Quantification of low density lipoprotein binding and cholesterol accumulation by single human fibroblasts using fluorescence microscopy

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Summary Using fluorescence microscopy, we have quantified low density lipoprotein (LDL) binding by indirect immunofluorescence and cellular cholesterol with the fluorescent sterol-binding polyene, filipin, in individual cultured human fibroblasts from normal subjects and from patients with heterozygous and homozygous familial hypercholesterolemia. LDL binding by fibroblasts from heterozygous patients was about 40% of that of the normal cells, and cholesterol accumulation upon incubation with LDL was decreased to a similar degree. Most fibroblasts from homozygous patients bound no detectable LDL and only rare cells demonstrated any accumulation of cholesterol after incubation with LDL.—**Kruth, H. S., and M. Vaughan.** Quantification of low density lipoprotein binding and cholesterol accumulation by single human fibroblasts using fluorescence microscopy. *J. Lipid Res.* 1980 **21:** 123–130.

Supplementary key words indirect immunofluorescence · familial hypercholesterolemia · filipin

Single cell fluorescence analysis is rapidly gaining importance as a sensitive analytical technique in basic research and clinical medicine (1). Such methodology has been used to quantify, for example, DNA, RNA, enzymes, amines and protein in single cells (2–6). The inherent sensitivity of quantitative fluorescence microscopy allows biochemical assays to be carried out in single cells, thus obviating the need for large numbers of cells required in most other biochemical assays. Cell-to-cell variation can be easily assessed, and simultaneous localization and quantification of biochemicals in individual cells is possible.

We have adapted an incident light fluorescence microscope to permit the measurement of fluorescence in single cells. Using this microscope fluorometer, we have quantified low density lipoprotein (LDL) binding and cholesterol in single cultured fibroblasts from a normal individual and from patients with heterozygous and homozygous familial hypercholesterolemia.

METHODS

The normal human fibroblasts derived from newborn foreskin were the same line used in other studies in this laboratory (7). Fibroblasts from patients homozygous (GM 701, 2000, 488, and 1915) or heterozygous (GM 700, 376, and 483) for familial hypercholesterolemia were obtained from the Institute for Medical Research, Camden, NJ. All cells were grown at 37°C in 75 cm^2 polystyrene flasks (Corning Glass Works, Corning, NY) sealed after gassing for 30 sec with a mixture of 95% air, 5% CO_2 and fed weekly with Eagle's minimal essential medium (MEM) supplemented with 10% (vol/vol) fetal calf serum (FCS) (North American Biologics, Inc., Miami, FL). Cultures were split 1:3 within 10 days before use. For experiments, a flask of cells was washed twice with 10 ml of Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) at 37°C, then incubated for 5–10 min at 37°C with 1 ml of DPBS containing 0.125% trypsin. Tryspinization was terminated by the addition of 10 ml of MEM with 10% FCS (37°C). After counting a sample of suspended cells in a Neubauer chamber, the suspension was diluted with MEM containing 10% FCS to a concentration of 5000 cells per 3 ml of medium. Samples (3 ml) of diluted cell suspension were transferred to 35-mm wells of culture trays (Falcon #3004, Oxnard, CA). Each well contained a 22 mm^2 glass coverslip heated (dry) at 180°C for 2 hr. Cultures were incubated at 37°C in humidified 95% air, 5% CO_2 for 24 hr or 48 hr, then washed twice with 2 ml of MEM (37°C), and further incubated with 3 ml MEM for 2–8 days before use in experiments.

LDL (d 1.019–1.063 g/ml) was prepared from human plasma by standard techniques (8, 9), then dialyzed for 48 hr against four changes of 41 of 0.85% saline containing 0.01% EDTA at 4°C, and filtered (sterile 0.45 μm Millipore) before storing at 4°C. Protein content was determined by the method of Lowry et al. (10).

For measurement of LDL binding, coverslips with attached cells were transferred to new 35-mm wells, washed three times with 2 ml of MEM (37°C) and incubated in 2 ml of MEM containing 75 μg/ml of LDL protein for 2 hr at 37°C in an atmosphere of 95% air, 5% CO_2. Thereafter dishes were kept on ice, and chilled solutions were used. Coverslips were washed three times with 2 ml of 0.15 M NaCl 50 mM Tris-chloride, pH 7.4, bovine albumin, 2 mg/ml (11), and incubated twice for 10 min with 3 ml of the same

Abbreviations: LDL, low density lipoproteins; MEM, Eagle's minimal essential medium; FCS, fetal calf serum; DPBS, Dulbecco's phosphate-buffered saline.

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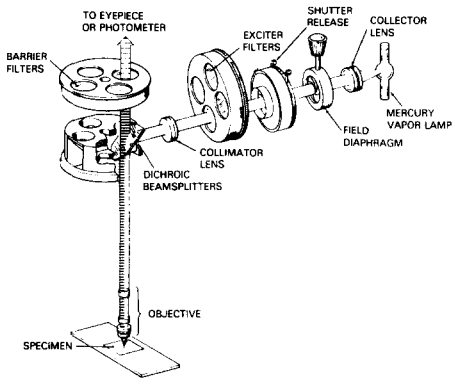

Fig. 1. Light path through the microscope fluorometer. Excitation radiation from the mercury vapor lamp passes through a collector lens, field diaphragm, mechanical shutter, exciter filter, and collimator lens before being reflected downward by the dichroic beamsplitter through the microscope objective to the specimen. Emitted fluorescence passes upward through the objective and, being of a longer wavelength than the exciting radiation, is transmitted (rather than reflected) by the dichroic beamsplitter through a barrier filter either to the eyepiece or to the photometer (modified and adapted from AO vertical illuminator reference manual with permission of American Optical Corporation, Buffalo, NY).

solution, followed by three washes with 2 ml of DPBS. They were incubated for 45 min in 1 ml of DPBS containing 64 μg (protein) of rabbit antiserum to human LDL (Miles Laboratories, Inc., Elkhart, IN), washed as outlined above, and incubated in the dark for 45 min in 1 ml of DPBS containing 30 μl of fluorescein isothiocyanate-labeled goat antibody against rabbit IgG (Miles Laboratories, Elkhart, IN). After washing three times with 2 ml of DPBS, cells were fixed in 10% phosphate-buffered formalin (pH 7.4) containing 0.1 M sucrose for 10 min, and washed twice with 5 ml of DPBS. Coverslips were mounted inverted on microscope slides in DPBS and sealed with nail polish. This procedure is very similar to that used by Brown, Ho, and Goldstein (11) to visualize bound LDL on normal fibroblasts and cells homozygous for familial hypercholesterolemia.

Cholesterol content was determined after filipin staining (12) of cells grown on coverslips and incubated at 37°C in 2 ml of MEM with or without 75 μg/ml of LDL protein for 8 to 36 hr. Coverslips were washed with 2 ml DPBS three times at room temperature, placed in 3 ml of 10% phosphate-buffered formalin (pH 7.4) containing 0.1 M sucrose and stored at 4°C until stained. For staining, coverslips were washed twice with 5 ml of DPBS and incubated at room temperature in 1 ml of DPBS containing 0.05% Triton X-100 for 5 min, washed twice in 5 ml DPBS for 5 min at room temperature, and incubated at 37°C for 2 hr in 2 ml of 0.1 M potassium phosphate buffer (pH 7.4) containing, where indicated, 400 μg of cholesterol esterase or 40 μg of cholesterol oxidase (Boehringer Mannheim, Indianapolis, IN). Coverslips were then washed twice in 5 ml of DPBS, stained for 30 min with 1 ml of DPBS containing 20 μl of filipin stock solution (2.5 mg filipin in 1 ml of dimethylformamide), and washed twice in 5 ml of DPBS for 5 min at room temperature. Coverslips were mounted in DPBS with the cells facing a microscope slide and sealed with nail polish.

An incident light fluorescence microscope (Model 2070, American Optical, Buffalo, NY) was modified essentially as described by Enerbäck and Johansson (13) to function as a microscope fluorometer. Excitation light was provided by either an Osram HBO 50 W mercury vapor lamp and power supply (Model 2054, American Optical, Buffalo, NY) or a 12 V tungsten halogen lamp and power supply (Model 2051, American Optical, Buffalo, NY). No voltage stabilizer was used. A mechanical camera shutter (Prontor II, Prontor-Work, Calmbach, Federal Republic of Germany) equipped with a flash synchronization tap placed in the path of the excitation light limited the duration of exposure of the specimen to light, thus minimizing fading of fluorescence. Routine exposure was $\frac{1}{25}$ sec. An FITC excitation filter was used in the LDL assay and a BG 12 excitation filter for the cholesterol assay (filters from American Optical). For both assays, 500-nm dichroic and 515-nm barrier filters were used. A photomultiplier with microphotometer system (Model 10-280, American Instrument Co., Inc., Silver Spring, MD) was positioned above a vertical tube (#1044, American Optical, Buffalo, NY) through which the specimen fluorescence passed.

To isolate a single cell for fluorescence measurement, the field was limited by placing an aperture disc in one eyepiece of the viewing binocular. This allowed simultaneous visualization of both the cell to be measured and the surrounding field. An identical aperture was placed in the vertical tube, restricting the emitted fluorescence seen by the photometer to the same field selected with the viewing binocular. Selection of cells and focussing were carried out with phase-contrast using a 45 X phase objective (N A = 0.66) and low intensity tungsten illumination with green filtration. This light was turned off and the same objective was used during measurement of fluorescence. To initiate measurements, the mechanical shutter was released. At complete opening of the shutter, a peak-detector circuit was activated via the synchronization tap of the shutter, causing the highest value detected by the photometer to be stored in a memory circuit and then displayed on a digital voltmeter (Model 7050 Multimeter, Fairchild, San Jose, CA). The principal components of the microscope are shown in **Fig. 1,** and the major elements of the fluores-

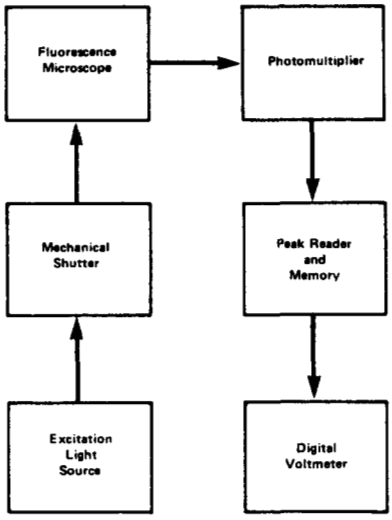

Fig. 2. Flow diagram of microscope fluorometer. Triggering the mechanical shutter allows passage of excitation light through the microscope to the specimen. Emitted fluorescence passes to the photomultiplier, and the peak fluorescence value is stored before display on a digital voltmeter.

cence excitation and quantification process are depicted schematically in **Fig. 2.**

Ektachrome 200 daylight color slide film (Eastman Kodak Co., Rochester, NY), which was "push-processed" to an ASA of 400, was used for fluorescence and phase photography. Where comparisons are made between experimental conditions at the same magnification, the exposure times were the same so that the relative fluorescence intensities are comparable.

RESULTS

Table 1 summarizes data from representative experiments on LDL binding by normal and heterozygous or homozygous fibroblasts. As fluorescence measurements were not standardized from one experiment to another, values for mean relative fluorescence are only comparable within each experiment. Values for cells not exposed to LDL were the same as those for cells that had been incubated with LDL but were treated with non-immune serum rather than anti-LDL antiserum before addition of the second antibody. For normal fibroblasts these values were usually ≦50% of those from cells exposed to LDL, followed by anti-LDL and second antibody in the complete standard procedure. The mean relative fluorescence of homozygous fibroblasts was little altered by the substitution of non-immune serum for anti-LDL antiserum.

LDL binding by heterozygous cells was intermediate between that of the normal and homozygous cells. To permit comparison of data from several experiments, LDL binding by heterozygous cells was expressed as a percentage of the difference between the values for normal and homozygous cells determined in the same experiment (sample calculation in legend for Table 1). As shown in **Table 2,** with fibroblasts GM 700 and GM 483, LDL binding was about 40% of normal with relatively little variation from one experiment to another. With line GM 376, for reasons that we have been unable to determine, binding was considerably more variable, although the mean (30.8 ± 8.2, n = 9) was not significantly different from that for the other heterozygous cells. Values for LDL binding at 37°C and at 0°C were similar (Table 2).

Cholesterol accumulation as a result of incubation of cells with LDL was quantified as the difference between the mean relative fluorescence of cells in replicate cultures incubated with and without LDL.

TABLE 1. LDL binding by normal fibroblasts and fibroblasts homozygous or heterozygous for familial hypercholesterolemia

Exp. No.	Cell Line	Mean Relative Fluorescence		
		Complete	No Anti-LDL	No LDL
3	Normal	1.28 ± 0.04	0.57 ± 0.01	0.60 ± 0.01
	GM 2000	0.62 ± 0.01		
	GM 376	0.89 ± 0.01		
4	Normal	1.49 ± 0.10	0.69 ± 0.01	0.71 ± 0.02
	GM 1915	0.82 ± 0.02		
	GM 483	1.16 ± 0.03		
12	Normal	1.46 ± 0.05	0.82 ± 0.01	
	GM 1915	0.94 ± 0.02	0.81 ± 0.02	
13	Normal	1.99 ± 0.08		
	GM 1915	1.05 ± 0.02	0.89 ± 0.02	
	GM 376	1.39 ± 0.04		

Cells (GM 2000 and 1915 homozygous, GM 376 and 483 heterozygous) were grown and prepared for quantitative fluorescence microscopy to assess LDL binding as described in Methods ("Complete"). Where indicated, replicate cultures were incubated without LDL before addition of anti-LDL antiserum followed by the second antibody or, after incubation with LDL, were exposed to non-immune serum followed by the second antibody. Relative fluorescence of each cell (selected using phase microscopy as described in Methods) was measured three times in succession and was highly reproducible. In each culture, the mean relative fluorescence of at least 25 cells was determined. The means of these values ± S.E. are reported. Fluorescence measurements were not standardized from experiment to experiment which accounts in large part for the differences in values in different experiments. (In three other successive experiments in which the microscope fluorometer settings were unchanged, mean relative fluorescence values of 1.5, 1.5, and 1.6 were obtained for normal fibroblasts.) To compare the LDL binding of heterozygous cells in different experiments (summarized in Table 2), the value for these cells was expressed as a percentage of that of the normal cells after subtracting from each the value for the homozygous cells in the same experiment. For example, in Exp. 3, LDL binding by GM 376 cells was $0.89 - 0.62/1.28 - 0.62 \times 100 = 41\%$ of normal by this calculation.

TABLE 2. LDL binding and cholesterol accumulation by fibroblasts heterozygous for familial hypercholesterolemia

Cells GM 700		Cells GM 483		Cells GM 376	
LDL	C	LDL	C	LDL	C
% normal		*% normal*		*% normal*	
29	57	33	33	5	30
33	36	38		15	11
41		40	34	37	44
44		40	45	41	68
50	48	51	21	46	68
58				61	
29[a]		36[a]		0[a]	
52[a]		41[a]		7[a]	
				65[a]	
Mean 42.0	47.0	39.9	33.2	30.8	44.2
SEM 3.9	6.1	2.1	4.9	8.2	11.0

[a] LDL binding carried out at 0°C. These values are included in the means.

Data from 12 experiments are presented. In each experiment, cells from one or more heterozygotes, from one or more homozygotes and from the normal fibroblast line were studied. In some experiments, LDL binding (LDL) and cholesterol accumulation (C) resulting from incubation with LDL were assessed using replicate subcultures; in others, only LDL binding was determined. Values for LDL binding and cholesterol accumulation by the heterozygous cells were calculated as described in the legends for Tables 1 and 3, respectively.

Data from seven experiments are shown in **Table 3.** There were no consistent differences between the mean relative fluorescence of normal, heterozygous, and homozygous cells incubated without LDL (Table 3). It will be noted that the variability in fluorescence of cells in the same culture stained for total cholesterol with filipin (Table 3) was considerably greater than the variability in LDL binding (Table 1). Nevertheless, there were clearly significant differences in accumulation of cholesterol by normal and heterozygous cells in the same experiment (Table 3). In most experiments, incubation with LDL produced no significant increase in relative fluorescence of the homozygous cells (Table 3). As shown in Table 2, cholesterol accumulation by heterozygous cells (calculated as described in the legend for Table 3) was about 40% that by normal cells. Because cholesterol accumulation by cells GM 376 (like LDL binding) seemed to be more variable from one experiment to another than it was with other heterozygous cells, we wonder whether they may represent a trait that differs in some way from the others.

LDL immunofluorescence was distributed in a punctate fashion over the entire surface of the normal fibroblasts (**Fig. 3b**), often apparently in linear arrays when viewed at higher magnification (e.g., Fig. 3h). There was, however, considerable cell-to-cell variability with regard to the amount of LDL bound. When normal nonimmune rabbit serum was substituted for immune (i.e., anti-LDL) rabbit serum or when incubation with LDL was omitted, the fibroblasts exhibited no fluorescence. (These negative controls which were repeated many times are not shown.) The great majority of cells in cultures of heterozygous fibroblasts bound less LDL than did the normal fibroblasts, but occasional cells appeared to have bound as much

TABLE 3. Cholesterol accumulation by normal fibroblasts and fibroblasts heterozygous or homozygous for familial hypercholesterolemia

Exp. No.	Control Cells			GM 700			GM 483			GM 376		
	−LDL	+LDL	Δ	−LDL	+LDL	Δ	−LDL	+LDL	Δ	−LDL	+LDL	Δ
1	0.27 ± 0.03	2.04 ± 0.16	1.77	0.20 ± 0.01	1.11 ± 0.08	0.91						
3	0.48 ± 0.06	3.12 ± 0.40	2.64							0.62 ± 0.05	2.49 ± 0.23	1.87
4	0.12 ± 0.01	2.13 ± 0.20	2.01				0.17 ± 0.02	0.71 ± 0.07	0.54			
5	0.19 ± 0.02	2.26 ± 0.26	2.07							0.28 ± 0.02	1.78 ± 0.13	1.50
10	0.22 ± 0.02	3.79 ± 0.26	3.57	0.30 ± 0.03	1.67 ± 0.13	1.37	0.35 ± 0.02	1.62 ± 0.11	1.27	0.23 ± 0.01	1.89 ± 0.15	1.66
11	0.14 ± 0.02	1.81 ± 0.26	1.67		1.10 ± 0.10		0.20 ± 0.02	0.83 ± 0.08	0.63	0.08 ± 0.01	0.36 ± 0.05	0.28
12	0.16 ± 0.01	1.67 ± 0.20	1.51	0.62 ± 0.07	1.51 ± 0.12	0.89	0.27 ± 0.03	0.98 ± 0.10	0.71	0.25 ± 0.02	0.75 ± 0.10	0.50

Cells (GM 700, 483, and 376 heterozygous, GM 2000, 1915, 701, and 488 homozygous) were grown, incubated with or without LDL, and stained for total cholesterol with filipin as described in Methods. In each culture, the mean relative fluorescence of at least 25 cells was determined. The means ± S.E. of these values are reported along with the difference (Δ) between the means for cells incubated with and without LDL. To compare cholesterol accumulation by heterozygous cells incubated with LDL in different experiments (summarized

LDL as an average normal cell (data not shown). In homozygous cultures, only rare fibroblasts had bound significant LDL (Fig. 3f, j); many had bound small amounts but most cells had no detectable bound LDL (Fig. 3d).

Normal fibroblasts incubated with LDL and stained with filipin following exposure to cholesterol esterase contained numerous fluorescent inclusions (**Fig. 4c, d**), although the number varied from cell to cell. Fewer fluorescent inclusions were seen in heterozygous cells after incubation with LDL (Fig. 4e, f). With rare exceptions (e.g., Fig. 4h) no fluorescent inclusions were found in the homozygous fibroblasts (Fig. 4g–j). Normal fibroblasts (Fig. 4a, b) and heterozygous or homozygous cells (not shown) incubated without LDL contained essentially no fluorescent inclusions.

In cultures of normal fibroblasts incubated with LDL and stained for free cholesterol (i.e., not incubated with cholesterol esterase before staining with filipin), there were cells with both fluorescent and nonfluorescent inclusions (**Fig. 5a, b**), only nonfluorescent inclusions (Fig. 5c, d), or inclusions that were essentially all fluorescent (Fig. 5e, f). When such cultures were stained for both free and esterified cholesterol (i.e., incubated with cholesterol esterase before filipin staining), all inclusions were fluorescent (Fig. 5g, h). Incubation, on the other hand, with cholesterol oxidase[2] before staining with filipin markedly reduced the fluorescence of inclusions in all cells (not shown). Fibroblasts not incubated with LDL contained no fluorescent or nonfluorescent inclusions.

[2] Oxidized cholesterol does not bind filipin (12).

DISCUSSION

The frequency of the heterozygous state of familial hypercholesterolemia is approximately 1 in 500 (14), making this genetic defect one of the more common in man. As persons with familial hypercholesterolemia are prone to premature atherosclerosis (15), detection of this genetic trait can be of clinical importance. We have used quantitative fluorescence microscopy to compare LDL binding and cholesterol accumulation on incubation with LDL by normal fibroblasts and fibroblasts from patients with heterozygous or homozygous familial hypercholesterolemia. Homozygous cells exhibited little or no LDL binding, whereas with cells of three heterozygous lines, LDL binding was quite reproducibly about 40% that of normal cells. This is similar to the heterozygote LDL binding of 40% of normal reported by Goldstein, Brown, and Stone (16) and Brown and Goldstein (17), who used radiolabeled LDL to measure binding. In our studies, the cells from four homozygous lines accumulated little or no cholesterol on incubation with LDL. Brown, Faust, and Goldstein (18) likewise found no significant increase in the chemically determined cholesterol content of homozygous cells incubated with LDL and provided evidence that the accumulation of cholesterol is dependent on the LDL receptor. Our ob-

GM 2000			GM 1915			GM 701			GM 488		
−LDL	+LDL	Δ	−LDL	+LDL	Δ	−LDL	+LDL	Δ	−LDL	+LDL	Δ
						0.17 ± 0.02	0.30 ± 0.03	0.13	0.13 ± 0.01	0.20 ± 0.02	0.07
0.27 ± 0.03	0.50 ± 0.04	0.23									
			0.22 ± 0.03	0.36 ± 0.04	0.14						
0.30 ± 0.02	0.59 ± 0.05	0.29									
									0.25 ± 0.01	0.41 ± 0.05	0.16
									0.16 ± 0.02	0.25 ± 0.02	0.09
			0.47 ± 0.06	0.53 ± 0.04	0.06						

in Table 2), the increment in fluorescence of these cells caused by incubation with LDL was expressed as a percentage of the increment in the normal cells after subtracting from each the increment observed in the homozygous cells in the same experiment. For example, in Exp. 3, cholesterol accumulation resulting from incubation with LDL by GM 376 cells was $1.87 - 0.23/2.64 - 0.23 \times 100 = 68\%$ of normal by this calculation.

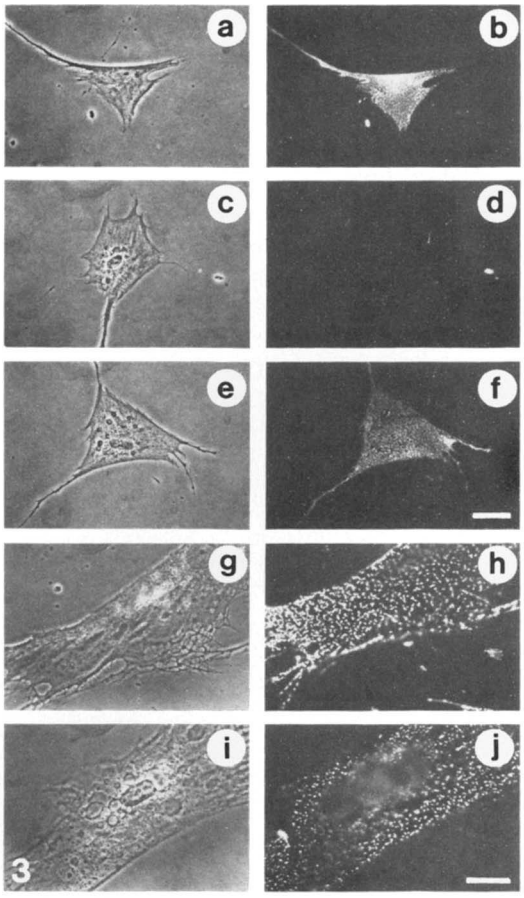

Fig. 3. LDL binding by normal and homozygous fibroblasts. Cells (5000 per well) were incubated in 3 ml of MEM with 10% FCS at 37°C for 24 hr, washed three times with 2 ml of MEM and incubated in 3 ml of MEM at 37°C for 48 hr. LDL binding (30 min, 37°C) and preparation for microscopy were then carried out as described in Methods. Phase (a, c, e, g, i) and fluorescence (b, d, f, h, j) micrographs of matching fields are shown. Bar in f, 40 μm, applies to a–f (×126). Bar in j, 20 μm, applies to g–j (×280). Normal cells (a, b, g, h); homozygous (GM 701) cells (c–f, i, j).

servation that the accumulation of cholesterol in heterozygous cells exposed to LDL was roughly 40% of normal is consistent with that view.

Although Goldstein and Brown (19) have postulated that homozygous receptor negative cells lack receptors for LDL, inspection of cultures of receptor negative homozygous fibroblasts with the fluorescence microscope revealed that some of the cells did bind LDL. This was not seen in all experiments, and in the homozygous cultures we never found cells that had accumulated the amounts of cholesterol seen in cells of heterozygous or normal cultures after incubation with LDL. The meaning of the observation that some receptor negative homozygous cells apparently bind LDL is unclear.

Examination of individual cells in normal cultures incubated with LDL and then stained for free or total (free plus esterified) cholesterol, revealed cells with predominantly free cholesterol-containing inclusions or essentially only cholesteryl ester-containing inclusions and cells with both types of inclusions. Thus, the chemical state of the cholesterol accumulated as well as the total amount and the extent of LDL binding differed considerably in individual cells in the same culture. Such differences are obscured when cholesterol or LDL binding determinations are made on whole cultures and only average values per cell can be ascertained. The possibility that at least some of these cell-to-cell differences are related to the cell cycle could be evaluated with studies of synchronized cultures, or by correlating DNA content with LDL binding and cholesterol in single cells.

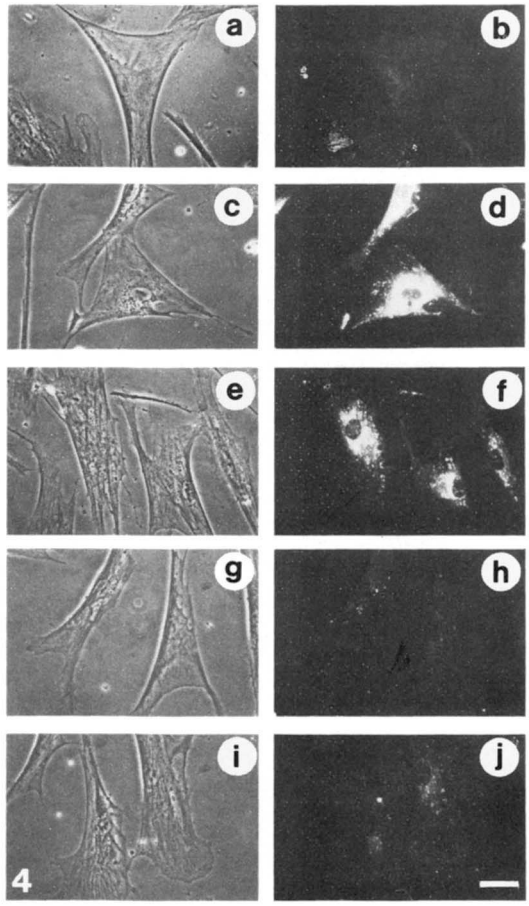

Fig. 4. Cholesterol accumulation in normal, heterozygous, and homozygous fibroblasts incubated with LDL. Cells (25,000 per well) were incubated in 3 ml of MEM with 10% FCS at 37°C for 24 hr, washed three times with 2 ml of MEM and incubated in 3 ml of MEM at 37°C for 48 hr. Cells were then incubated at 37°C for 24 hr in 2 ml of MEM (a, b) or MEM containing LDL, 75 μg/ml, (c–j), fixed and stained for total cholesterol with filipin as described in Methods. Phase (a, c, e, g, i) and fluorescence (b, d, f, h, j) micrographs of matching fields are shown. Bar in j, 20 μm, applies to all (×126). Normal cells incubated without (a, b) or with (c, d) LDL; heterozygous (GM 700) cells (e, f) and homozygous (GM 701, GM 488) cells (g, h, i, j) incubated with LDL.

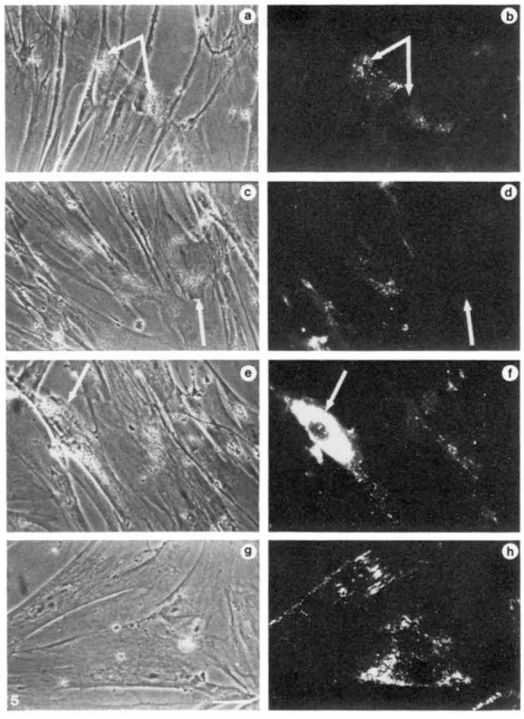

Fig. 5. Accumulation of free or total cholesterol in normal fibroblasts incubated with LDL. Cells (40,000 per well) were incubated in 3 ml of MEM with 10% FCS at 37°C for 24 hr, washed three times with 2 ml of MEM, and incubated in 3 ml of MEM at 37°C for 96 hr. Cells were then incubated at 37°C for 24 hr in 2 ml of MEM (not shown) or MEM containing LDL, 75 μg/ml, fixed and stained with filipin for free cholesterol (a–f) or free plus esterified cholesterol (g, h). Phase (a, c, e, g) and fluorescence (b, d, f, h) micrographs of matching fields are shown. Bar in g, 40 μm, applies to all (×300). Arrows indicate: (a, b) cell with both fluorescent and nonfluorescent inclusions, (c, d) cell with only nonfluorescent inclusions, and (e, f) cell with essentially all fluorescent inclusions.

Immunofluorescence techniques are used routinely in many clinical laboratories, and the reagents for immunofluorescence assays are, in general, more stable, less expensive, and safer than radiolabeled materials. The sensitivity of fluorescence methods permits measurements to be made on individual cells. The availability of flow microfluorometers with cell sorting capability (20), in which single cell fluorescence from up to 5000 cells per sec can be measured, makes feasible the analysis of large numbers of cells, which might reveal the presence of distinct subpopulations of cells in regard to LDL binding or cholesterol accumulation which could be separated for further investigation. Peripheral blood lymphocytes, which, like the cultured fibroblasts, express the receptor defect in familial hypercholesterolemia (21), are easily suspended in solution and could be readily studied using a flow microfluorometer. For analysis of cells such as fibroblasts which cannot conveniently be suspended for flow microfluorometry without trypsinization, which abolishes LDL binding (22), the microscope fluorometer can be utilized. Although we have adapted a standard laboratory fluorescence microscope for quantifying single cell fluorescence, other microscope fluorometers are commercially available.

Quantification of LDL binding in this way is advantageous, because, in addition, localization and specificity of binding may be assessed by inspection of cells being measured. As shown here, single cell fluorescence analysis provides an excellent means to differentiate normal cells from those with deficiencies in LDL binding and cholesterol accumulation. It should be valuable in clinical diagnosis and research related to hypercholesterolemia, as well as being more widely applicable for investigation of cellular binding of ligands other than LDL.

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