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Surface properties of LDL-binding lymphocytes in human peripheral blood

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Summary. Surface properties of low density lipoprotein (LDL)-binding lymphocytes were evaluated to determine whether LDL binds with a subpopulation of human peripheral blood lymphocytes (PBL). B- and T-cell rich fractions were prepared from PBL using E-rosette formation or nylon reticulum columns. Binding of FITC-labelled LDL with these cell fractions was determined with a fluorescent microscope and a fluorescence-activated cell sorter (FACS II). The specificity of the binding was evaluated by a dose-dependent inhibition of LDL binding with the addition of unlabelled lipoproteins. In parallel studies, surface properties including E-rosette formation, surface immunoglobulins, and receptors for IgG-Fc, as well as human and mouse C3 were examined. LDL binding lymphocytes were enriched in the B-cell rich fraction, and depleted in the T-cell rich fraction. In addition, FITC-LDL binding lymphocytes were selectively collected by the FACS II. These LDL binding cells restored surface immunoglobulins after incubation in serum-free medium following trypsinization. The majority of lymphocytes stimulated by PHA and PWM *in vitro* bound with LDL. It is concluded that LDL binds with B cells in fresh human PBL, while it binds with B and T cells in mitogen-stimulated lymphocytes. It is suggested that the selective collection of LDL binding lymphocytes by the FACS II can be applied to the evaluation of cellular interaction of these cells in various immunological reactions.

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INTRODUCTION

It has been demonstrated that lymphocytes have a low density lipoprotein (LDL) receptor and the LDL receptor controls intracellular cholesterol metabolism by regulating 3-hydroxy-3-methylglutaryl coenzyme A reductase activity (Ho, Brown, Kayden & Goldstein, 1976a; Ho, Brown, Bilheimer & Goldstein, 1976b). It has also been reported that LDL affects some immunological functions of T and B lymphocytes (Hagmann, Wiler & Waelti, 1979; Curtis & Edgington, 1979), but it remains obscure whether these different effects can be provoked via the LDL receptor. In this study, we analysed the surface properties of LDL binding lymphocytes in human peripheral blood. The results obtained indicated that LDL binds with B cells in fresh blood lymphocytes, and also binds with B and T cells in mitogen-stimulated lymphocytes.

MATERIALS AND METHODS

Cell separation

One hundred millilitres of heparinized venous blood was drawn from each subject after overnight fasting. Mononuclear cells were separated by Böyum's

method (1968), and adherent cells were removed by incubation in plastic petri dishes at 37° for 45 min. Peripheral blood lymphocytes (PBL) were pelleted with sheep erythrocytes, and after 60 min, the rosetting T cells were separated from the non-rosetting B-cell fraction on a second density gradient. The rosette forming T cells in the pellet were resuspended, and the erythrocytes were lysed with ammonium chloride buffer (Boyle, 1968). An aliquot of PBL was applied on nylon reticulum columns as described elsewhere (Daniels, Sakai, Cobb, Remmers, Sarles, Fish, Levin & Ritzmann, 1970). Cells eluted from the columns were also regarded as a T-cell rich fraction.

Surface markers

Surface immunoglobulin-positive cells were identified and enumerated by immunofluorescence by using fluorescein-conjugated $F(ab')_2$ fragments of goat anti-human IgG, IgA, and IgM (heavy chain specific, F/P ratio 2·9, Cappel Laboratories, Cochranville, Pa, Lot No. 10884). These antisera were diluted 1 : 10 with phosphate-buffered saline (pH 7·2) containing 2% bovine serum albumin (Sigma, St Louis, Mo, Lot No. 32C-0830), and centrifuged at 105,000 *g* for 30 min before use. The monospecificity of these antisera was examined by immunoelectrophoresis using normal human serum and purified human IgG, IgA and IgM (prepared in our laboratory from myeloma patients). E-rosette forming cells were determined by the method described by the International Union of Immunological Societies (IUIS, 1975). Receptors for ox erythrocytes (OE) conjugated with rabbit IgG antibodies (IgG-OE) were evaluated by OE coated with IgG fraction of anti-bovine red blood cell antibodies (Cappel Laboratories, Lot No. 6784). Receptors for human and mouse C3 were examined by the method described by Winchester & Ross (1976). To assure that rosettes represented binding to C3 receptors rather than to the IgM Fc receptors, cells to be assayed were pre-incubated in RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.) containing 1 mg/ml of purified human IgM.

Cell cultures

Aliquots of the cell suspension were cultured with mitogens. 2×10^5 cells were cultured in quadruplicate with or without 10 μg/ml of phytohaemagglutinin P (PHA) (GIBCO) or 10 μl/ml of pokeweed mitogen (PWM) (GIBCO) in 0·2 ml of 10% lipoprotein-deficient decomplemented normal human AB serum (prepared in our laboratory) in RPMI 1640 with humidified 5% CO_2–air at 37° for 3 days. LDL binding of the cultured cells was examined on the third day of the culture as described below. Transformation of lymphocytes was assayed by incorporation of 0·5 μCi tritiated thymidine (1·9 Ci/mM, New England Nuclear, Boston, Mass.) for 6 h. The cells were harvested by a semi-automated sample harvester (Microtiter, Dynatech, Billingshurst, England). The radioactivity in the cells was measured by a liquid scintillation spectrophotometer (Model 2425, Packard Corp, Downers Grove, Ill.)

Separation of lipoproteins

Human LDL (density 1·019–1·063) was obtained from serum of a healthy adult after overnight fasting. LDL was isolated by a differential ultracentrifugation (Hitachi, 65P-7, Tokyo) (Hatch & Lees, 1968). The purity of the separated LDL was examined by agarose gel electrophoresis and analytical ultracentrifugation. Trace amounts (less than 0·1 mg/ml) of albumin and IgG were detected in the LDL fractions by immunoelectrophoresis. However, trace amounts (less than 0·1 mg/ml) of fluorescein-conjugated human albumin and IgG (prepared in our laboratory) were incubated with normal human blood lymphocytes at 4° for 30 min, but there were no fluorescence-positive cells observed under an incident-type fluorescent microscope (Zeiss, Model 9901) using oil immersion. LDL was dialysed extensively at 4° with a solution containing 150 mM NaCl, and 0·5 mM EDTA-Na_2 overnight. Protein contents were 7 mg/dl as quantified by Lowry's method (1951). Cholesterol and triglyceride concentrations in this LDL were 163 mg/dl and 16 mg/dl, respectively. Very low density lipoprotein (VLDL, density 1·006–1·019) and high density lipoprotein (HDL, density 1·063–1·21) were also prepared by the same methods.

Labelling of LDL

Labelling of LDL with fluorescein isothiocyanate (FITC) (Sigma, St Louis, Mo, Lot No. 104C-5026) was performed using Marshall's method modified by Kawamura (1977). Free dyes were removed by dialysis for 48 h followed by column chromatography using Sephadex G-25 (Sigma, Lot No. 53C1030). The F/P ratio of the labelled LDL was 3·8.

Identification of LDL-binding lymphocytes

Lymphocytes were incubated at 4° for 30 min with FITC-labelled LDL in phosphate-buffered saline (pH 7·2) containing 2% bovine serum albumin. Cells were

washed three times, and LDL-binding cells were determined by an incident-type fluorescent microscope using oil immersion. Monocytes were identified by pre-incubation and phagocytosis of latex beads (0·81 μm in diameter, Difco, Detroit, Mich. Lot No. 608400). More than 250 cells were counted and the percentage of LDL-binding cells was calculated.

Analysis of LDL-binding lymphocytes by a fluorescence-activated cell sorter

LDL-binding lymphocytes were analysed by a fluorescence-activated cell sorter (FACS II, Becton Dickinson Electronics Lab, Mountain View, Ca). Lymphocytes were stained with FITC-labelled LDL, and were suspended at a concentration of 8×10^6 cells/ml in PBS(pH 7·2) containing 2% bovine serum albumin. The pressure of the sample input was 0·9 kg/cm^2. The cells were illuminated by a laser in 495 nm at 400 mV. The approximate rate of the cell flow was 1000 cell/s. The size of the cells was determined, and lymphocytes were gated for the subsequent analysis of fluorescence. Both monocytes and platelets were excluded electronically. The percentage of esterase-positive cells in the lymphocyte fraction gated by the FACS II was less than 1%.

Sorting of LDL-binding lymphocytes by the FACS II

LDL-binding lymphocytes were selectively sorted by the FACS II. The sorted cells were treated with 2·5 mg/ml trypsin (Serarac Lab. Berks, Lot No. 598) in PBS at 37° for 10 min. The cells were then washed three times with PBS and incubated in RPMI 1640 (GIBCO) at 37° for 4 h in humidified 5% CO_2–air. Cell-surface LDL was completely removed by this treatment as evaluated by fluorescent microscopy. Neither surface immunoglobulin-bearing cells nor E-rosette forming cells were recognized immediately after the trypsinization. The percentages of immunoglobulin-bearing cells restored in the sorted cells (LDL-positive cells, i.e. bright cells), and LDL-negative cells (i.e. dark cells) were determined by the immunofluorescent technique described above.

RESULTS

Surface markers on separated PBL

The results (Table 1) indicate that (a) the T-cell fraction separated by E-rosette formation contained less that 1% of cells bearing surface immunoglobulins (surface Ig) and C3 receptors, while that separated by a nylon reticulum column contained 2–4% of surface Ig-bearing cells and C3 receptor-bearing cells, (b) the B-cell fraction was substantially enriched for cells with surface Ig, receptors for IgG-OE and C3; and (c) the LDL-binding cells were enriched in the B-cell fraction, but they were markedly decreased in the T-cell fraction separated by a nylon reticulum column and completely absent in the T-cell fraction separated by E-rosette formation.

Identification of LDL-binding lymphocytes

Figure 1 shows an LDL-binding lymphocyte. LDL-binding lymphocytes were illuminated granually with FITC-LDL surrounding the cell surface and differentiated clearly from the dark cells which do not possess cell-surface LDL.

Table 1. Evaluation of surface markers on separated fractions of human peripheral blood lymphocytes

	E rosettes (%)	Surface Ig$^+$ (%)	IgG-OE receptor$^+$ (%)	C3 receptor$^+$* (%)	LDL-binding cells (%)
PBL	59·6 ± 6·1	14·4 ± 2·3	17·9 ± 2·2	8·0 ± 1·6	14·9 ± 3·7
T-cell fraction					
Rosette†	98·5 ± 1·2	0·2 ± 0·03	6·4 ± 1·3	0·2 ± 0·01	0
Nylon‡	91·1 ± 1·5	1·9 ± 0·2	6·0 ± 0·4	3·7 ± 0·2	0·6 ± 0·1
B-cell fraction	1·7 ± 0·6	65·4 ± 3·2	49·0 ± 5·8	21·8 ± 1·9	29·6 ± 4·2

Each figure represents the mean and standard error determined from single estimates on PBL from eight healthy individuals.

* Results from human and mouse complement were comparable.

† T-cell rich fraction separated by E-rosette formation followed by Ficoll–Hypaque and lysis of erythrocytes.

‡ T-cell rich fraction separated by nylon reticulum columns.

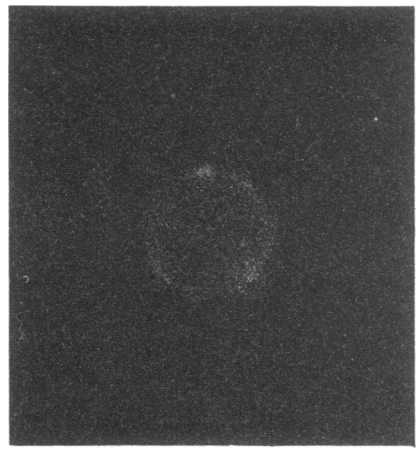

Figure 1. LDL-binding human peripheral blood small lymphocyte observed by fluorescent microscopy. 1000 × (phase-contrast oil immersion).

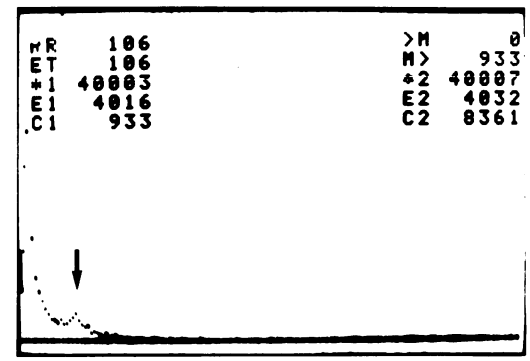

Figure 3. Fluorescence pattern of LDL-binding lymphocytes analysed by the FACS II. The ordinate indicates the amount of cells and the abscissa depicts the intensity of fluorescence. The arrow points to the single peak showing a population of LDL-binding lymphocytes. (See Materials and Methods for techniques used for the analysis by the FACS-II.)

Specificity of LDL binding on lymphocytes

As shown in Fig 2, binding of FITC-LDL on PBL was inhibited by addition of unlabelled LDL and VLDL, while HDL did not show such dose-dependent inhibition.

Fluorescence pattern of LDL-binding lymphocytes analysed by the FACS II

The fluorescence pattern of the LDL-binding lymphocyte analysed by the FACS II is shown in Fig. 3. LDL binding lymphocytes showed a single peak, indicating that the intensity of the fluorescence from these LDL-binding lymphocytes was within relatively narrow ranges. The cell count in this peak was 19·68 ± 1·38% of the total lymphocyte counts.

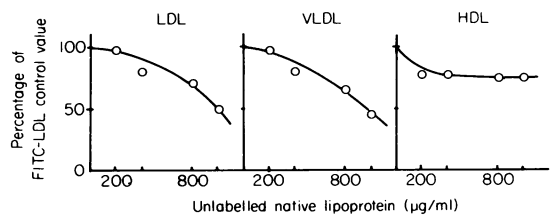

Figure 2. Inhibition of FITC-LDL-binding on PBL by unlabelled native lipoproteins. The ordinate indicates the percentage of FITC-LDL-binding lymphocytes in PBL. The percentage of LDL-binding lymphocytes without incubation with unlabelled native lipoproteins was adjusted to 100%, and those after incubation with various amounts of unlabelled native lipoproteins (abscissa) (left: LDL, centre: VLDL, right: HDL) are shown by circles. Each value represents a mean value from single estimates of PBL from three healthy individuals.

Surface markers on LDL-binding lymphocytes

FITC-LDL binding lymphocytes were selectively sorted by the FACS II, and surface markers of these LDL-binding lymphocytes (bright cells) and lymphocytes not bearing LDL (dark cells) were examined. The results (Table 2) indicate that (a) the bright cells were enriched for cells with surface Ig, while the dark cells did not contain these immunoglobulin-bearing cells; and (b) receptors for sheep red blood cells were not restored in either bright or dark cells after trypsinization followed by 4 h of incubation in serum-free culture medium.

LDL-binding cells in mitogen-stimulated cells

Table 3 shows that (a) there was a slight but significant ($P < 0.05$) increase in the percentage of LDL-binding cells in cells cultured in medium containing lipid-free serum without addition of mitogens; (b) there were prominent increases in the proportion of LDL binding cells in both PHA-stimulated and PWM-stimulated cultures; and (c) the percentage of LDL-binding cells and the degree of [^{3}H]-thymidine uptake were greater in cultures with PHA than in those with PWM.

Table 2. Surface markers of LDL binding lymphocytes

	IgG-bearing (%)	IgA-bearing (%)	IgM-bearing (%)	E rosette (%)
PBL	5·38 ± 1·06	1·47 ± 0·86	4·33 ± 1·18	59·9 ± 10·5
Bright cells	43·3 ± 10·2	10·4 ± 13·4	44·4 ± 13·4	0
Dark cells	< 0·1	< 0·1	< 0·1	0

Each figure represents the mean and standard error determined from five separate sortings by the FACS II using PBL from five healthy individuals.

Table 3. LDL-binding cells in mitogen-stimulated cells

	LDL-binding cells	c.p.m./culture
PBL	13·6 ± 3·8	Not done
Cultured cells without mitogen	18·2 ± 3·9	2·342 ± 608
PHA-stimulated cells	84·5 ± 6·7	96·192 ± 5·457
PWM-stimulated cells	52·9 ± 4·9	48·980 ± 4·621

Each figure represents the mean and standard error determined from single estimates on PBL from four healthy individuals.

DISCUSSION

In the present study our aim was to define B- and T-cell characteristics of LDL binding cells in human peripheral blood lymphocytes. Results from the study on surface markers on separated PBL indicated that LDL-binding lymphocytes were enriched in the B-cell fraction. LDL-binding cells were not observed in the T-cell fraction separated by E-rosette formation, and markedly diminished in that separated by nylon reticulum columns. The reason for employing two different methods for T-cell separation was to exclude the possibility that either E-rosette formation or filtration through nylon reticulum columns might damage the surface properties of T cells and thus might prevent binding with LDL. Although such a possibility could not be ruled out completely, the results from subsequent studies supported the proposition that LDL binding cells in fresh human PBL have B-cell characteristics. The B-cell fraction obtained in this study contained 4·5 times as many cells with surface Ig as PBL, while LDL binding cells were enriched only two-fold. The reason for this discrepancy in the enrichment is unknown.

It was easy to recognize FITC-LDL binding lymphocytes under a fluorescent microscope. The specificity of that binding was evaluated by inhibition of LDL-binding cells by the addition of unlabelled lipoproteins. Both LDL and VLDL showed dose-dependent inhibition on LDL binding of lymphocytes presumably due to the presence of apoprotein B in these lipoproteins. HDL did not show dose-dependent inhibition. A slight but significant inhibition of LDL binding by a small amount of HDL might be due to some non-specific cross-reactions between lipoproteins. The recognition of FITC-LDL binding lymphocytes by the FACS II made it feasible to separate these cells selectively. LDL-binding cells (bright cells) consisted of IgG, IgA and IgM-bearing lymphocytes. The proportion of IgG, IgA and IgM-bearing cells in the trypsinized bright cells was approximately identical to that in fresh PBL, indicating a lack of class bias of surface immunoglobulins in the LDL-binding lymphocytes. Although dark cells might represent T cells, this was not proven because receptors for sheep red blood cells were not restored during 4 h of incubation following the trypsinization. The increase in the percentage of LDL-binding cells during culture in medium containing lipoprotein-deficient serum was compatible with the observation of Ho and his associates (1976b). Since more than 80% of cells from PHA-stimulated cultures bound with LDL, it is indicated that T cells also bound with LDL under such conditions. A similar emergence of binding sites on both B and T cells after *in vitro* stimulation with mitogens have been observed for insulin (Helderman & Strom, 1978). However, our

results indicate that LDL binds exclusively to B cells in fresh PBL. Since the detection of LDL binding cells using fluorescence is less sensitive than that using isotopes, it is possible that small amounts of LDL might bind to T cells. However, the recognition and sorting of FITC-LDL-binding cells from fresh human PBL makes it feasible to elucidate the immunological functions of LDL binding B lymphocytes. The biological significance of the localization of LDL receptors on fresh PBL is unknown. Cholesterol as well as phospholipids are important materials for the formation and maintenance of membranes. Lymphocytes obtain cholesterol from serum LDL because these cells do not synthesize enough cholesterol to support their membranes (Ho, Smith, Brown & Goldstein, 1978). B cells are rich in microvillae and have larger surface areas than T cells. It is speculated that B cells in fresh PBL require more LDL receptors than T cells in order to obtain more cholesterol.

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