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Cholesterol regulates the cell surface expression of glycophospholipid-anchored CD14 antigen on human monocytes

Mojtaba Esfahani [a,b], Robert D. Bigler [b], John L. Alfieri [a], Sissel Lund-Katz [c], Jonathan D. Baum [a] and Louis Scerbo [a]

[a] *Department of Biological Chemistry, Hahnemann University School of Medicine, Philadelphia, PA (USA),* [b] *Department of Neoplastic Diseases, Hahnemann University School of Medicine, Philadelphia, PA (USA) and* [c] *Department of Biochemistry, Medical College of Pennsylvania, Philadelphia, PA (USA)*

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The CD14 antigen which is expressed on human monocytes and macrophages is a phosphatidylinositol-linked surface protein. We investigated the effects of cellular cholesterol depletion and repletion on cell surface expression of this glycoprotein. Adherent normal human monocytes were cultured for four days in media containing delipidated fetal calf serum which depleted cellular cholesterol. Immunofluorescence analysis demonstrated a markedly diminished surface expression of CD14 on cells cultured in delipidated serum compared to normal serum. Expression of CD64 (high-affinity Fc receptors, FcγRI) also was reduced under these conditions. This inhibition of CD14 expression was overcome by addition to the culture medium of cholesterol, low density lipoprotein, or very low density lipoprotein. All of these supplements replenished cellular cholesterol. Expression of CD64(FcγRI) was not restored by addition of cholesterol. These observations indicate that cholesterol can regulate the surface expression of some phosphatidylinositol-anchored glycoproteins.

Introduction

The structure and function of any biological membrane is determined by the dynamics of its components. Cholesterol is one of the major components of the mammalian plasma membrane [1]. In Chinese hamster ovary cells, rat hepatocytes and fibroblasts, 80–94% of the total free cholesterol has been located in the plasma membrane [2]. Cholesterol interdigitates between pairs of phospholipid acyl chains increasing the fluidity when they are in the rigid state and increases the order when the molecules flex and twist in the liquid-crystalline state [3–5]. Cholesterol also has been reported to modulate other components of membrane dynamics, such as acetylcholine receptor activity [6], pinocytosis [7], phorbol-ester-induced adhesion [8], cellular morphology [9], vertical displacement of membrane proteins [10] and some membrane-bound enzymes [11]. This sterol also controls the clustering of the glycophosphatidylinositol-linked (GPI-linked) plasma membrane receptors for 5-methyltetrahydrofolate into uncoated pits (caveolae) of MA104 cells [12]. This effect indicates that interaction between the sterol and acyl chains of the anchor phospholipid directs lateral diffusion of the glycoprotein. The studies reported here show that the surface expression of a second GPI-linked glycoprotein, the CD14 antigen of human monocyte/macrophages, is also regulated by cholesterol. Thus, our studies add an additional dimension to the control of the dynamics of GPI-linked glycoproteins of mammalian plasma membrane by this sterol.

CD14 is a 55-kDa glycoprotein [13]. The gene encoding this antigen maps on chromosome 5 in a region containing genes for a number of growth factors and receptors [14] and has been cloned [14,15]. Its chromosomal location, as well as its frequent deletion in certain myeloid leukemias have prompted the suggestion that CD14 may represent a new receptor important for myeloid differentiation [14]. More recently, CD14 has been implicated in Gram-negative endotoxin-induced shock syndrome [16]. It has been proposed that lipopolysaccharide (LPS) molecules derived

Correspondence to: M. Esfahani, Department of Biological Chemistry, Hahnemann University, Mail Stop No. 411, Broad and Vine, Philadelphia, PA 19102-1192, USA.

Abbreviations: FCS, fetal calf serum; DFCS, delipidated fetal calf serum; FcγRI, high-affinity Fc receptors; VLDL, very low density lipoprotein; GPI-linked, glycosphingolipid-linked; S.E., standard error of mean; PBS, phosphate-buffered saline; MFI, mean fluorescence intensity; BSA, bovine serum albumin; LPS, lipopolysaccharide; LBP, lipopolysaccharide binding protein.

from Gram-negative bacteria bind to a plasma glycoprotein called lipopolysaccharide-binding protein (LBP) and are then presented to macrophages through binding to CD14 [16]. LBP also opsonizes LPS-bearing particles such as Gram-negative bacteria or LPS-coated erythrocytes [17]. The LPS-LBP complexes bind to CD14 eliciting the rapid synthesis and release of tumor necrosis factor-α (TNF-α). Excess secretion of TNF-α is an important factor in endotoxic shock, a frequent fatal complication of Gram-negative bacteria infection [18]. CD14 also plays a role in the clearance of Gram-negative pathogens [16]. Thus, this antigen represents an important plasma membrane receptor whose dynamics could be regulated by membrane lipids, such as cholesterol, which may play a role in the endotoxic shock syndrome.

Materials and Methods

Reagents. Human interferon-γ in PBS (IFN-γ 10^6 U/mg of protein, adjusted to a total protein concentration of 2–4 mg/ml with human serum albumin), BSA, vitamin E and cholesterol were products of Sigma (St. Louis, MO). Heat-inactivated FCS and RPMI 1640 with L-glutamine were obtained from Gibco (Grand Island, NY). Delipidated FCS (DFCS) was prepared as described [17]. Ficoll-Paque was obtained from Pharmacia (Piscataway, NJ). All other chemicals were reagent grade and obtained from various commercial sources.

Immunological reagents. Anti-CD64 (32.2) against the high affinity Fc receptor (FcγRI) [18] and anti-CDw32 (IV.3) against the low avidity Fc receptors (FcRγRII) [19] were gifts from Dr. Paul M. Guyre of Dartmouth Medical School. Other antibodies: anti-CD2 (Leu 5b), anti-CD19 (Leu 16) and anti-CD14 (Leu M3) were products of Becton Dickinson (San Jose, CA). Fluorescein-conjugated goat $F(ab')_2$ anti-murine IgG was obtained from Tago (Burlingame, CA).

Isolation of human monocytes. The procedure of Passwell et al. [20] with modification was used for isolation of human monocytes. Approx. 60 ml of heparinized blood from a healthy individual was separated by Ficoll-Paque density gradient centrifugation. The mononuclear cells were washed, resuspended in RPMI containing 10% FCS and aliquots of 4–5 ml were transferred to 50 ml culture flasks (for flow cytometry studies) or 20 ml to 250 ml flasks (for cholesterol analysis). After 1 h of incubation at 37°C in a CO_2 incubator, the non-adherent cells were discarded and the adherent cells were washed two or three times with PBS. 5 ml of the indicated growth media (see below) were added to monolayers in 50 ml flasks and 20 ml were added to those in 250 ml flasks. The flasks were incubated for four days at 37°C in a 5% CO_2 incubator.

Growth media. Basal growth media consisted of RPMI 1640 with L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/ml), streptomycin (78 U/ml), IFN-γ (500 U/ml), and vitamin E (100 μM). Neither IFN-γ nor vitamin E had any significant effects on CD14 antigen expression. Vitamin E was added to prevent lipid oxidation [21] and IFN-γ was added to augment FcRI expression [22]. This medium was supplemented with 10% heat-inactivated FCS, 10% DFCS, or 10% DFCS supplemented with 10.6 μg/ml of cholesterol added as an ethanol/BSA mixture [7]. The concentration of ethanol never exceeded 0.3%. Where indicated, the medium containing DFCS was supplemented with LDL or VLDL, as described below.

Preparation of lipoproteins. LDL ($1.019 < d < 1.063$ g/ml) and very low density lipoprotein (VLDL; $d < 1.006$ g/ml) were isolated from plasma of healthy male donors by sequential floatation in KBr [23]. *N*-Ethylmaleimide(5 mM) was added to plasma. The purity of lipoprotein samples was assessed by agarose gel electrophoresis and Sudan black staining [24]. The lipoprotein solutions in KBr containing 0.24 mM EDTA to inhibit oxidation were stored under nitrogen at 4°C for less than two weeks [25]. To prevent oxidation during dialysis, the lipoproteins were dialyzed against RPMI 1640 which contains 1 mg/l reduced glutathione. Prior to use, the lipoprotein fractions were dialyzed once or twice at 4°C against 200 volumes of RPMI 1640. They were then passed through a 0.45 μ filter prior to protein determination. Lipoproteins were then mixed with growth medium containing 10% DFCS. This medium was sterilized by filtration through a 0.45-μ filter and stored at 4°C. Protein was determined according to a modified Lowry procedure [26].

Immunofluorescence and flow cytometry. Antibody labeling and flow cytometry were carried out as described previously [27], except that the adherent cells were harvested with a rubber policeman and nonspecific binding of antibody was blocked by adding 50 μl of 50% human serum in PBS followed by incubation at 4°C for 20 min. Due to the cell damage that can occur with the processing of adherent cells in these studies, viability was determined in 11 of the 12 experiments and was found to be $72 \pm 4\%$ by Trypan blue exclusion. Additionally, the flow cytometer was gated on populations having light scatter characteristics of viable cells. Values for the percentage of reactive cells and fluorescence intensity are expressed as mean $\pm$S.E. unless otherwise noted.

Lipid extraction and cholesterol analysis. Cells were harvested with a rubber policeman and washed twice with PBS at 4°C. Those cells that were incubated with cholesterol were harvested and resuspended in 3 ml of PBS, 3 ml of petroleum ether was added and the mixture was gently agitated for 1 min to remove noncellular cholesterol. The organic layer was discarded,

TABLE I

Free cholesterol content of human monocytes cultured in supplemented media

Lipoprotein supplementation was 0.2 mg protein/ml and cholesterol supplementation was 10.6 μg/ml. For further details, please see Materials and Methods.

Growth medium	Cholesterol [a] (μg/mg protein)
10% FCS	18 ± 2
10% DFCS	8 ± 4
10% DFCS + VLDL	34 ± 1
10% DFCS + LDL	32 ± 3
10% DFCS + cholesterol	82 ± 2

[a] Mean ± mean deviation.

and cells were harvested and washed twice with PBS. Lipids were extracted and cholesterol was quantitated by gas-liquid chromatography using cholestane as internal standard [27,28]. All monocyte cultures that were used for lipid analysis were monitored for purity by staining with anti-CD2 as a T-cell marker, anti-CD19 as a B-cell marker, and anti-CDw32 (IV.3) as a monocyte marker. At least 84% of the cells in each flask were IV.3-reactive.

Results

Altered expression of CD14 on cells upon cholesterol deprivation and supplemention

Initial experiments were performed to study the effects of cellular cholesterol depletion on the expression of CD14. Incubation of monocytes for four days in medium in which FCS was replaced by DFCS depleted cellular cholesterol by 56% (Table I). The effect that this cholesterol depletion had on the expression of CD14 was monitored by indirect immunofluorescence and flow cytometry using LeuM3 (Fig. 1). Expression of CD14 on these adherent cells decreased from 93 ± 1% (mean ± mean deviation of duplicates) in normal FCS-containing medium to 31 ± 6% in DFCS-containing medium (Fig. 2A). This decrease in the fraction of cells expressing CD14 was parallel to a decrease in the average number of molecules per cell, as indicated by a reduction in the mean fluorescence intensity (MFI) from 883 ± 17 to 172 ± 18 (Fig. 2B).

When cholesterol was added to the DFCS-containing medium at a concentration of 10.6 μg/ml, cellular cholesterol content increased to 82 ± 2 μg/mg protein (Table I). This increase in cellular cholesterol almost completely restored the percentage of cells expressing CD14 (Fig. 3A). This restoration of cholesterol also increased the expression of CD14 as indicated by a 2.6-fold increase in MFI for cells cultured in DFCS alone (252 ± 8) compared to cholesterol-supplemented

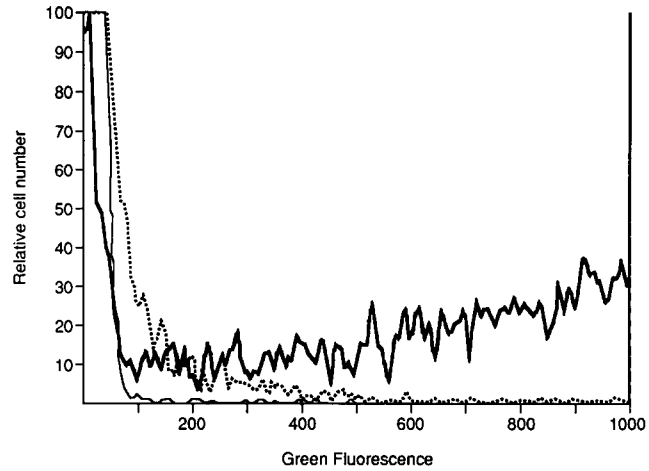

Fig. 1. Fluorescence histograms of cells labeled with mAb LeuM3. Cells were incubated for four days in the growth medium containing fetal calf serum (FCS), or delipidated fetal calf serum (DFCS), and then stained for LeuM3 binding by indirect immunofluorescence. (———), Negative control; (▬▬), fetal calf serum; (· · · · · ·), delipidated fetal calf serum.

medium (660 ± 42) (Fig. 3B). This increase, however, did not reach the normal level of 895 ± 55 noted for cells cultured in FCS medium.

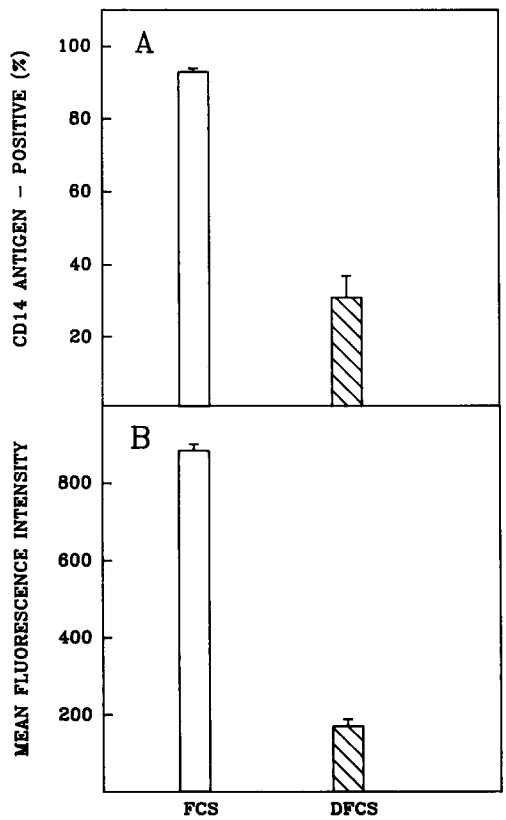

Fig. 2. Effect of incubation of monocytes in medium containing DFCS on the expression of CD14. Monocytes were incubated for four days in the growth medium containing FCS or DFCS and then stained for LeuM3 binding by indirect immunofluorescence. Monocytes from two donors were used. Results from two experiments are presented. Two replicates were analyzed. Mean ± mean deviation.

Restoration of antigen expression by LDL and VLDL

Due to the ability of LDL and VLDL to donate cholesterol to human monocytes (see Table I and Ref. 29), the ability of these two lipoproteins to regulate the expression of CD14 was tested. Supplementation of DFCS medium with 0.02 or 0.2 mg LDL protein per ml fully restored expression of CD14 both in terms of percentage of $CD14^+$ cells (Fig. 4A) and MFI (Fig. 4B). The effects of supplementation of the same medium with VLDL on expression of CD14 also were investigated. As with LDL, supplementation of DFCS media with either 0.02 or 0.2 mg VLDL protein per ml fully resorted the expression of CD14 on cells incubated in this medium (Fig. 5A,B). VLDL is taken up by monocyte/macrophages through the classical LDL receptor pathway [30] and its ability to restore CD14 expression is most likely related to its ability to supply cholesterol to these cells. The values of $CD14^+$ cells shown in Figs. 4A (56 ± 6) and 5A (57 ± 10) are somewhat higher than those seen in Fig. 2A due to the use of different donors and different experiments. The MFI values, however, were quite similar.

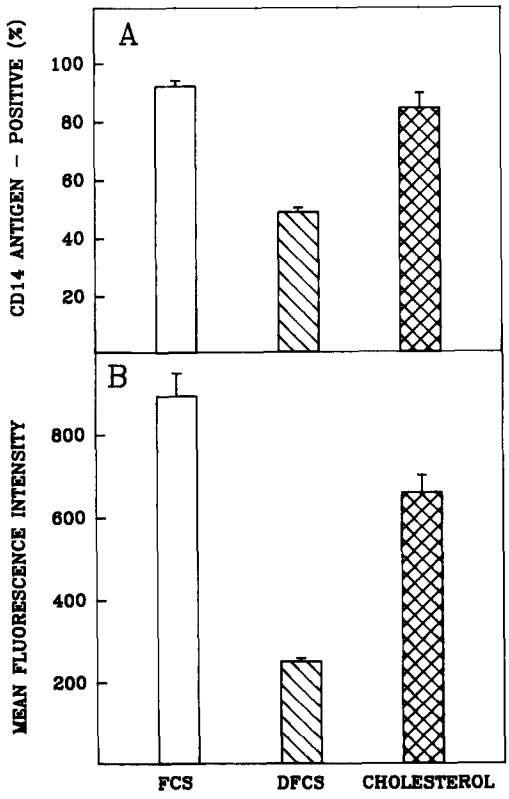

Fig. 3. Effect of cholesterol supplementation of DFCS medium on CD14 expression. Monocytes were incubated for four days in growth medium containing FCS, DFCS, or DFCS plus cholesterol (10.6 μg/ml) and then stained for LeuM3 binding by indirect immunofluorescence. Monocytes from three donors were used. Results of three experiments are presented. Three replicates were analyzed. Mean ± S.E.

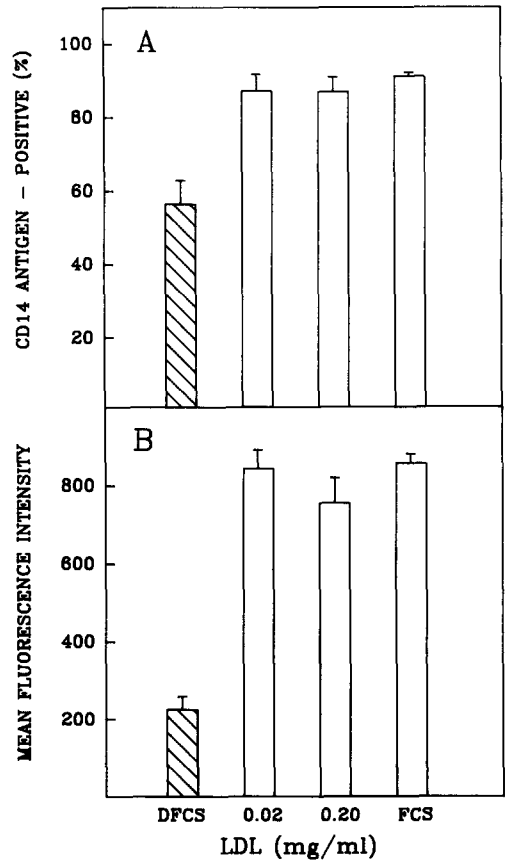

Fig. 4. Effect of LDL supplementation of DFCS medium on CD14 expression. Monocytes were incubated for four days in growth medium containing FCS, DFCS, or DFCS plus LDL and then stained for LeuM3 binding by indirect immunofluorescence. Monocytes from four donors were used. Results of seven experiments are presented. Seven replicates were analyzed. Mean ± S.E.

Failure of cholesterol to restore expression of high affinity Fc receptors (FcγRI)

To demonstrate that the cholesterol effect on CD14 expression was not a general membrane phenomenon, expression of CD64 (FcγRI) was measured. Cells from the same cultures used to measure CD14 expression were used to measure FcγRI expression in media containing FCS, DFCS, or DFCS supplemented with cholesterol (Fig. 6A,B). Cholesterol-depleted cells showed an impairment of FcγRI expression both in terms of percentage of receptor-positive cells (Fig. 6A) and MFI (Fig. 6B). FcγRI was present on $88 \pm 2\%$ of cells cultured in FCS medium but was detected on only $54 \pm 11\%$ of cells cultured in DFCS and $37 \pm 13\%$ of cells cultured in DFCS plus cholesterol. The corresponding values for MFI were 386 ± 97, 246 ± 66 and 113 ± 17, respectively. Thus, cholesterol failed to re-

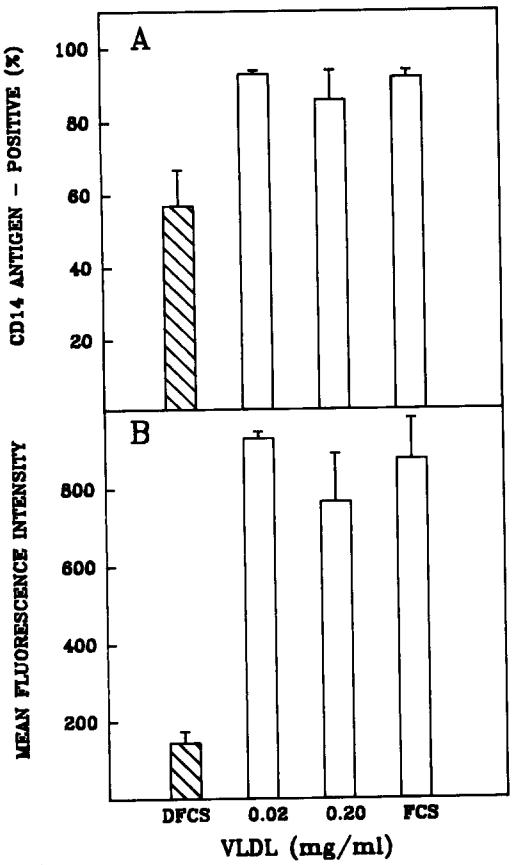

Fig. 5. Effect of VLDL supplementation of DFCS medium on CD14 expression. Monocytes were incubated for four days in growth medium containing FCS, DFCS, or DFCS plus VLDL and then stained for LeuM3 binding by indirect immunofluorescence. Monocytes from two donors were used. Results of two experiments are presented. Two replicates were analyzed. Mean ± mean deviation.

store FcγRI expression in the same cultures that it did restore CD14 expression.

Discussion

The CD14 antigen has been shown to be a GPI-linked glycoprotein which is expressed primarily on myelomonocytic cells [13–17,31]. The gene encoding this antigen maps in a region containing genes for a number of receptors and growth factors [14]. Its chromosomal location has prompted the suggestion that CD14 may represent an important receptor for myeloid differentiation [14]. This glycoprotein has also been implicated in Gram-negative endotoxin-induced shock syndrome [16] and it plays a major role in clearance of Gram-negative pathogens [17]. In this study the expression of CD14 on normal human monocytes has been shown to be regulated by cellular cholesterol content. Despite earlier studies demonstrating the ability of monocytes to synthesize cholesterol [32], the current method consistently depleted cellular cholesterol during the culture interval. The decrease in both the percent of monocytes expressing CD14 and the average number of CD14 molecules detected per cell as measured by the mean fluorescence intensity indicates a pronounced effect on the expression of this surface molecule when monocytes are cholesterol depleted. Since pure cholesterol restored expression of CD14, this lipid must play an important role in the cellular regulation of CD14. The ability of LDL and VLDL to similarly restore surface expression is presumably related to the delivery of cholesterol to the cells. However, the possibility that these lipoproteins deliver components in addition to cholesterol, as previously suggested for FcγRI regulation in U937 cell [27,28] cannot be eliminated.

The decreased expression of this one monocyte surface molecule is not a reflection of a non-specific cellular membrane or metabolic effect on all surface molecules. Like CD14, the expression of FcγRI was decreased during culture but there were no detrimental effects on the percentage of cells expressing FcγRII by any culture conditions or supplements we used (data not shown). FcγRII was used as a control in all experiments to confirm the identity of the analyzed population. These observations in normal monocytes support our previous observations on the monocytic cell line

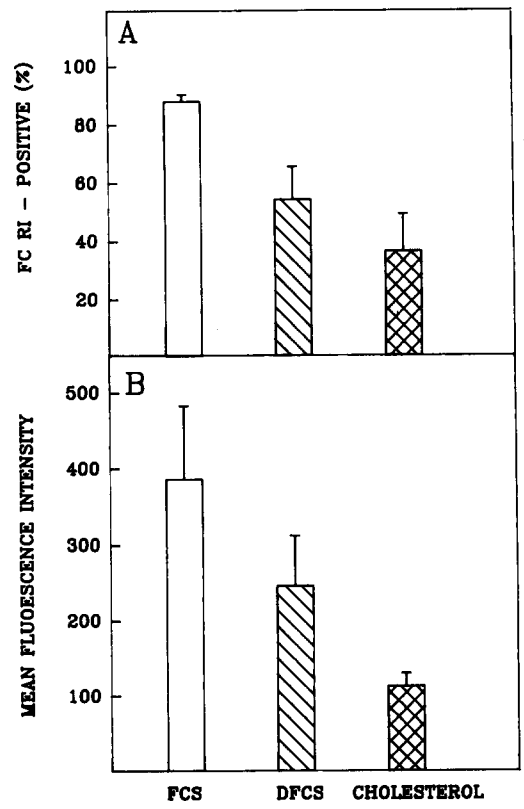

Fig. 6. Effect on FcγRI expression of cholesterol supplementation of medium containing DFCS. For experimental conditions see legend to Fig. 3. Monocytes from three donors were used. Results of three experiments are presented. Three replicates were analyzed. Mean ± S.E.

U937 that surface antigens are differentially regulated by cholesterol and lipoproteins [27]. Cholesterol failed to restore expression of FcγRI which has been shown to be an integral membrane-spanning glycoprotein [33]. Rothberg et al. [12] demonstrated a similar difference between the LDL and 5-methyltetrahydrafolate receptor. Their study demonstrated that lateral diffusion of the protein-anchored LDL receptor was not modulated by cholesterol depletion. The clustering of the GPI-linked 5-methyltetrahydrafolate receptor, however, was cholesterol-dependent. This study used microscopic methods to evaluate the alteration in membrane receptor distribution which cannot be directly compared to the immunofluorescence measurements performed in our study. These two studies, nevertheless, do suggest that GPI-linked and membrane spanning receptors may be differentially regulated by cholesterol.

The mechanism regulating the expression of CD14, and possibly other GPI-linked membrane proteins, is unknown. Several potential sites of control can be considered. One site could be regulation of surface membrane expression by activation of membrane phospholipases [34]. The presence of a soluble form of CD14 which has a structure consistent with phospholipase cleavage has been demonstrated [31]. Modulation of the activity of membrane Na^+/K^+-ATPase by cholesterol also supports this possibility [11]. The intracellular trafficking of the CD14 molecules could be defective to alter the extent or efficiency of the phospholipid linkage, transport of the GPI-linked molecule to the surface membrane, or membrane fusion [12,35]. Since cholesterol also has been shown to regulate the genetic expression of the LDL receptor [36], the possibility of control at the transcriptional or translational level of the CD14 gene also can be considered.

This study demonstrating the regulation of CD14 by cholesterol, the study of Rothberg et al. [12] showing differences in local clustering of surface molecules controlled by cholesterol, and our previous studies indicating lipoprotein regulation of FcγRI expression and function in the U937 monocytic cell line [27,28] all serve to confirm that cholesterol and lipoproteins play an important role in the regulation of cell surface molecules and the cellular functions of those molecules.

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