Methodological Aspects of Flow Cytometry to Measure Low-Density Lipoprotein Receptor Expression on Peripheral Monocytes

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ABSTRACT

Conflicting reports question the value of low-density lipoprotein (LDL) cholesterol receptor expression assays by flow cytometry to diagnose individual cases of heterozygous familial hypercholesterolemia (FH). We used genetically proven index patients with FH to assess an optimized LDL receptor expression assay on mononuclear cells.

Assay conditions (preincubation period, staining procedure, mode of data calculation) were investigated for their impact on test reproducibility and plausibility in 20 patients with clinically diagnosed FH and 6 control subjects. This approach was then validated in 4 genetically characterized index patients.

Good discrimination of controls and patients was observed after 4 and 7 days but not after 2 days of preincubation. Further studies showed that test reproducibility was better after 6 days of preincubation than after 7 ($P<0.05$). The index patients were assessed repeatedly on 4 different dates. Only mean LDL-receptor expression of all 4 measurements was, in all instances, below 80% of normal (mean 73%).

Our data indicate that the expression of LDL receptors changes markedly as mononuclear cells mature. Six days of preincubation appear optimal for the detection of patients with heterozygous FH. Reports claiming that the LDL-receptor test is not suitable for diagnosing heterozygous FH may be explained by lack of intra-assay controls, short cell preincubation periods, and clinical rather than molecular characterization of patients.

INTRODUCTION

Patients with familial hypercholesterolemia (FH) are commonly characterized by a mutation in the gene encoding the receptor for low-density lipoprotein (LDL) choles-

Table 1. Physical Characterization of Healthy Controls, Clinically Diagnosed Cases of Familial Hypercholesterolemia (FH), and Genetically Characterized Index Patients With FH

	Age [y]	Sex [F/M]	Height [m]	Body mass [kg]	Chol.[*] [mmol/L]	TG[†] [mmol/L]	VLDL[‡] [mmol/L]	LDL[§] [mmol/l]	HDL[‖] [mmol/l]
Controls									
Mean	46	4/2	1.69	67	6.3	1.4	0.4	4.4	1.5
SD	3	0.07	11	2.2	0.8	0.2	2.2	0.3	
Clinical FH									
Mean	42	9/11	1.69	75	8.2	1.7	0.6	6.1	1.4
SD	12	0.10	14	3.5	0.9	0.5	3.5		0.6
Index cases									
Mean	53	3/1	1.64	72	8.0	1.1	0.6	6.2	1.5
SD	10	0.08	3	1.5	0.5	0.6	1.2	0.4	

*cholesterol, †triglycerides, ‡very low density lipoproteins, §low density lipoproteins, ‖high density lipoproteins

terol, resulting in hypercholesterolemia, premature atherosclerosis, and coronary heart disease. Unlike sporadic hypercholesterolemia, FH is associated with such high coronary heart disease risk that drug treatment is always necessary.[1,2]

More than 300 different mutations have been identified, which can be diagnosed by molecular techniques. Since no routine test of LDL receptor expression in vivo is presently available, the LDL receptor is assessed ex vivo by activity assays. The labor-intensive analysis of LDL binding, uptake, and internalization from patient-derived skin fibroblasts is widely accepted for research use. However, no routine assay has been established.

The measurement of LDL receptor activity can be performed on peripheral blood mononuclear cells using flow cytometry and fluorescent dye-conjugated LDL-receptor specific antibodies. Conflicting reports question the value of these methods to diagnose individual cases of heterozygous FH. Several investigators noted that LDL-receptor studies are possible for homozygous FH, but result in a large overlap between patients with heterozygous FH and normal subjects.[3-5] In this respect, it is also of concern that studies relied on clinically diagnosed rather than genetically typed cases of heterozygous FH to assess the value of LDL-receptor assays.

We assessed assay conditions such as preincubation period, staining procedure, and mode of data calculation, and investigated their impact on test reproducibility and plausibility in control subjects and clinically diagnosed cases of heterozygous FH. Based on these experiments we assessed patients with characterized mutation of the LDL receptor gene. Our results indicate that standardization of cell preincubation time and repeated testing significantly improves methods currently used to distinguish healthy subjects from patients with heterozygous FH.

METHODS

Serum triglycerides and total cholesterol concentrations were measured colorimetrically on a Vitros V 950 IRC analyzer (Ortho Diagnostic System GmbH, Neckargemünd, Germany). Standard lipid electrophoresis was performed according to manufacturer's instructions using the Lipidophor system (Immuno AG, Vienna, Austria) for quantitation of very low density lipoprotein (VLDL) cholesterol, LDL, and high density lipoprotein (HDL) cholesterol.

Table 2. **Genetic Characterization of Index Patients With Heterozygous Familial Hypercholesterolemia**

Patient	Mutation Name	Mutation	Exon	AA* position	Reference
1	TCT Ins CC	232 Ins CC	3	195 Ins 2	Geisel et al[23]
2	313 + 1 Ins G	-	3-4	313 + 1	Ebhardt et al[9]
3	V[415]->A	T[1307]->C	9	V[436]->A	Lombardi et al[24]
4	A370T	G[1171]->A	8	V[391]->T	Kotze et al[25]

*amino acid; nomenclature of mutations according to Universal LDLR-Mutation database (http://www.ucl.ac.uk/fh/) and the LDLR Database (http://www.umd.necker.fr/)

Experiments

Several experiments were performed to optimize the following variables of the LDL-receptor assay: separation medium, fluorescent dye, preincubation period, test reproducibility, and test sensitivity to detect differences between healthy subjects and patients with clinically diagnosed FH. Finally, a validation study of 4 genetically diagnosed patients with heterozygous FH was performed. The design of each study was based on the results of the foregoing experiment.

Preliminary Experiments were performed on unselected healthy subjects (local laboratory personnel) to assess different separation media and fluorescent dyes.

Preincubation Experiment: Five normo- and 1 hypercholesterolemic control subject (an otherwise healthy staff member of the lab and not meeting the criteria for FH) served as a control group (Table 1). Lipid profiles were performed on those whose lipid levels had never been measured or were unavailable. Control subjects were not on medication of any kind. LDL-receptor expression of monocytes was assessed after 2, 4, 7, and 9 days of preincubation (see also next section).

Clinical FH Experiment: Twenty patients with clinically diagnosed heterozygous familial hypercholesterolemia at the local lipid clinic were studied (Table 1). Diagnosis of heterozygous FH was based on the presence of primary hypercholesterolemia (compared with reference values),

increased LDL cholesterol, and a positive family history of coronary heart disease and/or at least 1 first- degree relative with hypercholesterolemia. Presence of tendinous xanthoma, arcus lipoides corneae, or xanthelasma was optional. All patients with clinically diagnosed FH were on lipid-lowering therapy for at least 2 weeks, 15 receiving statin therapy. LDL-receptor expression of monocytes was assessed after 2, 4, and 7 days of preincubation and compared with controls.

Precision experiment: All control subjects were assessed together and repeatedly on 4 different dates to assess short-term biological variation of LDL-receptor expression as well as test reproducibility. LDL-receptor expression was assessed after 6 and 7 days of preincubation, respectively.

Validation experiment: Index patients had no indication of defective apoB, were genetically characterized (Table 2), and received all lipid-lowering therapy including atorvastatin (Lipitor). The patients and 1 intra-assay control were studied together on 4 different dates. LDL expression was assessed after 5 and 6 days of preincubation.

Mononuclear Cell Isolation and Culture

Ten mL of EDTA blood (S-Monovette, 1.6 mg K_2-EDTA/mL blood, Sarstedt AG & Co, Nümbrecht, Germany) and 1 mL of autologous serum from each patient and control subject were obtained from an antecubital vein after an overnight fast. The EDTA blood was diluted 1:2 with phosphate-

buffered saline (PBS; Gibco, Eggenstein, Germany) and carefully layered on 15-mL Ficoll-Hypaque 1.077 (Lymphosep CCPro, Neustadt, Germany) in a 50-mL polypropylene tube (Sarstedt, Germany). Tubes were centrifuged for 30 minutes at 400 x g at room temperature. After removal of the upper layer the cells above the gel were carefully removed using a siliconized Pasteur pipette. After addition of 20 mL sterile PBS the tube was vortexed and then centrifuged for 10 minutes at 300 x g at room temperature. The cell pellett was reconstituted in 12-mL cell culture medium (RPMI 1640 from Gibco; supplemented with 10 mM Glutamine, 10 mM HEPES, 2g/L $NaHCO_3$, 100 U penicillin/mL, and 100 μg Streptomycin/mL), centrifuged again at 200 g for 10 minutes at room temperature and finally counted with a hemacytometer chamber. The cell concentrate was adjusted to 0.5 x 10^6 cells/mL culture medium and then transferred to tissue culture plates (Greiner, Nürtingen, Germany). Either 0.2 mL of lipoprotein deficient serum (LPDS)[6] or 0.2 mL autologous serum were added to 10^6 cells/1.8 mL cell culture medium, each. Up to a concentration of 2.5 x 10^6 cells/mL, the rate of LDL-degradation has been shown to be linearly proportional to the number of cells.[7] All procedural steps involving the handling of cells were performed in a safety work bench. Preincubation was performed for up to 9 days at 37°C in a humidified CO_2 (5%) incubator.

Flow Cytometry and Data Evaluation
The mononuclear cell suspension was pipetted up and down 2 to 3 times and then transferred into 5-mL polystyrene Falcon tubes (12 x 75mm; Becton Dickinson, Heidelberg, Germany). The cells from each plate were then divided into 2 or 4 equal aliquots and washed twice with cold PBS supplemented with 0.5% bovine serum albumin. The tubes were centrifuged (5 minutes at 250 x g, 4°C) and the supernatants removed. Four micrograms of mouse anti-LDL receptor monoclonal antibody (order no. RPN 537,

Amersham Buchler, Braunschweig, Germany) was added to the test sample and incubated for 30 minutes at 4°C. No antibody was added to the control tube. The cell suspension was then washed 3 times and incubated with biotinylated goat anti-mouse IgG (Becton Dickinson, Heidelberg, Germany). After another wash procedure, streptavidin-phycoerythrin (Becton Dickinson, Heidelberg, Germany) was added and the tube again incubated for 30 minutes in an ice bath, and covered to prevent exposure to light. Eventually 2 mL of washing solution was added to the tubes, which were vortexed and then centrifuged (5 minutes, 250 g, room temperature). The supernatant was aspirated, leaving behind approximately 50 μL of fluid. After addition of 200 μL of cold washing solution the tubes were placed in a covered ice bath and measured within 30 minutes.

Labeled samples were analyzed on a FACScan flow cytometer using the Lysis II software (Becton Dickinson, Heidelberg, Germany). Fluorescent labeled beads of known fluorescence intensity (CaliBRITE; Becton Dickinson, Heidelberg, Germany) were run at the beginning of each experiment to ensure a proper standardization of the instrument. Fluorescence signals were recorded to produce a histogram of cells counted versus relative fluorescence intensity (AU, arbitrary units) after logarithmic amplification. Forward-scatter and side-scatter readings (FSC/SSC) were captured from each single cell and were used to exclude cell debris or aggregates as well as to delineate lymphocyte and monocyte populations. A gate was set around the population of monocytes and the mean fluorescence intensity of the selected cells was estimated for each sample, with a total of 10,000 cells per experiment being analyzed.

The receptor expression on monocytes, which had been preincubated in LPDS, was compared with the expression on cells after culturing in human autologous serum (factorial method)[8] or compared with monocytic autofluorescence. In this case, background
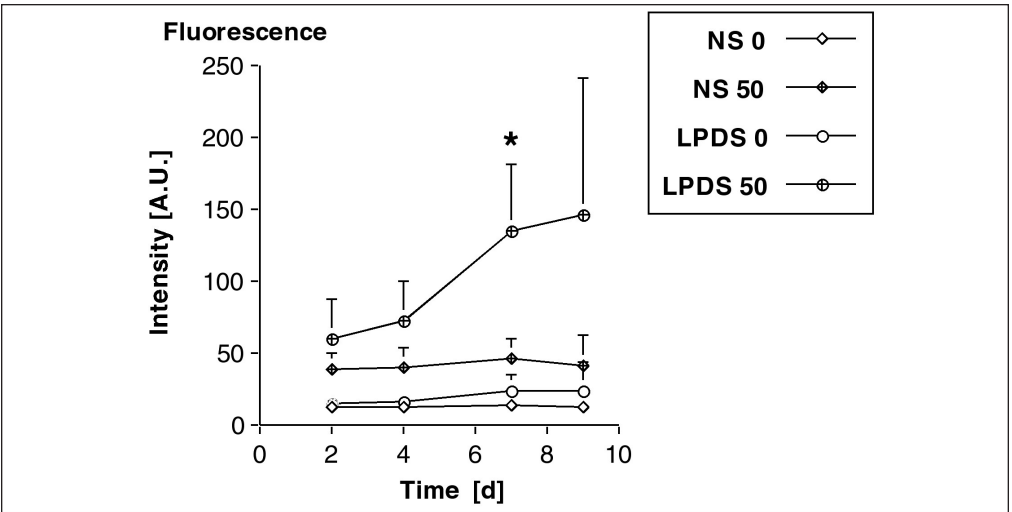
Figure 1. Fluorescence intensity on monocytes after 2,4,7, and 9 days of stimulation with LPDS with (LPDS 50) or without (LPDS 0) anti-LDL receptor antibody, and after preincubation with autologous serum with (NS 50) or without (NS 0) anti-LDL receptor antibody (n = 6); * $P<0.05$ vs 2 days. Data presented as mean ± SD.
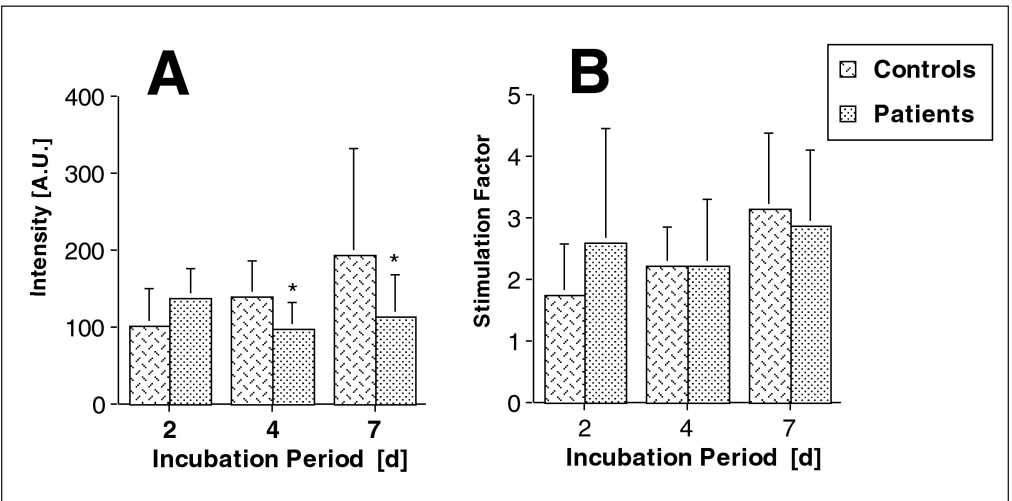

Figure 2. Average LDL receptor expression on monocytes of healthy control subjects and clinically diagnosed cases of heterozygous FH calculated according to the differential (Figure 2A) and factorial (Figure 2B) method (see "Methods" section) after 2, 4, and 7 days of stimulation with LPDS. Significant differences between the groups were observed only with the differential method after 4 and 7 days of stimulation (*$P<0.05$).

fluorescence due to cell autofluorescence and nonspecific binding of antibodies is subtracted to give a net mean fluorescence (differential method), which is a reflection of the average quantity of receptor protein present on a single cell in the population.

DNA Amplification, SSCP Analysis, and DNA Sequencing

DNA was isolated from whole blood by standard procedures.[9] The exons of the LDLR gene were amplified using the primers described by Hobbs et al[10] except for exons 12, 17, and 18 for which primers
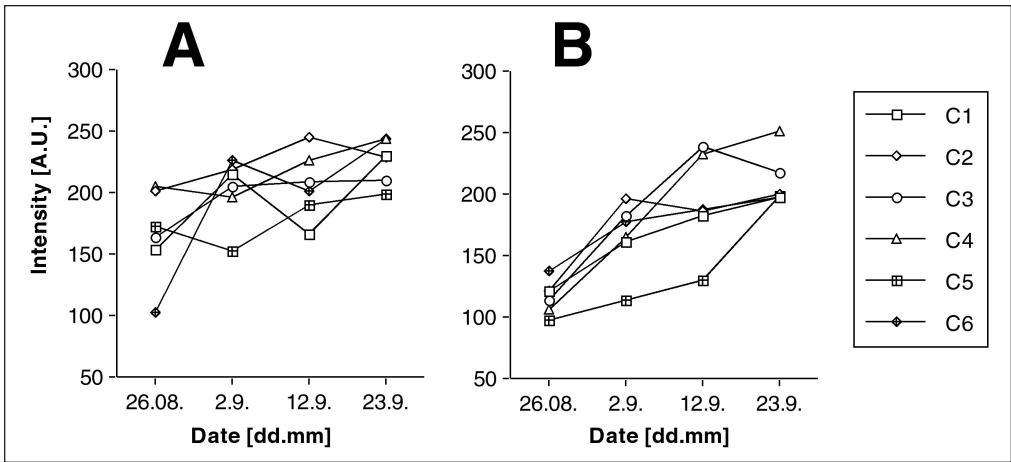
Figure 3. Four repeated measurements of LDL receptor expression on monocytes after 6 (A) and 7 (B) days of LPDS stimulation, respectively, of control subjects. Note the covariance of the results depending on the date of investigation.
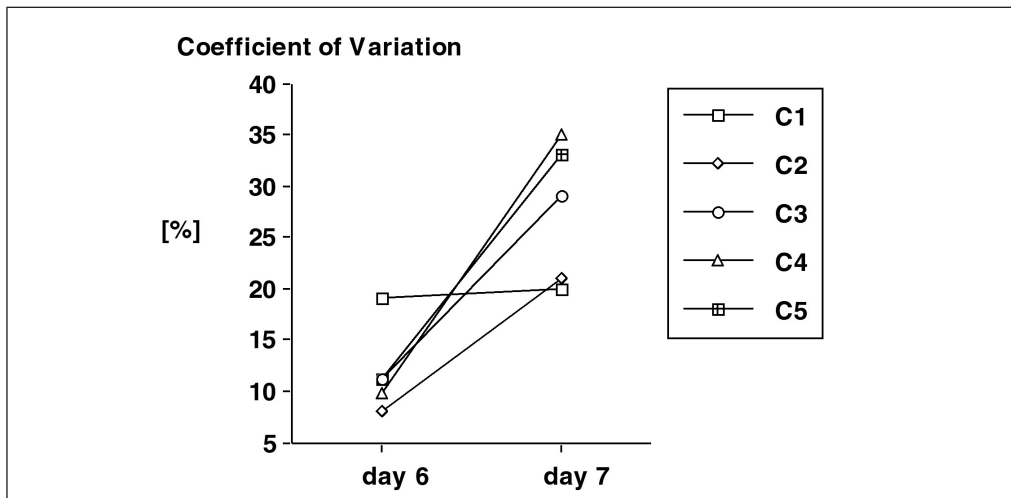

Figure 4. Coefficient of variation of the differential method for 4 repeated measurements of LDL-receptor expression after 6 and 7 days of LPDS stimulation, respectively, in 5 healthy control subjects ($P<0.05$). Results of one control subject with unexplained hypercholesterolemia (C6) are not shown (see "Methods" section).

were used, which previously has been described by Nissen et al.[11] Single stranded conformational polymorphism (SSCP) analysis was performed according to Hobbs et al.[10] To make bands visible, the gels were silver stained after electrophoresis. Samples showing band shifts were sequenced by radioactive labeling with the Sequenase 2.0 PCR Product Sequencing Kit

(Amersham/USB, Braunschweig, Germany) or by fluorescence labeling with the big dye terminator mix of ABI-Perkin Elmer (ABI-Perkin Elmer, Weiterstadt, Germany), according to the manufacturer's instructions.

Results of the genetic characterization of the index patients are shown in Table 2. Defects of LDL-receptor internalization and recycling, which cannot be detected by
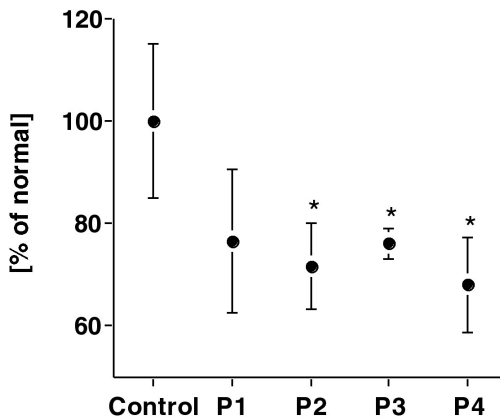
Figure 5. LDL receptor expression on monocytes of 4 index patients (P1-P4) with heterozygous FH compared to controls measurements 6 days of stimulation with LPDS. LDL-receptor expression of the index patients was on average 73% of normal. All single measurements were averaged and then expressed as percent of the mean of the 4 control measurements performed in the same analytical runs; results shown are mean ± SD values of all four runs (*$P<0.05$ vs control).

LDL-receptor binding assays, appear to account for only a small number of patients and were not present in our index cases.[10]

Statistics

Results were assessed for normal distribution and expressed as means ± standard deviation (SD). The significance of intergroup comparisons was established by the unpaired Student's t-test. Multiple comparisons of repeated measurements were assessed by analysis of variance and Bonferroni correction. Correlation between the factorial and differential method to calculate LDL-receptor expression was analyzed by linear regression. A probability of 0.05 or less was considered statistically significant.

RESULTS

Preliminary Experiments: Preliminary experiments assessed different cell separation media, such as LeucoPREP , Percoll, Nycoprep, and Ficoll, which have been shown to exert a differential immunological activation of monocytes.[12] These different media had no obvious effects on LDL-

receptor expression of monocytes (data not shown). More reproducible results for cell recovery were obtained with faster cell processing using the least number of separation steps. Two different fluorescent dyes—fluorescein-isothiocyanate (FITC) and phyco-erythrin(PE)—were also compared; PE labeling showed generally stronger signals and remained the method of choice.

Preincubation experiment: Measurements on monocytes of control subjects (Table 1) were performed after 2, 4, 7, and 9 days of preincubation in LPDS. We observed a significantly higher fluorescence intensity compared with 2-day values on day 7 (Figure 1). Whereas a preincubation for 9 days resulted in increased variability, a shorter preincubation period of 4 days was associated with lower LPDS-induced LDL-receptor expression (Figure 1).

Clinical FH experiment: Based on the results of the preincubation experiment, we compared results obtained in clinically diagnosed patients with heterozygous FH with those of healthy control subjects. Calculation and expression of data by the factorial method, that is, with reference to

cells cultured in autologous serum (see method section), did not result in any discrimination between controls and patients (Figure 2). However, if the mean fluorescence values of the gated monocytes were corrected for auto-fluorescence they were significantly different for controls and patients after 4 and 7 days but not after 2 days of stimulation (Figure 2). There was a poor but significant positive correlation between the results obtained by the factorial and differential method calculated from the same set of raw data (r = 0.43, $P<0.01$; data not shown).

Precision Experiment: To further optimize duration of the preincubation period we assessed the between-series reproducibility for measurements in all control subjects (Table 1). Figure 3 shows the results of this experiment for 6 and 7 days of preincubation indicating covariance of LDL receptor expression on monocytes depending on the date of analysis. The same data set was used for calculation of the coefficient of variation (CV) of LDL-receptor expression after 6 and 7 days of preincubation, respectively (Figure 4). Precision was better after 6 days of preincubation than after 7 days ($P<0.05$).

Validation experiment: Four treated index patients of heterozygous FH were assessed repeatedly on 4 different dates. LDL receptor expression was similar after 5 and 6 days of preincubation but the results tended to be more reproducible after 6 days (coefficient of variation 16.7 ± 7.6% vs. 12.0 ± 6.2%, not significant; data not shown). For all index patients average LDL-receptor expression after 6 days of preincubation was below 80% of normal (mean 73%).

DISCUSSION

This study assessed optimal conditions and timing of mononuclear cell culture as well as subsequent fluorescence activated cell scan (FACS) analysis for the study of LDL receptor expression. Our data indicate that the expression of specific LDL receptors change markedly as mononuclear cells

mature, and that preincubation periods of 6 days are optimal for the assessment of LDL receptor activity on monocytes. In healthy control subjects LDL-receptor expression is increased after 7 days or preincubation with better reproducibility after 6 days of preincubation (Figures 1 and 3). In contrast, monocytes of patients with true or suspected heterozygous FH do not further increase LDL-receptor expression after 2 days of stimulation with LPDS (Figure 2). The sensitivity for differences between healthy subjects and patients with FH is highest after approximately 6 days of preincubation. Patients with clinically diagnosed heterozygous FH had significantly lower LDL-receptor expression than controls after 4 days of stimulation, and this difference increased for another 3 days (Figure 2A).

Our observation is in line with previous reports by Knight and Soutar,[13] who observed that maximum ^{125}I-LDL degradation by monocytes produced by preincubation with LPDS increases between day 2 and 5 in control subjects but is rather stable or decreasing in patients with FH following 2 days of stimulation. Similarly, LDL receptor activity on lymphocytes has been reported to plateau after 5 to 6 days of stimulation by interleukin-2 or anti-CD3 antibody.[14]

Marked interassay variation is known to compromise the value of the 125I-LDL degradation method as well as the FACS analysis methods of LDL-receptor activity.[15,16] For this reason, Löhne et al used frozen standard cells and showed that these standard cells exhibited a between-series coefficient of variation of 31%.[16] The source of this variation remains unknown. As a consequence, most but not all investigators normalize data of each experiment to the activity of controls tested in the same run. Our data show that optimizing preincubation times reduces but does not eliminate interassay covariation (Figures 3 and 5). Thus, it is important to test at least 2 controls in the same run of each experiment. We believe that conflicting results claiming that the

LDL-receptor test is not suitable in identifying individual cases of heterozygous FH, may be explained by lack of such internal control measurements as well as short cell preincubation periods.[3,17]

Preincubation with LPDS causes a dramatic increase of LDL high-affinity degradation by monocytes, and this effect is much lower in lymphocytes.[15,18] Higher levels of LDL receptor expression may be observed in proliferating lymphocytes.[5] For this reason, stimulation of lymphocytes with phytohemagglutinin (PHA) has been propagated. Although one report claims significant improvement of assay performance using PHA stimulation, other reports question this approach: Suzuki et al,[14] Løhne et al,[16] and Benhamamouch et al[19] found that PHA stimulation is associated with the occurrence of significant cellular debris and cell aggregation, which precludes accurate cell counts by flow cytometry.[14,16,19] Further, proliferating lymphocytes created a lower increase in fluorescence compared with monocytes. After PHA stimulation of lymphocytes, no homogenous LDL receptor active population can be identified and LDL receptor binding varies widely. Recent evidence suggests that lymphocyte subsets differ significantly in LDL uptake, causing distribution-dependent inaccuracies.[20] These observations support the concept that LPDS- stimulated monocytes have to be regarded as the assay of choice.

All of our index patients were receiving cholesterol-lowering therapy. This therapy is known to enhance the capacity of LDL receptor expression in clinically diagnosed cases of heterozygous FH.[15] Cuthbert et al. used an assay based on LDL-dependent, mitogenic stimulation of lymphocytes and reported that the LDL-receptor activity is restored to normal levels in some heterozygous FH patients.[21] We cannot exclude that any in-vivo effect of statin therapy was lost in tissue culture during preincubation. However, our data appear to indicate that in genetically diagnosed cases of heterozygous FH cholesterol-lowering therapy does not restore LDL receptor expression to normal levels: our index patients all had lower than normal LDL-receptor expression despite cholesterol lowering therapy (Figure 5).

Lack of genetic classification (false diagnosis of FH, presence of undetectable defects) may explain earlier reports of normal LDL-receptor expression in up to 35% of patients with clinically diagnosed heterozygous FH.[22] In addition, the use of an antibody to stain LDL receptor on the cell surface is also not adequate to detect rare class 3 and 4 LDL-receptor mutations (e.g. receptor internalization and intracellular processing defects), which can be detected by use of fluorescence dye labeled LDL molecules.

A major problem with the clinical diagnosis of FH is that clinical criteria cannot be well defined. If xanthoma in the patient or first degree relatives is required for the diagnosis, one will underestimate the prevalence of the disease. On the other hand, if diagnosis relies only on the presence of hypercholesterolemia and a positive family history, the prevalence will be overestimated.

Our study shows that patients with heterozygous FH (due to defective LDL receptor expression) can be reliably diagnosed by flow cytometry using LDL-receptor specific antibodies if (1) prolonged stimulation periods are applied, (2) intra-assay controls are used, and (3) patients are repeatedly investigated. The source of poor precision of the LDL receptor assay, however, remains unknown and warrants further investigations.

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