

# An improved method for detection of low density lipoprotein receptor defects in human T lymphocytes

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**Abstract** Familial hypercholesterolemia (FH) results from an inherited functional defect of the low density lipoprotein (LDL) receptor and is complicated by premature atherosclerosis. FH diagnosis is obtained by sophisticated techniques or is suggested by clinical criteria. We have developed a technique based on flow cytometry for the measurement of DiI-labeled LDL uptake in human peripheral blood T lymphocytes left for 2 days in a lipoprotein-deficient culture medium. Flow cytometry allowed us to discriminate the uptake of DiI-LDL by T lymphocytes subpopulation from the uptake by the whole mononuclear population using a T cell-specific anti-CD3 antibody. The method appeared to be highly specific for the receptor-mediated pathway of LDL uptake as DiI-LDL uptake was inhibited in the presence of a 10-fold excess of unlabeled LDL and by EDTA. A good relationship was found between the uptake of DiI-LDL and <sup>125</sup>I-labeled LDL degradation. The test was applied in three groups of patients: patients with normal cholesterol levels, patients with heterozygous FH, and patients with high cholesterol levels but without clinical criteria of FH. The mean fluorescence intensities were  $23.1 \pm 8.9$ ,  $6.3 \pm 1.7$ , and  $17.1 \pm 3.5$  (mean  $\pm$  standard deviation), respectively. The ability to measure the fluorescence in T lymphocytes improved the discrimination between FH and non-FH subjects when compared with values obtained from the whole mononuclear cell population. These results suggest that our method could be useful for LDL receptor defects screening.—**Verhoeye, F. R., O. Descamps, B. Husson, J-C. Hondekijn, M-F. Ronveaux-Dupal, J. F. Lontie, and F. R. Heller.** An improved method for detection of low density lipoprotein receptor defects in human T lymphocytes. *J. Lipid Res.* 1996. **37**: 1377-1384.

**Supplementary key words** flow cytometry • familial hypercholesterolemia • fluorescence labeling • dioctadecylindocarbocyanine

Familial hypercholesterolemia (FH) results from a genetically determined functional defect of the low density lipoprotein (LDL) receptor leading to hypercholesterolemia, tendon xanthomas, and premature atherosclerosis (1). Early identification of such patients is important as drugs and diet that lower plasma cholesterol may prevent the development of atherosclerosis (2).

Diagnosis of FH is usually suggested on the basis of a cluster of findings such as LDL cholesterol levels higher than 190 mg/dl in association with normal triglycerides, early coronary heart disease, and presence of tendon xanthomas (3). This last sign is not specific as it can be found in other defects such as apolipoprotein B mutations. On the other hand, it is not present in all FH patients (4).

Many techniques have been developed to identify LDL receptor-defective patients. The identification of the specific mutation sites by genetic techniques is considered to be the standard for the diagnosis of FH. However, this method requires the screening of a large number of mutations (5). Thus far, the functional evaluation of LDL receptors in cell culture is recognized as the most sensitive technique for diagnosing FH. When used mainly with fibroblasts, this technique allows a definition of the metabolic step responsible for the defect: synthesis, binding, internalization, lysosomal digestion, or exocytosis (6-8). Evaluation of LDL receptor activity on peripheral blood mononuclear cells (PBMC) is of interest given the easy accessibility and culture processing of these cells (9). However, PBMC contain heterogeneous cell types presenting differences in the expression and the regulation of their LDL receptors. That might explain the great dispersion of LDL receptor activity values among individuals and frequent overlaps in LDL receptor activity between FH and non-FH individuals when studied in total PBMC (10).

We have developed an improved technique for evaluating the activity of the LDL receptor pathway on T

Abbreviations: FH, familial hypercholesterolemia; LDL, low density lipoprotein; PBMC, peripheral blood mononuclear cells; DiI, 3,3'-dioctadecylindocarbocyanine.

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TABLE 1. Plasma lipid profiles and clinical data of subjects (mean  $\pm$  standard deviation)

	Total Cholesterol	HDL Cholesterol	LDL Cholesterol	Triglycerides	Tendon Xanthoma	Coronary Heart Disease
	<i>mg/dl</i>					
Normocholesterolemic subjects (n = 24)	196 $\pm$ 27	50 $\pm$ 16	124 $\pm$ 25	107 $\pm$ 47	0/24	0/24
FH subjects (n = 18)	393 $\pm$ 62	47 $\pm$ 14	317 $\pm$ 60	141 $\pm$ 47	9/18	12/18
Non-FH subjects (n = 10)	328 $\pm$ 40	55 $\pm$ 11	232 $\pm$ 38	200 $\pm$ 68	0/10	0/10

lymphocytes by using LDL labeled with 3,3'-dioc-tadecylindocarbocyanine (DiI).

## MATERIALS AND METHODS

### Subjects

The method was performed on a population of 24 healthy normocholesterolemic volunteers (mean age: 37 years; 28–50 years) and 18 hypercholesterolemic patients with clinical criteria of FH subjects (mean age: 44 years; 16–65 years). Clinical criteria used for FH were: LDL-cholesterol levels higher than 190 mg/dl with normal triglycerides, tendon xanthomas, and (or) coronary heart disease before 50 years in the patient or, at least, in one first-degree or second-degree relative. Another population of 10 patients was selected on the basis of high serum levels of cholesterol not associated with clinical criteria of FH and the absence of high cholesterol levels in their relatives (non-FH subjects) (mean age: 50 years; 29–71 years). Lipid profiles and clinical data are given in Table 1. None of the FH patients were apoB-100-defective as confirmed by genetic screening using polymerase chain reaction (11). All volunteers and patients gave their informed consent. The homozygous FH patients were two 10-year-old male subjects, both proved to be LDL receptor-negative by fibroblast analysis and had serum LDL-cholesterol levels of approximately 600 mg/dl.

### LDL isolation and labeling with DiI

LDL subfraction of d 1.019–1.063 g/ml was obtained from a pool of healthy individuals and prepared by a two-step ultracentrifugation technique using a Ti80 rotor and an L8-80 Beckman ultracentrifuge (12). Briefly, two successive runs were performed at 68000 rpm for, respectively, 16 h and 3 h. Butylated hydroxytoluene (BHT) was present during all steps of preparation in order to prevent lipid peroxidation. Lipoproteins were then dialyzed against a 0.15 M NaCl buffer containing 0.1 g/l EDTA. LDL protein was estimated by the method of Lowry et al. (13) with an albumin standard (Sigma, Bornem, Belgium).

A stock solution of the fluorescent probe DiI (Molecular Probes, Eugene, OR) was prepared by dissolving 15 mg DiI in 1 ml dimethylsulfoxide (DMSO). For LDL labeling, 10  $\mu$ l of the DiI solution was added per mg LDL protein, thus giving a final concentration of 150  $\mu$ g DiI per mg LDL. This mixture was then incubated for 16 h at 37°C (14, 15). A final ratio of 150  $\mu$ g DiI to 1 mg LDL protein was reached and the mixture was then incubated for 16 h at 37°C (14, 15). Labeled LDL were removed by ultracentrifugation and dialyzed against EDTA (0.01%) containing NaCl (150 mM) buffer. The level and quality of DiI incorporation into LDL was assessed by comparing their fluorescence intensities (expressed as fluorescent arbitrary units (A.U.) per mg LDL protein) to commercially available DiI-LDL batches (Molecular Probes, Eugene, OR). DiI-LDL standard curve was obtained after extracting DiI with isopropanol as described by Stephan and Yurachek (15). Measurements of DiI fluorescence were performed on an LS-30 Perkin-Elmer spectrofluorometer set at excitation and emission wavelengths of 520 nm and 578 nm, respectively.

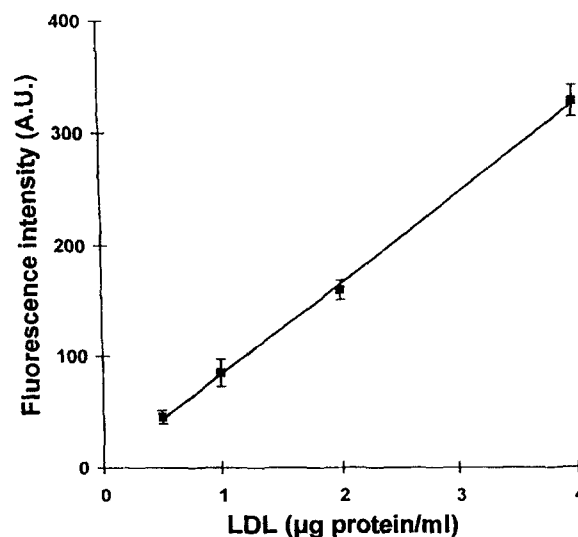
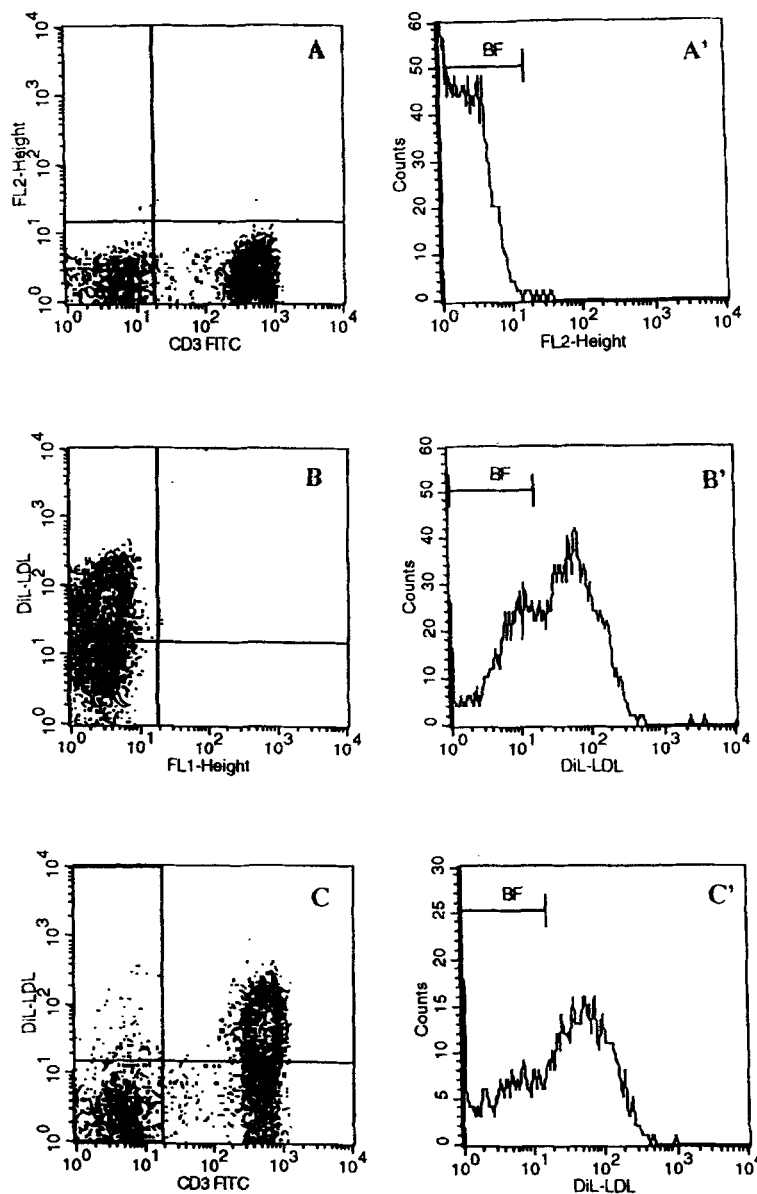


Fig. 1. Representative experiment of LDL amount-dependent fluorescence intensity of DiI-LDL. DiI was extracted with isopropanol and fluorescence was measured on an LS-30 Perkin-Elmer spectrofluorometer set at excitation and emission wavelengths of 520 and 578 nm respectively.

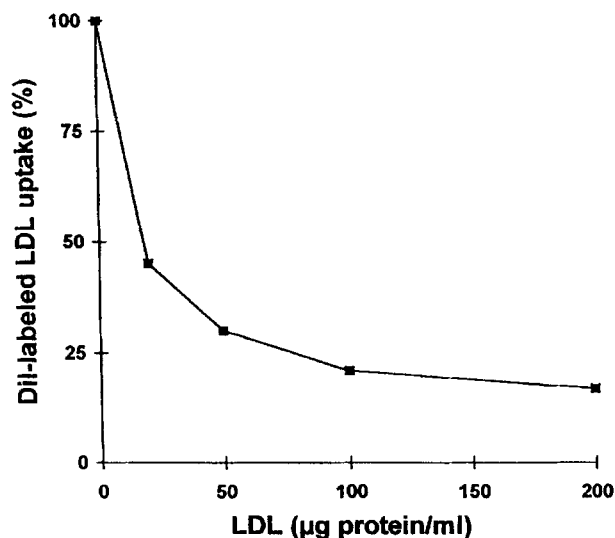
## Culture of human peripheral blood lymphocytes

Mononuclear cells were collected from fasting subjects. Hypercholesterolemic patients were free of hypolipidemic drugs for at least 2 days. Freshly collected human mononuclear cells from EDTA-containing blood were isolated using Lymphoprep gradient (Nycomed) (16). The mononuclear cell layer was then cultured in

25-cm<sup>2</sup> flasks (NUNC) at 37°C for 44 h in glutamine-containing RPMI-1640 medium (Gibco, Merelbeke, Belgium) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% delipidated heat-inactivated fetal calf serum (P.A.A., Eurobiochem, Bierges, Belgium). The phenotyping of cell suspensions at the end of the 44 h incubation showed more than 80% T lymphocytes and less than 10% monocytes.



**Fig. 2.** Representative flow cytometric dot plots (A, B, and C) and histograms (A', B', and C') of lymphocytes from a normocholesterolemic volunteer in different conditions of incubation. Cells ( $5 \times 10^5$ ) were preincubated for 2 days in a lipoprotein-deficient medium. A and A': FITC-labeled anti-CD3 fluorescence of T lymphocytes incubated without exposure to DiL-LDL. B and B': DiL-LDL uptake by lymphocytes without subsequent exposure to FITC-labeled anti-CD3. C and C': Flow cytometric dot plot showing combined DiL-LDL uptake and FITC-labeled anti-CD3 fluorescence. In panels A', B', and C', background fluorescence (BF) means the range of fluorescence of cells incubated with FITC-conjugated anti-CD3 alone.



**Fig. 3.** Representative measurement of the effect of the addition of unlabeled LDL on the uptake of DiI-labeled LDL by normal PBMC. PBMC were cultured for 44 h in the absence of lipoproteins. Thereafter, the cells were labeled with 10 µg DiI-LDL in the presence of increasing concentrations of unlabeled LDL at 37°C for 1 h and then with FITC-labeled anti-CD3 for another 20 min. Results are expressed as percentage of the fluorescence intensity measured without addition of unlabeled LDL and to which a 100% value is assigned.

#### Evaluation of LDL receptor activity in flow cytometry

After 44 h incubation, a volume of DiI-labeled LDL solution equivalent to 10 µg LDL protein was added to the suspensions of PBMC,  $5 \times 10^5$  cells/500 µl medium, and the incubation was continued for an additional hour at 37°C. Then PBMC were washed twice in phosphate-buffered saline (pH 7.2) and cells were incubated for

another 20 min at room temperature with an FITC-conjugated anti-CD3 (Becton Dickinson, Erembodegem, Belgium). For each experiment, at least one control blood was tested simultaneously with the hypercholesterolemic patients.

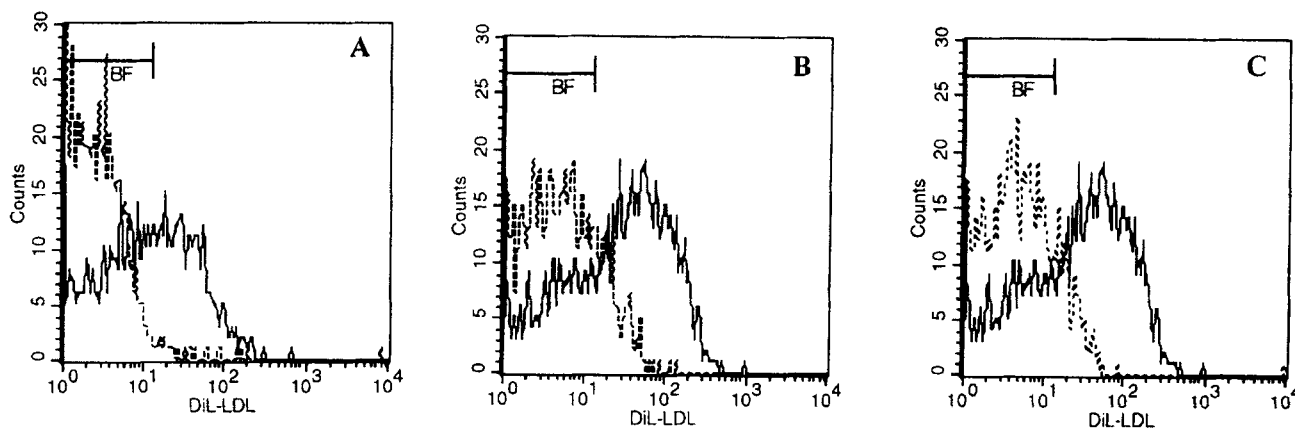
Samples were analyzed on a FACScan (Becton Dickinson, Erembodegem, Belgium) equipped with an argon laser that emitted at 488 nm and allowed simultaneous acquisition of green fluorescence (from FITC) at 515–540 nm and red fluorescence (from DiI) at greater than 620 nm. The software used was the LYSYS II (Becton Dickinson, Erembodegem, Belgium).

The cells were first preanalyzed in order to set the gates that defined the different PBMC populations, including the lymphocyte subset, and to exclude dead cells. A cell acquisition program was then run to accumulate PBMC data until at least 5000 lymphocytes were collected. Thereafter, T lymphocytes were selected according to their CD3 phenotype. CD3<sup>+</sup> lymphocytes were analyzed using logarithmic amplification. For each sample, we estimated the mean fluorescence intensity.

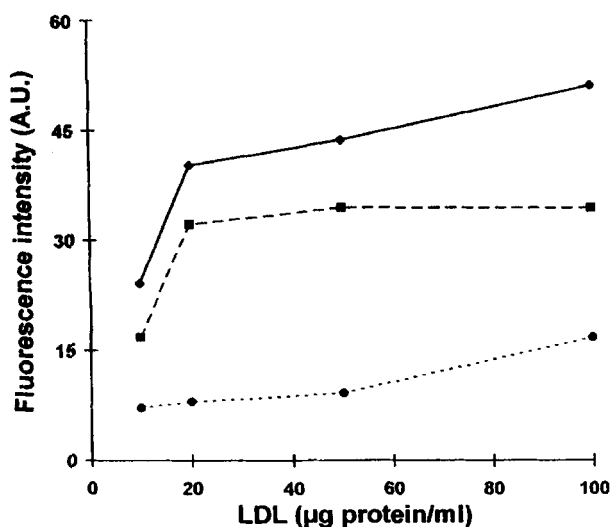
Nonspecific uptake of LDL was assessed either in the presence of increasing concentrations (2-, 5-, 10-, and 20-fold) of unlabeled LDL particles or in the presence of 12.5 mM EDTA. A zero reference was obtained for each sample by measurement of the mean fluorescence values of lymphocytes incubated in presence of 10 µg/ml unlabeled LDL alone.

#### <sup>125</sup>I-labeled LDL degradation

The degradation of <sup>125</sup>I-labeled LDL was assayed by the method of Bilheimer and coworkers (17). Briefly, the cells ( $2 \times 10^6$ /ml) were cultured in lipoprotein-defi-



**Fig. 4.** Representative LDL uptake by T lymphocytes preincubated for 2 days with FITC-conjugated CD3 antibody. Cells ( $5 \times 10^5$ ) were incubated with 10 µg DiI-LDL. After 1 h incubation, cells were washed and reincubated 20 min with FITC-conjugated anti-CD3. Background fluorescence (BF) means the range of fluorescence of cells incubated with FITC-conjugated anti-CD3 alone. Figure 4A shows overlay histograms from a normocholesterolemic volunteer (?) and an FH hypercholesterolemic patient (—). In Fig. 4B, lymphocytes from a normocholesterolemic volunteer were incubated with DiI-LDL alone (?) or in the presence of a 10-fold excess of unlabeled LDL (—). Panel 4C shows DiI-LDL uptake by CD3<sup>+</sup> lymphocytes from a normocholesterolemic subject in the presence (—) or the absence (?) of 12.5 mM EDTA. Results are expressed as fluorescence arbitrary units.



**Fig. 5.** Saturation curve for DiI-LDL uptake by T lymphocytes preincubated 2 days in lipoprotein-deficient medium. Cells ( $5 \times 10^5$ ) in a volume of 500  $\mu$ l medium were incubated at 37°C during 1 h in the presence of increasing concentrations of DiI-LDL alone (—) or together with 10-fold unlabeled LDL (.....). Then the cells were washed and reincubated for 20 min with FITC-labeled anti-CD3. The specific curve (---) was obtained by subtracting nonspecific DiI-LDL uptake. Results are expressed in arbitrary fluorescence values (A.U.).

cient medium under the same conditions as described above. After 72 h incubation, 20  $\mu$ g  $^{125}$ I-labeled LDL (83 cpm/ng LDL protein) were added to the cultures and cells were incubated for another 6 h at 37°C. The cell-free supernatant was treated with 15% trichloroacetic acid and then with KI (0.4%) and hydrogen peroxide (1.2%). After chloroform extraction to remove free iodine, the aqueous phase was counted in an LKB gamma counter. Radioactivity recorded in cpm was converted to ng LDL protein degradation per mg cell protein. Receptor-mediated degradation of  $^{125}$ I-labeled LDL was calculated by subtracting the  $^{125}$ I-LDL degraded in presence of 25-fold excess unlabeled LDL.

#### Statistics

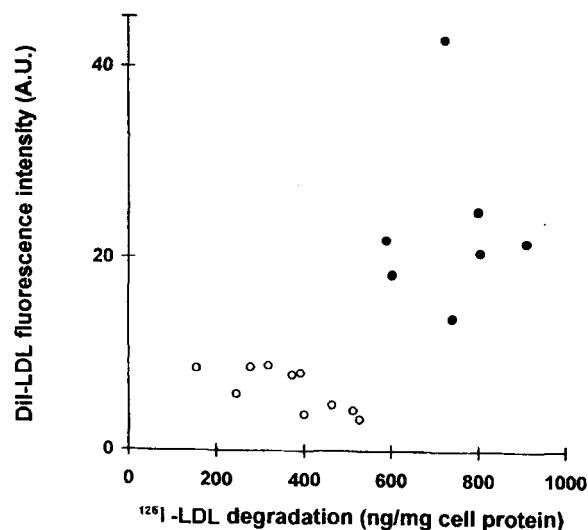
The significance of the differences between values was estimated from Student's *t*-test.

### RESULTS

**Figure 1** shows a linear relation between LDL protein and its fluorescence intensity measured upon isopropanol extraction of our DiI-LDL stock. Labeling efficiency is estimated at 27 ng DiI/ $\mu$ g LDL protein. The results are equivalent to those observed with the commonly used commercial DiI-LDL preparations according to previous articles (14, 15, 18).

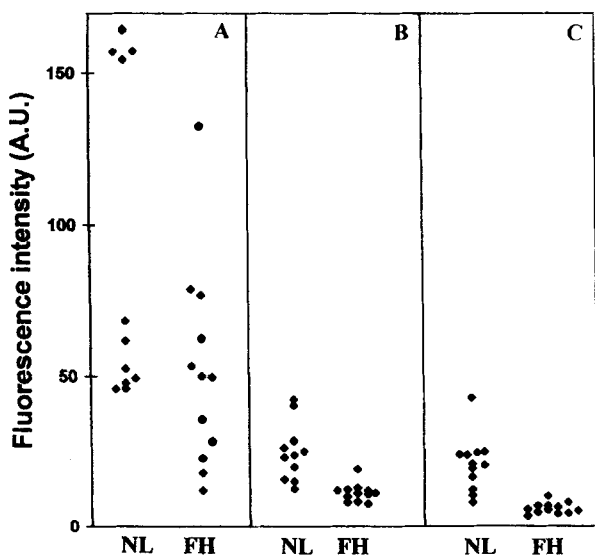
Our method was set up for the measurement of LDL receptor activity using freshly isolated PBMC from normal subjects. All measurements were performed on PBMC incubated 2 days in an LDL-free medium. These conditions are known to increase the expression of LDL receptor on the cell surface. These cells avidly took up DiI-LDL and accumulated DiI in a linear fashion over time. One hour incubation of PBMC with DiI-LDL appeared enough to clearly distinguish DiI fluorescence emission from autofluorescence (data not shown). To discriminate T lymphocytes in the whole PBMC, we used physical criteria (small size and low granularity) and antigenic properties (CD3 antigen). For this purpose, specific FITC-labeled anti-CD3 were added at the end of the incubation with DiI-LDL. **Figure 2** shows FACS dot plots and histograms of CD3 background fluorescence alone (Figs. 2A and 2A'), DiI fluorescence alone (Figs. 2B and 2B') and combined FITC/DiI fluorescence (Figs. 2C and 2C'). It is important to note that the simultaneous use of the second fluorescent molecule did not interfere with the DiI fluorescence estimation.

Results from different experiments performed with the simultaneous use of DiI-LDL and FITC-labeled anti-CD3 demonstrated that the uptake of DiI-LDL showed the same properties as unlabeled LDL. First, human PBMC from a normocholesterolemic volunteer were exposed to increasing concentrations of unlabeled LDL along with a fixed concentration of DiI-LDL (10  $\mu$ g/ml). As shown in **Fig. 3**, there was a clear competition between labeled LDL and unlabeled LDL. At 50% inhi-



**Fig. 6.** DiI-LDL uptake by T lymphocytes preincubated 2 days in lipoprotein-deficient medium from patients identified as normocholesterolemic (●) or FH hypercholesterolemic (○) on the basis of the  $^{125}$ I-labeled LDL degradation by PBMC preincubated 72 h in lipoprotein-deficient medium. DiI-LDL uptake values are expressed in arbitrary fluorescence intensities (A.U.) and  $^{125}$ I-labeled LDL degradation products as ng/mg cell protein.





**Fig. 7.** Specific uptake of DiI-labeled LDL in human PBMC isolated from 12 normocholesterolemic (NL) and 12 heterozygous FH patients (FH), when using the whole mononuclear cell fraction (A), the total lymphocyte population (B), and the T lymphocyte fraction (C) from each patient of the two groups. Values are expressed in arbitrary fluorescence intensities (A.U.).

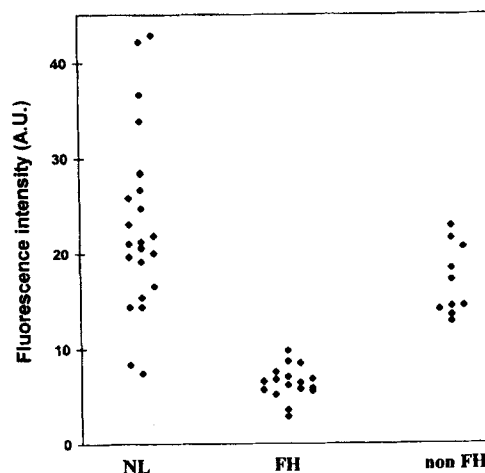
hibition, the concentration of unlabeled LDL was equal to that of the DiI-LDL present in the medium ( $10 \mu\text{g}$  protein/ml). In the presence of a 10-fold excess of unlabeled LDL, cellular DiI fluorescence dropped to background intensity (as can be seen in Fig. 3 and Fig. 4B). Similar results were obtained in three other normocholesterolemic volunteers. Second, experimental data showed that the uptake of DiI-LDL was receptor specific-mediated. Indeed, in five normocholesterolemic volunteers, the presence of the calcium chelator EDTA (representative experiment shown in Fig. 4C) inhibited the uptake of DiI-LDL. Moreover, by performing saturation curves for DiI-LDL uptake by T lymphocytes preincubated 2 days in lipoprotein-deficient culture medium, it could be shown that mean fluorescence intensities increased in relation to the dose of DiI-labeled LDL and reached a plateau value around  $50 \mu\text{g}$  DiI-LDL protein/ml (see Fig. 5). Finally, T lymphocytes collected from the two homozygous FH patients displayed very low mean fluorescence intensities of 3.02 and 3.21, respectively.

We also assessed mean DiI-LDL uptake by flow cytometric analysis in normocholesterolemic patients ( $n = 7$ ) and in patients with heterozygous FH ( $n = 10$ ) and compared the results obtained by the standard technique measuring  $^{125}\text{I}$ -labeled LDL degradation in lymphocyte cultures (17). The two groups of patients were completely discriminated on the basis of either technique used (Fig. 6).

We next measured the LDL receptor activity in 12 normocholesterolemic subjects and in 12 heterozygous FH patients by using the whole PBMC population, the total lymphocyte population, and the T lymphocyte subpopulation (see Fig. 7). When using the whole PBMC population, values obtained in FH hypercholesterolemic patients and normocholesterolemic volunteers overlapped to a large degree ( $51.4 \pm 32.8$  and  $87.9 \pm 57.9$ , respectively). When using the whole population of lymphocytes or the T lymphocytes population, a better discrimination was obtained between normocholesterolemic volunteers and the FH patients, particularly when using T lymphocytes ( $20.8 \pm 8.7$  and  $5.1 \pm 2.0$ ).

From repeated experiments on five healthy subjects (one tested 4 times and the others tested 3 times), we established that the mean coefficient of variation of the method was 16.8%.

Figure 8 exhibits the values of fluorescence intensities measured in T lymphocytes obtained from four groups of patients including normocholesterolemic patients (NL), hypercholesterolemic patients without signs of FH (non FH), and hypercholesterolemic patients with heterozygous FH (FH). In heterozygous FH subjects, mean fluorescence intensity values ( $6.3 \pm 1.7$ ) were significantly lower ( $P < 0.001$ ) than those of normocholesterolemic volunteers ( $23.1 \pm 8.9$ ). Intermediate fluorescence intensity values were recorded in the non-FH hypercholesterolemic patients ( $17.1 \pm 3.5$ ) which overlapped those of the normocholesterolemic group. From these data, we chose a cut-off value of 11.5 for the



**Fig. 8.** Receptor-mediated uptake of DiI-labeled LDL in human peripheral blood T lymphocytes isolated from normocholesterolemic (NL), heterozygous familial hypercholesterolemic (FH), and non-FH patients (non FH). Cells ( $5 \times 10^5$ ) were incubated in a volume of  $500 \mu\text{l}$  medium at  $37^\circ\text{C}$  during 1 h in the presence of  $10 \mu\text{g}$  DiI-LDL, then washed and reincubated for 20 min with FITC-labeled anti-CD3 before FACScan analysis. Values are expressed in arbitrary fluorescence intensities (A.U.).

diagnosis of FH that was the highest mean fluorescence intensity of the FH patients.

Finally, LDL receptor activity was measured in a group of 62 consecutive subjects referred for dyslipidemia, suspicion of FH, or evaluation of an eventual cardiovascular risk. The assay was performed without the knowledge of the clinical diagnosis. Using our discriminating cut-off for flow cytometric analysis, we observed that 8/42 clinically diagnosed FH displayed mean fluorescence intensities above 11.5, and 2/10 normocholesterolemic patients and 1/10 of the non-FH group presented DiI-LDL uptake values below 11.5. When considering only patients with coronary heart disease and xanthoma in the FH group, none had mean fluorescence intensities above 11.5.

## DISCUSSION

Lymphocytes have been frequently used to screen genetic LDL receptor defects amongst hypercholesterolemic patients because these cells are easily obtainable (8).

In the present study, LDL particles were labeled with the DiI probe and the uptake was analyzed by flow cytometry. Flow cytometry and DiI-labeled LDL have already been used to estimate the LDL receptor activity in different subsets of lymphocytes (19, 20) and monocytes (10). However, only monocytes were used as a diagnostic tool for the identification of FH (10). Labeling with DiI seems to preserve LDL specific binding sites of the apolipoprotein B-100 (18, 21). That DiI-labeled LDL are taken up by a receptor-mediated mechanism is also suggested by our study. Indeed, no significant uptake of DiI-labeled LDL was observed in the presence of a calcium chelator (EDTA). The uptake was competitively inhibited in the presence of unlabeled LDL, confirming previous studies (22, 23). Moreover, DiI-LDL appear to behave similarly to  $^{125}\text{I}$ -labeled LDL as shown by our own experiments and previous studies (14, 15). Further, results obtained from saturation curves showed dose-dependent increase in fluorescent LDL uptake reaching a plateau between 20 and 50  $\mu\text{g}$  LDL protein/ml. These data were in accordance with several articles (24, 25). Finally, very low uptake of DiI-LDL was found in two patients who proved to be homozygous FH.

Flow cytometry and the use of two different fluorescent molecules to label LDL and CD3 antibody allow the measurement of DiI-labeled LDL uptake in T cells identified by their CD3 antigen. By this technique, DiI-labeled LDL uptake can be measured on a single cell basis and, therefore, a more selective and a more homogeneous subpopulation can be analyzed with a lower dispersion of the results. As a consequence, a better discrimi-

nation was obtained between FH patients on one hand and normocholesterolemic volunteers and non-FH hypercholesterolemic patients on the other hand. Thus, T cells are more useful than the entire mononuclear cells to detect LDL receptor defects.

From a technical point of view, our method is relatively easy to handle and appears less laborious than radiolabeling techniques. Moreover, our technique is less time-consuming than other described techniques as the incubation time necessary to allow full expression of the LDL receptor is shorter (44 h versus 72 h) (26). Finally, it requires only 1 h incubation in contrast to previous studies with longer incubation times (22, 27). Although our technique showed results similar to those of the standard technique using  $^{125}\text{I}$ -labeled LDL, normocholesterolemic subjects and heterozygous FH patients diagnosed on a clinical basis were not completely discriminated. Several explanations are possible: some FH patients could have been falsely classified by clinical criteria. On the other hand, some discrepancy could be observed when measuring LDL receptor activity in different types of cells, as demonstrated for fibroblasts and hepatocytes (28). Moreover, one cannot exclude that the LDL receptor activity of lymphocytes can be altered by the functional state of these blood cells depending on the degree of immunological stimulation. This could partially explain the fact that some FH patients displayed normal LDL receptor activity. A previous study using an indirect immunocytofluorimetric assay of LDL binding on lymphocytes showed that approximately 25% of clinically diagnosed FH patients had LDL receptor activity values falling in the normal range (29).

In conclusion, our technique based on the DiI-LDL uptake in T lymphocytes leads to some improvement in the detection of LDL receptor activity defects. ■

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