46, 47). The corresponding sensitivity and specificity were then read off the chart

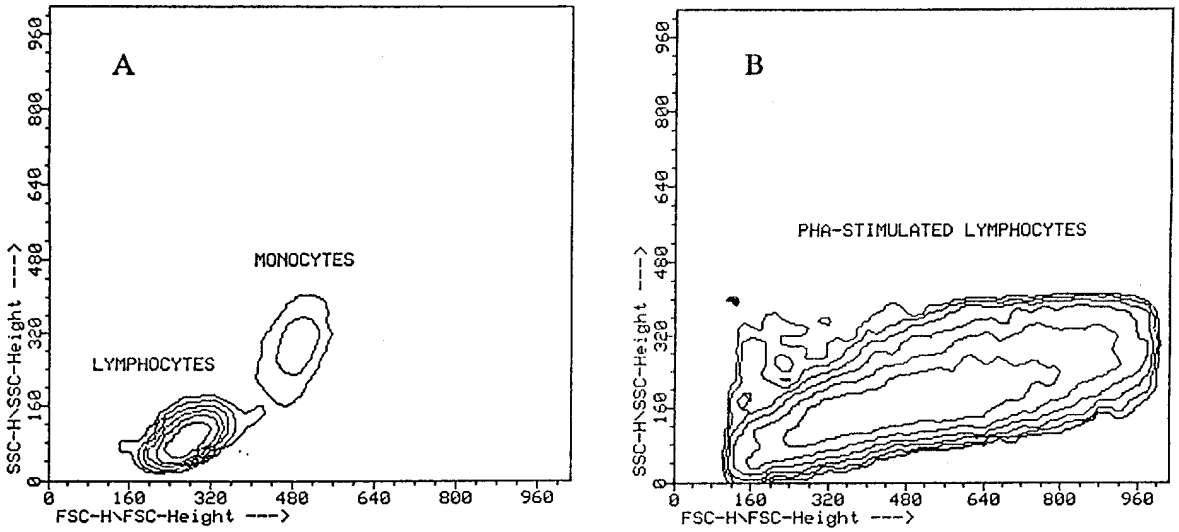

Fig. 1. Forward-scatter (FSC) versus side-scatter (SSC) plots for cultured mononuclear cells. (A) Size (FSC) and granularity (SSC) characteristics of lymphocytes and monocytes cultured in 10% lipoprotein-deficient serum (LPDS) for 3 days; the lymphocyte and monocyte populations could easily be separated or gated on the FSC versus SSC plot. At the same time, cell debris or aggregates could also be excluded. (B) Lymphocytes cultured in 10% LPDS plus 1% PHA for 3 days; the monocyte population could not be readily separated from the lymphocytes due to blast formation.

accordingly. Ninety-five percent confidence intervals for sensitivity and specificity, q, were calculated as $q \pm 1.96$ (SE) (48), where SE (standard error) $= [q(1 - q)/N]^{1/2}$.

RESULTS

Labeling of PHA-stimulated lymphocytes by anti-LDL-R antibody and DiI-LDL

The optimal concentrations of anti-LDL-R antibody and DiI-LDL for labeling peripheral blood lymphocytes cultured in RPMI supplemented with 10% LPDS and 1% PHA for 3 days was determined by assessing the dose response. Receptor saturation occurred at about 0.5 μg/mL of the antibody and essentially plateaued between 0.5 and 6 μg/mL, the upper dose used. To avoid any untoward volume dilution in the labeling procedure, 2 μg/mL was chosen for all subsequent experiments. The concentration dependency of DiI-LDL uptake was also determined under the above culture conditions. Both total and nonspecific uptake were concentration dependent and increased as the concentration of DiI-LDL increased. Specific uptake, which is the difference between total and nonspecific uptake, plateaued at less than 30 μg/mL protein of DiI-LDL. To minimize nonspecific uptake, 30 μg/mL of the DiI-LDL was used in all subsequent experiments.

PHA- versus LPDS-mediated LDL-R up-regulation

To ensure that assaying LDL-R in PHA-stimulated lymphocytes does offer an advantage over LPDS up-regulation alone (regardless of cell type), the LDL-R expression was monitored in lymphocytes and monocytes cultured in 10% LPDS and in lymphocytes cultured in 10% LPDS plus 1% PHA over a period of 96 hs (**Fig. 2**). The LDL-R expression was barely detectable in freshly isolated mononuclear cells (zero hour). The LDL-R expression in both the lymphocytes and monocytes cultured in LPDS without PHA increased steadily and after 72 h reached a maxi-

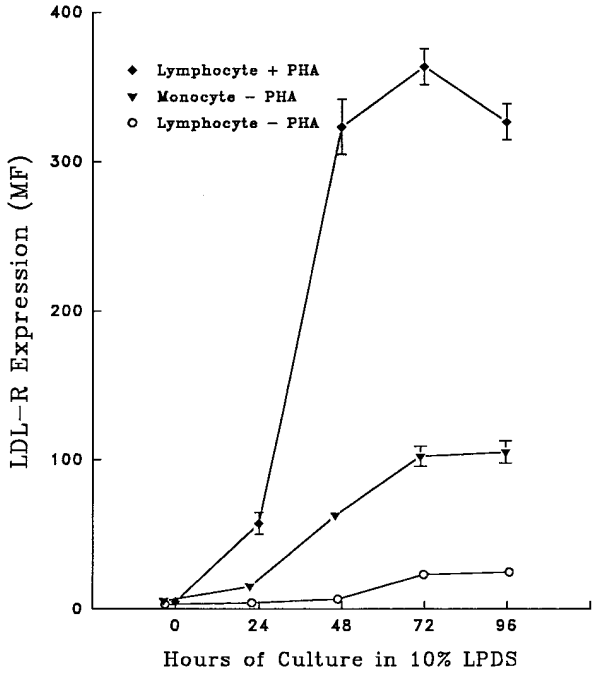

Fig. 2. Temporal expression of LDL-R in mononuclear cells cultured in 10% LPDS with and without 1% PHA. At the indicated times, LDL-R expression was determined by an anti-LDL-R antibody as described in Methods in lymphocytes (○) and monocytes (▼) cultured in LPDS without PHA, and in lymphocytes (◆) cultured in 10% LPDS plus 1% PHA. Results represent mean ± SEM from triplicate determinations in five individuals.

mum of 5- and 17-fold, respectively, above that at zero hour, with the increase in monocytes equating to a 4-fold higher LDL-R expression than in lymphocytes. In lymphocytes cultured in LPDS plus PHA, the LDL-R surface expression increased from 0 to 72 h of culture by more than 72-fold and dropped thereafter. The LDL-R surface expression of lymphocytes cultured in LPDS plus PHA for 72 h was 15.2-fold and 3.6-fold higher than that of the lymphocytes and monocytes cultured in LPDS (without PHA), respectively.

To discriminate the effect of PHA (mitogenesis) from LPDS (sterol deprivation) on LDL-R expression of LDL-R, surface expression was examined in media deficient in lipoproteins (LPDS), media deficient in lipoproteins (LPDS) with PHA, and LPDS media with reconstituted LDL and PHA, and LDL-R expression was measured 72 h after culture. LDL, at 20 and 103 μg/mL, reduced the LDL-R surface expression by 85% and 97%, respectively, when compared to cultures in LPDS plus PHA. However, even at 103 μg/mL of LDL, the level of surface LDL-R expression was still more than 2-fold higher than the freshly isolated lymphocytes. Thus, abrogating the sterol-dependent effect of LPDS by the addition of LDL did not completely prevent the increase in surface LDL-R expression in PHA-stimulated lymphocytes. On the other hand, addition of LDL, even at a low concentration (20 μg/ml) completely abolished the increase in surface LDL-R expression of lymphocytes grown in LPDS without PHA (results not shown).

Correlation between LDL-R expression and mitogenic response

The temporal relationship between the LDL-R surface expression and the mitogenic response as measured by FSC, IL2-R (CD25)expression and SI in PHA-stimulated lymphocytes is shown in **Fig. 3**. FSC and SI did not increase significantly until after 28 h of PHA treatment. By 50 h, the increase in FSC had reached the maximum and plateaued thereafter. SI, on the other hand, did not peak until 72 h. The IL2-R and LDL-R expression started to increase in parallel as early as 16 h. The greatest increase occurred between 28 and 50 h of culture. By 72 h, both had reached peak expression and declined afterwards.

The relationship between surface LDL-R expression and mitogenic response as measured by FSC, surface IL2-R expression, or SI was examined by simple linear regression analyses. Different levels of mitogenic response in PHA-stimulated lymphocytes were generated by varying the length of exposure to PHA (0–4 days). In addition, the mitogen and LDL-R expression was varied by adding graded concentrations of lovastatin (0.1–5.0 μmol/L) to the PHA-stimulated lymphocytes and the relationship between mitogen response and LDL-R expression was examined at 72 h of culture. The results were pooled and are presented in **Fig. 4A–C**. Each measure of mitogenic response viz. FSC, IL2-R surface expression, and SI was highly correlated with the surface LDL-R expression ($r > 0.9$ and $P < 0.001$ in each case). Over 80% (r^2) of the changes in LDL-R expression was related to any one of

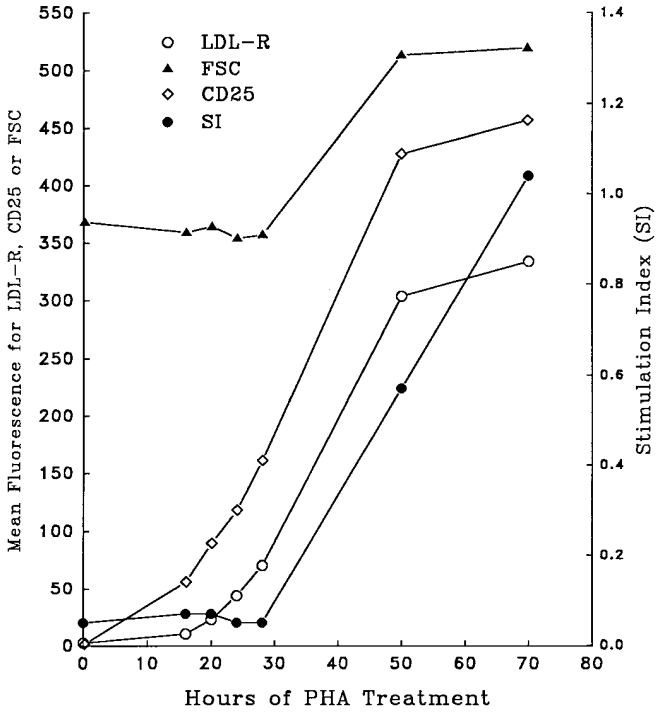

Fig. 3. Temporal changes in LDL-R expression and mitogenic responses during PHA stimulation. Lymphocytes were cultured in 10% LPDS and 1% PHA for 72 h. LDL-R expression (○) was determined by immunocytofluorimetry using an anti-LDL-R antibody and mitogenic response was assessed by: *a*) blasts formation as indicated by changes in cell size (FSC) (▲), *b*) expression of interleukin-2 receptor (IL2-R or CD25) (◇) and *c*) Stimulation Index (SI) (●). Each value represents the mean of duplicate determinations from two individuals.

the changes in FSC, IL2-R expression or SI. While the relationship between mitogenesis and LDL-R expression was maintained in the presence of lovastatin, the response was graded.

Mitogenic responses in FH and normolipemic subjects

As the LDL-R expression in PHA-stimulated lymphocytes is related to the degree of mitogenic response, it is important to note that any difference in the LDL-R expression found between FH and normolipemic subjects is not due to changes in the mitogenic response. Therefore, FSC, IL2-R expression, and SI in 3-day PHA-stimulated lymphocytes were compared between 30 FH and 42 normolipemic subjects. Differences in the means between the two groups were tested using the unpaired Student's *t* test. None of the three measures of mitogenic response demonstrated any significant differences between the FH and the control groups ($P > 0.05$ in each case). To ensure that different levels of the LDL-R expression among individuals were not due to differences in mitogenic response, the IL2-R surface expression and FSC of each sample were used as an internal control, i.e., result of the LDL-R expression would be rejected if the IL2-R surface expression and FSC were greater than 3 standard deviations of all samples assayed within an experiment.

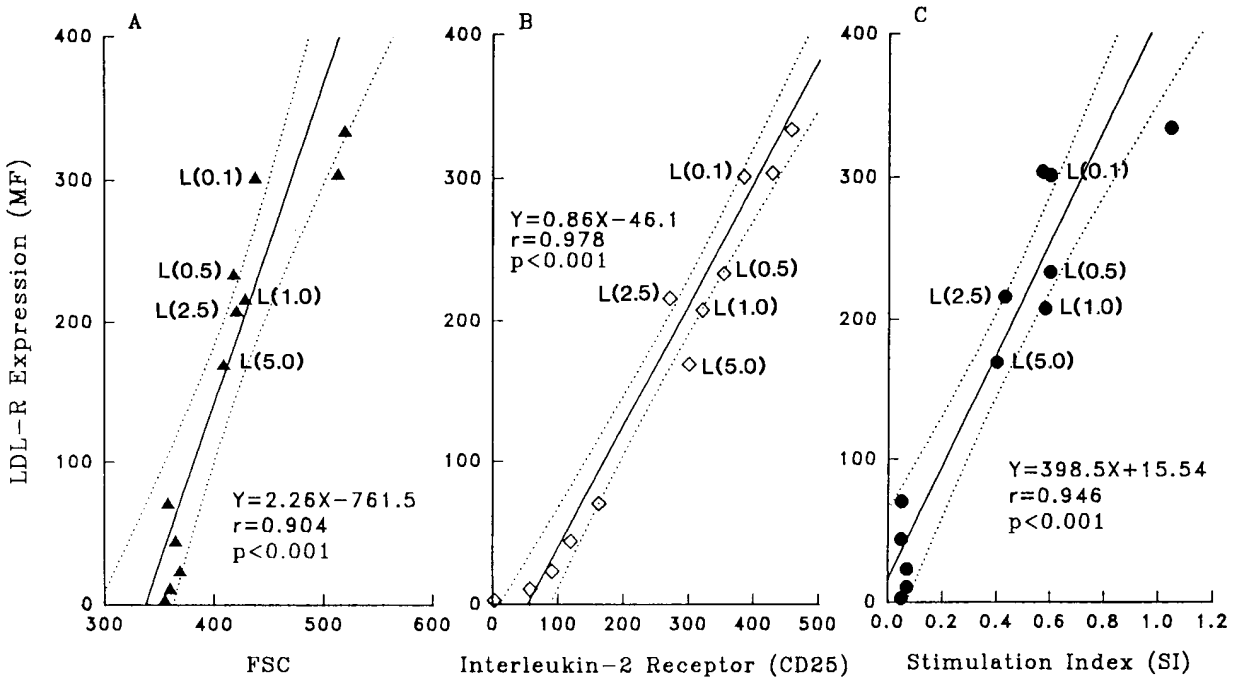

Fig. 4. Correlation between LDL-R expression and mitogenic response. Different levels of mitogenic response in PHA-stimulated lymphocytes were generated by varying the length of exposure to PHA (0–4 days) or by supplementing the cultures with varying concentrations (0.1–5.0 μmol/L) lovastatin at zero hour. Surface LDL-R expression as measured by anti-LDL-R antibody and mitogenic response as measured by FSC, surface IL2-R expression or SI were monitored simultaneously as described in Methods. The results of the two treatments were pooled and presented in (A) LDL-R versus FSC, (B) LDL-R versus IL2-R (CD25) and (C) LDL-R versus SI. The solid line represents the best-fit line while the dotted lines in each plot indicate the 95% confidence limits. The "L" next to data symbols indicates treatment with lovastatin while the number in brackets indicates the concentration of lovastatin (μmol/L) used.

LDL-R expression in PHA-stimulated lymphocytes from FH and normolipemic controls

The ability of the anti-LDL-R antibody assay to detect LDL-R deficiencies was assessed by comparing it with the specific uptake of DiI-LDL in 3-day PHA-stimulated lymphocytes from seven previously diagnosed FH subjects (**Table 1**). Three separate experiments with three normolipemic controls each were performed. Results for both the antibody binding and the specific DiI-LDL uptake were expressed as a percentage of the mean of control MFs in each experiment. With the antibody method, FH subjects demonstrated an average LDL-R expression of 39.5 ± 12.5% (mean ± SD) of the controls while with the DiI-LDL method, the specific uptake was 45.7 ± 8.6% of the controls. As the results from both methods were not significantly different (Student's *t* test, $P > 0.05$) and the receptor status as predicted by both methods were the same, either method could be used for detecting LDL-R deficiencies.

Using the LDL-R antibody, the surface LDL-R expression was determined in 3-day PHA-stimulated lymphocytes from a cohort of 1 homozygous, 30 heterozygous FH, 7

TABLE 1. Determination of LDL-R binding and activity with anti-receptor antibody and DiI-labeled LDL in 3-day PHA-stimulated lymphocytes

	DiI-LDL, 37°C		Anti-LDL-R, 4°C	
	Binding (MF)	%LDL-R	Binding (MF)	%LDL-R
Control (n = 3)	86.9 ± 11.3	100 ± 13.0%	483.4 ± 72.1	100 ± 14.9%
Subject 1	52.6	60.5%	204.7	42.3%
Subject 2	38.1	43.8%	257.7	53.3%
Control (n = 3)	157.1 ± 9.4	100 ± 6.0%	387.6 ± 34.8	100 ± 9.0%
Subject 3	62.8	40.0%	103.9	26.8%
Subject 4	70.0	44.6%	222.9	57.5%
Subject 5	54.9	34.9%	155.2	40.0%
Control (n = 3)	69.4 ± 6.2	100 ± 8.9%	370.3 ± 40.9	100 ± 11.0%
Subject 6	37.3	53.7%	105.2	28.4%
Subject 7	29.7	42.8%	104.7	28.3%

MF, mean fluorescence; %LDL-R, the expression of LDL-R MF as a percentage of the mean of LDL-R MF from controls of the same experiment. Results represent an average of duplicate determinations. Values from control subjects represent mean ± SEM.

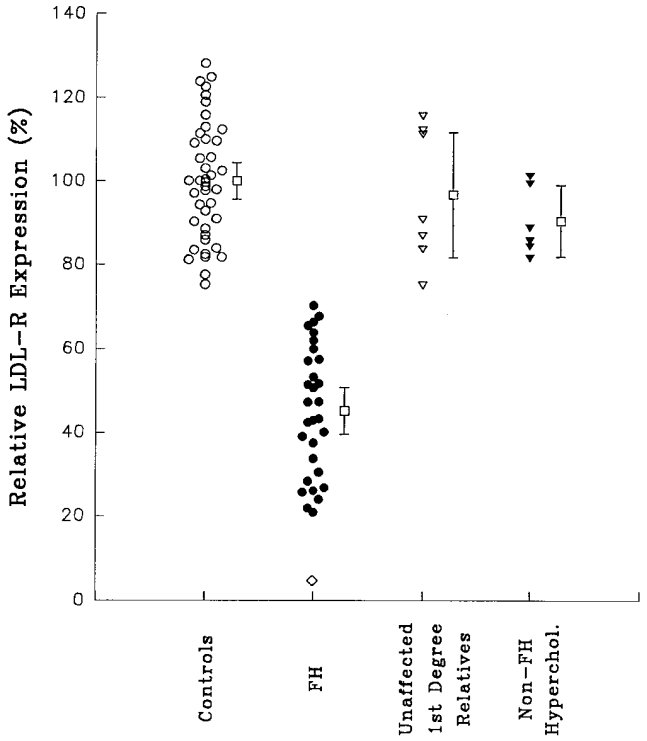

Fig. 5. LDL-R surface expression in familial hypercholesterolemic and control subjects. The LDL-R surface expression as measured by monoclonal antibody was determined in 3-day PHA-stimulated lymphocytes from a cohort of 42 normolipemic controls (○), 1 homozygote (◇), 30 clinically diagnosed heterozygotes for FH (●), 7 normocholesterolemic first degree relatives (▽) and 6 non-FH hypercholesterolemic (▼) subjects.

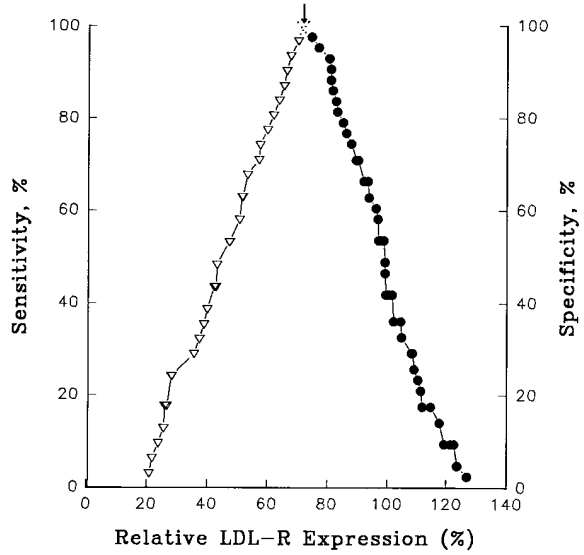

Fig. 6. Cumulative distribution analysis graph for the LDL-R assay. The diagnostic efficacy of the LDL-R assay in PHA-stimulated lymphocytes is presented in a CDA graph. Sensitivity (▽) and specificity (●) are calculated from the FH and control groups, respectively. ↓ indicates the cutoff value for discriminating FHs, derived from the intersection of the extrapolated sensitivity and specificity curves (. . . . .).

unaffected first degree relatives, 42 normolipemic (control), and 6 non-FH hypercholesterolemic individuals. The results are presented in **Fig. 5**. Analyses using the Kolmogorov-Smirnov two-sample test indicated that the FH population could not have come from the same parent distribution as the other three groups (DN = 1, $P < 0.001$ in each case). The LDL-R expression in clinically diagnosed heterozygous FHs (45.2 ± 15.3%, mean ± SD) was significantly lower than the unaffected first degree relatives (96.6 ± 16.2%), control subjects (100 ± 14.1%), or the non-FH hypercholesterolemic individuals (90.3 ± 8.2%) (unpaired *t* test, $P < 0.001$ in each case). The expression in the homozygous FH was less than 5%. The reproducibility of the assay was assessed in two individuals by determining the LDL-R expression on seven different occasions. The average variation (CV) was 9.9%.

For additional verification of the absence of LDL-R defects in the six non-FH hypercholesterolemic subjects mentioned above, DiI-LDL uptake was also performed in 3-day LPDS- and PHA-stimulated lymphocytes. All six subjects demonstrated greater than 80% DiI-LDL uptake when compared to normolipemic controls.

Diagnostic performance of the LDL-R assay

A CDA graph was constructed using results from the FH and the control populations and is shown in **Fig. 6**. The curves for sensitivity (true positive rate) and specificity (true negative rate) at various levels of LDL-R expression did not overlap. Extrapolating the two curves to the point of intersection yielded a value of 73% in LDL-R expression which corresponded to a sensitivity (and specificity) of about 98.5%. The SE for the sensitivity and specificity are then calculated to be 2.2% and 1.5%, respectively, and the 95% confidence intervals estimated to be 94.2–102.9% and 94.8–102.2%, respectively. The efficiency of the assay (i.e., % of correct identifications) is 97%.

Interestingly, when lymphocytes from the 30 FH subjects were cultured in LPDS without PHA, 5 demonstrated levels of LDL-R expression considered to be in the normal range, i.e., above the cut-off of 70% when compared to normolipemic controls (2 displayed borderline LDL-R expression at 70–75%, 2 had levels greater than 80%, and 1 above 90%). The false negative rate of 17% (5/30) translates into a sensitivity of 83.3%.

DISCUSSION

In the current study, classical FH subjects (index cases) meeting the most stringent diagnostic criteria and one homozygote were used to validate a flow cytometric assay for detecting FH. The utility of the assay was further examined by analyzing a cohort of first degree relatives (both affected and unaffected) and non-FH hypercholesterolemic subjects in a blinded fashion. All the 15 index cases and 16 out of 23 first degree relatives demonstrated abnormal LDL-R expression. Subsequent examination revealed elevations in plasma LDL cholesterol levels in all 16 individuals, therefore meeting the commonly used criteria for a diagnosis of FH (see Subjects under Methods).

The 7 first degree relatives who did not demonstrate abnormal LDL-R expression were also found to have normal LDL levels. Of the 6 non-FH hypercholesterolemic subjects who demonstrated normal LDL-R expression, 4 had an initial diagnosis of FH; all had LDL levels above the 95th percentile for age and sex and a family history of CAD. Detailed study of medical history revealed the presence of mildly elevated triglyceride level before the onset of lipid-lowering treatment and a family history of multiple lipoprotein phenotype, diabetes, and/or autoimmune diseases in 3 of the subjects while the 4th one had no first degree relative (5 examined) with hypercholesterolemia. These subjects were diagnosed as familial combined hyperlipidemia and polygenic hypercholesterolemia, respectively. The rest of the non-FH hypercholesterolemic subjects who demonstrated normal LDL-R expression had only mildly elevated plasma cholesterol level and no family history of hypercholesterolemia. Specific causes for hypercholesterolemia could not be pinpointed in these individuals. Apparently, detailed family history plays a major role in the clinical diagnosis of FH.

The high levels of LDL-R expression in PHA-stimulated lymphocytes (15.2- and 3.6-fold higher than unstimulated lymphocytes and monocytes, respectively) make it a very attractive cellular system for the detection of LDL-R abnormalities. The high correlation of the LDL-R expression with FSC and IL2-R expression, which could be determined concurrently, allows precise and convenient control over fluctuations in lymphocyte activation and proliferation. As no significant difference in mitogenic responses as measured by FSC, IL2-R expression, or SI was observed between FH and control subjects, the low LDL-R expression in the FH group reflects the underlying genetic defect.

It has been known for sometime that mitogen stimulation of lymphocytes increased the LDL-R expression (26, 28, 49, 50). However, what exactly caused the up-regulation is not clear. PHA activates lymphocytes by binding to surface receptors that are different from antigen recognition receptor, leading to biochemical changes such as hydrolysis of phosphatidylinositol-4,5-bisphosphate, generation of diacyglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3), increases in intracellular calcium and sodium, protein phosphorylation, activation of protein kinase C (PKC) and adenylate cyclase, expression of a whole host of activation molecules and synthesis of protein, RNA and DNA (51). Interestingly, transcription of the LDL-R gene, as demonstrated in other cell lines, appears to involve at least some of these biochemical changes including activation of PKC, hydrolysis of IP_3, increase in intracellular calcium level, and activation of adenylate cyclase (52). It is possible that both the cell stimulation by PHA and the up-regulation of the LDL-R by growth-mediated mechanisms share some common signal transduction pathways.

Experiments were done to clarify the role of LDL sterol-mediated (no LDL) or PHA (mitogen-mediated) regulation of on LDL-R expression. Lymphocytes cultured for 72 h in LPDS without PHA demonstrated a 5-fold increase in LDL-R expression. As cell proliferation is known to induce LDL-R expression in lymphocytes, we examined the combination of LPDS plus PHA on LDL-R expression. LPDS and PHA caused a 72-fold increase in the surface expression of LDL-R expression, a far superior response than to LPDS alone. We next compared the response of lipoprotein sufficient serum without and with PHA. Addition of as little as 20 μg/mL of LDL to LPDS without PHA completely prevented the 5-fold increase in LDL-R expression seen with LPDS alone. This was not the case with lipoprotein-sufficient serum (addition of LDL) and PHA. While the response was severely curtailed with a high concentration of LDL (103 μg/mL), LDL-R expression at 72 h of culture was still 2-fold elevated above LPDS alone. The latter 2-fold elevation in LDL-R expression in the presence of a high concentration of LDL is attributed to the stimulation effect of PHA. The huge up-regulation of the LDL-R expression observed in LPDS plus PHA suggests a synergistic effect of PHA stimulation and sterol deprivation (LPDS) on the surface expression of LDL-R. While it has been suggested that PHA stimulation activates the LDL-R expression through sterol-independent means (28, 53), transfection studies have indicated that the sterol-responsive elements of the LDL-R gene are also responsive to growth activation (54). Further studies are required to elucidate the interplay of the two mechanisms involved.

It has been reported that LDL-R expression in resting lymphocytes increases with age (44, 55) while the mitogen-induced proliferative response decreases (47). In the current study, we had 2 FH and 2 control subjects over the age of 60 and no difference in any aspects regarding the proliferation or the LDL-R expression that would affect their classification was observed. As the major target group for FH detection is those under the age of 55, the impact of increase in LDL-R expression and decrease in proliferative response to mitogen in the aged should be minimal.

Although it has been suggested that monocytes grown in LPDS have a higher expression (mean fluorescence) than in PHA-stimulated lymphocytes (20), our observation could not support it. The discrepancy may be explained by the fact that the LDL-R expression is dependent on a number of factors such as cell density (56), batches of lipoprotein-deficient serum (57), and cell proliferation (15). In the PHA system, the LDL-R is highly correlated with the mitogenic response which, in turn, has been shown to be extremely sensitive to the culture conditions such as cell density (58, 59), length of culture, the type and concentration of PHA used, the geometry of the culture flasks, and even the techniques of harvesting cells (60–62). The culture conditions not only affect the maximum response attainable but also determine when maximum expression occurs. One day earlier or later may yield very different results.

In lymphocytes cultured in LPDS without PHA, an increased false negative rate of 17% (5/30) was observed. The false negative rate was somewhat comparable to previous reports that 22–32% of FH patients were shown to have normal LDL-R function in LPDS-up-regulated (no

PHA) lymphocyte systems using DiI-LDL (21, 25). On the other hand, if we were not careful in examining previous medical and family history, four of our non-FH hypercholesterolemic subjects would have been placed in the FH category, pushing the false negative rate to 12% (4/34). Although the use of antibody for LDL-R assay has been reported to give false negative results in individuals with the internalization-defective (type 4A) mutations (20, 42), our extremely low false negative results suggest that either the incidence of these mutations is very low in our FH population or the assay system is capable of detecting the mutations. In the former, DiI-LDL uptake at 37°C will be a useful adjunct study while for the latter, further study is required to confirm the observation. While the exact cause for normal LDL-R expression in FH patient is not clear, the high rate of clinically diagnosed FH patients showing normal LDL-R activity reported in other studies may be an overestimation. Overall, we believe that maximizing the LDL-R expression in LPDS- and PHA-stimulated lymphocytes provides superior sensitivity in the detection of LDL-R anomalies and will likely to reduce the number of clinical FH patients with normal LDL-R activity.

In summary, measuring LDL-R expression by anti-LDL-R antibody in 3-day PHA-stimulated lymphocytes represents a simple and effective method with improved sensitivity for detecting FH subjects. Such assay will be most useful in *1*) the differential diagnosis of FHs, especially in the absence of previous medical and/or family history, from other causes of hypercholesterolemia such as familial defective apoB (FDB) (63, 64), phytosterolemia (65, 66), familial combined hyperlipidemia (67), autoantibody to LDL-R (68), elevated synthesis of LDL (69), and polygenic hypercholesterolemia; *2*) diagnosing FH from those with borderline cholesterol levels and/or without a traceable family history; and *3*) assessing CHD risk.

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