Macrophage uptake of low-density lipoprotein bound to aggregated C-reactive protein: possible mechanism of foam-cell formation in atherosclerotic lesions

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Foam cells found in atherosclerotic lesions are believed to derive from macrophages that take up aggregated low-density lipoprotein (LDL) particles bound to the extracellular matrix of arterial walls. C-reactive protein (CRP) is an acute-phase protein found in atherosclerotic lesions, which when immobilized on a solid phase, can bind and cluster LDL particles in a calcium-dependent manner. In the present study, we examined whether CRP-bound aggregated LDL could be taken up by macrophages in culture. CRP molecules were aggregated in the presence of calcium and immobilized on the surface of polystyrene microtitre wells. Human LDL added to the wells bound to and aggregated on the immobilized CRP, also in a calcium-dependent manner. On incubation with macrophages, the immobilized CRP-bound LDL aggregates were readily taken up by the cells, as demonstrated

by immunofluorescence microscopy, by the cellular accumulation of cholesterol and by the overexpression of adipophilin. Immunofluorescence microscopy and flow-cytometry analysis established that the uptake of the LDL–CRP complex was not mediated by the CRP receptor CD32. These observations with immobilized CRP and LDL, approximating the conditions that exist in the extracellular matrix of the arterial wall, thus suggest that CRP may contribute to the formation of foam cells in atherosclerotic lesions by causing the aggregation of LDL molecules that are then taken up by macrophages through a CD32-independent pathway.

Key words: adipophilin, calcium, CD32, cholesterol.

INTRODUCTION

Foam cells found in atherosclerotic lesions are primarily macrophages laden with cholesterol ester-rich cytoplasmic lipid inclusions that originate from blood circulation [1,2]. In response to poorly defined signals, circulating monocytes become attached to the endothelial surface of elastic and muscular arteries and, in response to chemotactic factors in the arterial intima and media layers, penetrate the sub-endothelial space and differentiate into macrophages [2]. The cholesterol-rich lipid inclusions of the foam cells are derived from plasma lipoproteins, mainly the low-density lipoproteins (LDL), which filter into the intima and media layers of the arteries where they are retained in the extracellular matrix and are eventually taken up by the monocyte-derived macrophages [3]. Microscopy studies have provided evidence that the LDL retained in the arterial wall form aggregates [4,5] and it has been hypothesized that foam cells are formed when such LDL aggregates are taken up by the macrophages. Support for this hypothesis is derived from studies using macrophages in culture, which demonstrated that the uptake of aggregated LDL particles, but not the unclustered LDL, results in the formation of foam cells [6]. The mechanisms responsible for LDL aggregation in the arterial wall are not known. On the basis of *in vitro* studies, a variety of agents have been postulated to be responsible for the aggregation of LDL, including phospholipase A₂, phospholipase C, sphingomyelinase, proteolytic enzymes, as well as oxidation [1]. Despite their heterogeneity of action, all these agents cause the clustering of LDL particles with their shared ability to produce irreversible changes in the phospholipid and/or apoprotein B-100 compositions on the surface of the lipoproteins. Whether such changes occur *in vivo* and are, in fact, required for the aggregation

of LDL in the arterial wall has not been conclusively demonstrated.

C-reactive protein (CRP) is an acute-phase protein present in the plasma [7], which has also been demonstrated to be present in the intima and media layers of human atherosclerotic arteries [8,9] as well as on the surface of foam cells [10]. CRP is composed of five identical subunits, each of which can bind two molecules of calcium with high affinity as well as a variety of ligands, including plasma lipoproteins [11]. CRP is synthesized in the liver and, to explain its presence in the arterial wall, it has generally been assumed that it derives from circulating CRP that filters into the sub-endothelial space in response to or in conjunction with inflammation of the arteries. A recent study [12] has provided the evidence that CRP can be synthesized by macrophages and smooth muscle-like cells present in human atherosclerotic plaques. The function CRP performs in atherosclerotic lesions is not presently known. Recently, Zwaka et al. [13] suggested that CRP might play a role in the formation of foam cells by opsonizing native LDL. These authors provided evidence that LDL complexed to CRP can be taken up by macrophages in culture via the CRP receptor CD32 [14]. Significantly, this proposed mechanism of LDL uptake by the macrophages does not involve changes in the composition of the lipoproteins. It is noteworthy, however, that in the study by Zwaka et al. [13] the LDL/CRP complexes were formed in solution and suspended in a culture medium, which was then added to macrophages. Recent reports [15,16] have indicated, however, that the mechanism of macrophage uptake of LDL suspended in a fluid phase differs substantially from the uptake of LDL immobilized on a solid phase. Since CRP present in the arterial wall is presumably immobilized in the extracellular matrix, in the present study we examined whether macrophage uptake of LDL complexed to

Abbreviations used: CRP, C-reactive protein; HBSS, Hanks balanced salt solution; LDL, low-density lipoprotein.

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immobilized CRP could lead to the formation of foam cells and whether CD32 is involved in the process.

MATERIALS AND METHODS

Materials

Tissue culture reagents were purchased from Gibco, and tissue culture flasks and 96-well microtitre plates from Fisher Scientific (Fair Lawn, NJ, U.S.A.). LDL, PMA, EDTA, cholesterol assay kits, mouse anti-(human CRP) monoclonal antibody and mouse antibody raised against human β -actin were obtained from Sigma Chemical Co. Immobilized *p*-aminophenyl phosphorylcholine was obtained from Pierce (Rockford, IL, U.S.A.). Coomassie Blue protein assay kits were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). A Spectra Max 190 microtitre plate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) was used in all cholesterol and protein assays. Affinity-purified goat anti-(human apo B-100) antibodies were obtained from Biodesign International. Mouse anti-(human cathepsin D) monoclonal antibody and goat anti-(human cathepsin D) polyclonal antibodies were from Santa Cruz Biotechnology (New York, NY, U.S.A.). Rat anti-(goat IgG) conjugated with rhodamine, rabbit anti-(mouse IgG) conjugated with FITC, sheep anti-(mouse IgG) conjugated with rhodamine and rabbit anti-(goat IgG) conjugated with FITC were from ICN Biomedicals (Irvine, CA, U.S.A.). Monoclonal antibody raised against human adipophilin was obtained from ProGen Immuno-Diagnostics (Heidelberg, Germany). [^{14}C]Oleic acid was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.).

CRP

CRP was purified from human ascites fluid by affinity chromatography on phosphorylcholine-agarose beads essentially as described by Du Clos et al. [17], and its purity was assessed by SDS/PAGE under reducing conditions.

Attachment of CRP on to polystyrene microtitre plates

CRP (160 $\mu\text{g}/\text{ml}$) dissolved in PBS containing calcium chloride in the indicated concentrations was added to 96-well polystyrene microtitre plates (100 $\mu\text{l}/\text{well}$) and incubated overnight at 4 °C. The incubation solution was then removed by aspiration and the wells were washed twice with Hanks balanced salt solution (HBSS). The amounts of CRP bound to the polystyrene wells were determined using a Coomassie Brilliant Blue G-250 protein assay kit (Bio-Rad).

Attachment of LDL to the immobilized CRP

PBS (100 μl) containing LDL and calcium chloride was added, in the concentrations indicated, to microtitre wells containing the immobilized CRP. After 4 h incubation at 37 °C, the solution was removed by aspiration and the wells washed twice with HBSS. The LDL attached to CRP was measured as a function of the cholesterol retained in the wells, following the addition of 0.5 % (v/v) Triton X-100 in PBS (100 μl) to the wells to solubilize the lipoproteins. LDL cholesterol was measured using the InfinityTM cholesterol reagent kit from Sigma.

Cell culture

Human U937 monocytic cells were obtained from A.T.C.C. (Manassas, VA, U.S.A.) and maintained in RPMI 1640 medium containing 10 % heat-inactivated fetal bovine serum (Life

Technologies, Gaithersburg, MD, U.S.A.) and amphotericin B (1.25 $\mu\text{g}/\text{ml}$). Differentiation of U937 monocytes into macrophages was induced by adding PMA to the culture medium at a concentration of 0.2 $\mu\text{g}/\text{ml}$ and incubating the cells for 72 h in a 5 % CO_2 tissue culture incubator at 37 °C. When indicated, the differentiated macrophages were detached from the tissue culture flasks by incubation with PBS containing 0.2 % EDTA for 5 min at room temperature. The detached cells were then washed twice with HBSS by centrifugation at 1000 rev./min for 5 min using a Beckman GPR centrifuge and used for further experimentation.

Cellular uptake of LDL

Macrophages washed as described above were plated at a density of 2×10^5 cells/well on to microtitre wells containing immobilized CRP/LDL and incubated for the indicated times at 37 °C in HBSS. Incubations were terminated by the addition of 0.2 % EDTA in PBS. EDTA caused the detachment of the cells, the disaggregation of the LDL/CRP complex, as well as the disaggregation of the CRP molecules. The cells were then washed twice with PBS by centrifugation using a microplate carrier for Beckman GH 3.7 rotors, for 5 min each time. The LDL associated with the macrophages was then quantified by measuring the total cholesterol content of the cells. For this purpose, the cells were solubilized by incubating overnight at 4 °C in the presence of 0.5 % (v/v) Triton X-100 (100 $\mu\text{l}/\text{well}$), and their cholesterol content measured using the InfinityTM cholesterol reagent kit from Sigma.

Incorporation of [^{14}C]oleate into [^{14}C]cholesteryl oleate

This procedure was performed essentially as described previously [18]. Briefly, macrophages were cultured for 12 h in RPMI 1640 medium containing 0.5 μCi of [^{14}C]oleic acid. The labelled cells were then washed as described above, plated at a density of 2×10^5 cells/well on to microtitre wells containing immobilized CRP/LDL or immobilized CRP only, and incubated for up to 8 h. At 2 h intervals, the media was removed from the microtitre wells, the cell lipids were extracted and fractionated by TLC, as described previously [18]. The [^{14}C]oleate incorporated into [^{14}C]cholesteryl oleate was then quantified by scintillation counting.

Immunofluorescence staining

For immunofluorescence staining, macrophages were incubated with immobilized CRP/LDL for 4 h at 37 °C, washed twice, as described above, and transferred into 15 ml plastic tubes. Cells were then fixed with 4 % formaldehyde for 15 min, washed with PBS, incubated with 50 mM NH_4Cl for 10 min to quench autofluorescence and permeabilized with 0.5 % (v/v) Triton X-100 for 20 min at room temperature. After washing with PBS, the cells were adhered to poly-L-lysine-coated glass coverslips for 30 min at room temperature. The cells were blocked with 0.2 % fish skin gelatin in PBS and probed with mouse anti-(human CRP) antibody (1:100 dilution), goat anti-(human apo B-100) antibody (1:200 dilution in the blocking solution) and/or goat or mouse anti-(human cathepsin D) antibodies (1:100 dilution). For the detection of CRP, the secondary antibody used was sheep anti-(mouse IgG) conjugated with FITC. For the detection of apo B, the secondary antibodies used were rabbit anti-(goat IgG) conjugated with FITC or rat anti-(goat IgG) conjugated with rhodamine (1:200 dilution) in PBS containing 0.05 % fish skin gelatin. For the staining of intracellular cathepsin D, the secondary antibodies used were rabbit anti-(mouse IgG) conjugated

with FITC or rat anti-(goat IgG) conjugated with rhodamine. When indicated, cell localization of CD32 was performed using monoclonal FITC-conjugated anti-CD32 (Pharmingen).

Microscopy and image analysis

Fluorescence microscopy and digital image collection were performed on an Olympus IX inverted fluorescence microscope and a Photometrix cooled CCD camera (CH350/LCCD) driven by DeltaVision software from Applied Precision Inc. (Seattle, WA, U.S.A.). Forty 400 nm optical sections were taken with the depth of the cells and Delta Vision software (softWoRx) was used to deconvolve these images and construct three-dimensional volume views [19,20]. Background (or non-specific) signal was subtracted by imaging areas that had no cells.

Immunoblotting

For immunoblotting analysis, macrophages were incubated with or without CRP/LDL for 12 h at 37 °C, harvested as described above and resuspended in extraction buffer [20 mM HEPES/HCl (pH 7.4)/10 mM KCl/1.5 mM MgCl₂/1 mM EGTA/250 mM sucrose/10 mM succinate/0.1 mM PMSF/2.0 µg/ml aprotinin/2.0 µg/ml pepstatin]. The total cell protein extract (50 µg) was boiled for 5 min and loaded and run on SDS/PAGE (10 % gel). Separated proteins were then blotted on to a 0.45 µm nitrocellulose membrane (Bio-Rad) using a Bio-Rad blotter.

The membranes were blocked for 3 h in TBST [10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.1 % (v/v) Tween 20] containing 5 % non-fat dried milk and then probed overnight with a mouse monoclonal antibody against human adipophilin (1:500 dilution in TBST) or a mouse monoclonal antibody against human β -actin (1:5000 dilution in TBST). Reactions were detected with goat anti-mouse secondary antibody (1:1000 dilution in TBST) conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.) using ECL® (Amersham Pharmacia Biotech).

Flow cytometry

Macrophages cultured under various conditions were detached from the polystyrene wells and washed as described above. They were stained for CD32 using monoclonal FITC-conjugated anti-CD32 (Pharmingen, San Diego, CA, U.S.A.) at a 1:100 dilution, and for CD14 using biotinylated-conjugated anti-CD14 (Pharmingen) at a 1:100 dilution followed with avidin-R-phycoerythrin secondary antibody at 1:100 dilution. Cells were analysed using a Becton Dickinson FACSsort flow cytometer and CellQuest software. Forward and side-scatter was used to gate cell population and to exclude cell debris. At least 25000 positively stained cells were analysed. Irrelevant anti-mouse isotype-matched antibodies were used as controls. The instrument settings were adjusted such that the control distribution curve centred within the first decade.

RESULTS

Binding of CRP on to polystyrene microtitre plates

CRP can be immobilized by adsorption on the surface of polystyrene microtitre plates [21]. In the course of the present study, we observed that the efficiency of CRP binding to polystyrene was influenced by the concentration of calcium in the incubation solution. We also noted that calcium caused the CRP to aggregate, as indicated by the appearance of a slight opalescence in the incubation solution. The results in Figure 1 show the effects of calcium on the aggregation of the CRP, as well as

Figure 1 Attachment of CRP on to polystyrene microtitre plates

CRP dissolved in PBS (160 µg/ml), and containing calcium chloride in the indicated concentrations was added to 96-well polystyrene microtitre plates (100 µl/well) and incubated overnight at 4 °C. Turbidity was measured by spectrophotometric absorbance and CRP by a protein micro assay kit, as described in the Materials and methods section. Each point is the combined means $\pm$ S.D. of triplicate samples from three separate experiments.

on the efficiency of CRP binding to the polystyrene microtitre plates. A progressive increase in CRP aggregation, as measured by changes in the absorbance, was observed with increasing calcium concentrations in the incubation medium, reaching a plateau at 10 mM. Binding of CRP to the microtitre plates also increased in the presence of calcium but maximum binding was observed at 4 mM. Significantly less binding occurred at higher calcium concentrations. Similar results were observed in the microtitre wells precoated with collagen (results not shown). We interpreted these observations to indicate that CRP adsorbed on to polystyrene most efficiently when in the form of small calcium-induced aggregates.

LDL binding to immobilized and aggregated CRP

Previous work demonstrated that CRP aggregated and immobilized on Sepharose beads could bind LDL in a calcium-dependent manner [22]. We examined whether CRP aggregated and immobilized on a polystyrene surface could also bind LDL, and how calcium might affect the lipoprotein binding. Figure 2 shows that, in the absence of calcium, the addition of increasing amounts of LDL to microtitre wells containing immobilized CRP resulted in a progressive increase in LDL binding to the CRP. In wells containing no CRP, no LDL binding could be detected (results not shown). At the highest LDL concentration examined (100 µg cholesterol/well), only a small fraction (1 µg cholesterol) became bound to the immobilized CRP. Similar results were observed when 2 mM calcium was present in the incubation medium. However, in the presence of 4 mM calcium, significantly higher amounts of LDL bound to the CRP. At the lowest LDL concentration added to the microtitre wells (6.25 µg cholesterol/well), approx. 10 times more LDL bound to the CRP

Figure 2 Attachment of LDL to immobilized CRP

CRP was immobilized by adsorption to 96-well polystyrene microtitre plates, in the presence of 4 mM calcium chloride, as described in the legend for Figure 1. After removal of unbound CRP, the wells were washed twice with HBSS. PBS (100 µl) containing 2 mM (●), 4 mM (○) or no (□) calcium chloride and LDL in the indicated concentrations was then added to the wells and incubated for 4 h at 37 °C. After removal of the unbound LDL and washing the wells as described in the Materials and methods section, the cholesterol of the bound lipoproteins was measured as described in the Materials and methods section. Each point is the means $\pm$ S.D. for four measurements.

compared with LDL incubated in the absence of calcium. The effects of higher calcium concentrations on LDL binding were not examined because this would have resulted in a significant decrease in the amounts of CRP adsorbed to the polystyrene surface (Figure 1). The results in Figure 2 were obtained 4 h after the addition of LDL to the CRP-containing microtitre wells. Longer incubation times did not result in further increases in LDL binding to the immobilized CRP (results not shown).

Macrophage uptake of LDL bound to immobilized CRP aggregates

Figure 3 shows the results obtained when macrophages were seeded in microtitre wells containing LDL bound to immobilized CRP, or immobilized CRP only, and the total cholesterol content of the cells measured at increasing time intervals. The first time point examined was 2 h after seeding of the cells to allow them sufficient time to become adherent to the culture-well surface. Within 2 h of plating the macrophages, cholesterol associated with the cells incubated with CRP-bound LDL was twice that of the cells incubated in the absence of LDL. Thereafter, there was further increase in the cholesterol content of the cells, and after 8 h of incubation, the cholesterol recovered from the cells incubated with CRP-bound LDL was 3 times more than that of

Figure 3 Macrophage uptake of LDL bound to immobilized CRP

CRP was immobilized by adsorption to 96-well polystyrene microtitre plates, in the presence of 4 mM calcium chloride, as described in the legend for Figure 1. PBS (100 µl) containing LDL (50 µg cholesterol) and 4 mM calcium chloride was then added to the CRP-containing wells and incubated for 4 h at 37 °C. After removal of unbound LDL as described in the legend for Figure 2, the wells were plated with macrophages (2×10^5 cells) and incubated at 37 °C for the indicated times. The wells were then washed and the cell-associated cholesterol measured as described in the Materials and methods section. Each bar is the means $\pm$ S.D. for four determinations.

the cells incubated in the absence of LDL (3.45 ± 0.22 versus 1.1 ± 0.12 µg/ 10^5 cells). Because two-thirds of the total LDL cholesterol originally present in the microtitre well had become associated with the cells after 8 h (Figure 2), more prolonged incubations were not performed.

To determine whether the observed increases in macrophage cholesterol content (Figure 3) resulted from the cellular internalization of LDL, we used deconvolution fluorescence microscopy [19,20] to detect the presence of apo B-100 in the cells. Macrophages were seeded in microtitre plates containing LDL bound to immobilized CRP and after incubation for 4 h, processed for microscopy, as described in the Materials and methods section. The choice of 4 h for incubation was made to (a) ensure sufficient cellular uptake of LDL and (b) minimize the potential degradation of internalized apo B-100 that could occur with more prolonged incubations. Cells were stained for apo B-100, CRP and for cathepsin D, a marker for lysosomes, as described in the Materials and methods section. Apo B, as well as CRP, co-localized with cathepsin D (results not shown), demonstrating that CRP-aggregated LDL had indeed been internalized by the macrophages and transported to lysosomes. We next examined the metabolic fate of the LDL cholesterol taken up by the macrophages. Macrophages prelabelled with [$1\text{-}^{14}\text{C}$]oleate, as described in the Materials and methods section, were incubated with LDL bound to aggregated CRP, under

Figure 4 Synthesis of [¹⁻¹⁴C]cholesteryl oleate

Macrophages prelabelled with [¹⁻¹⁴C]oleate were incubated with immobilized aggregated CRP alone (○) or immobilized aggregated CRP/LDL (●). After extraction, the cell lipids were fractionated by TLC and the [¹⁻¹⁴C]cholesteryl oleate formed quantified by scintillation counting. Each point is the mean of three separate lipid extractions.

Figure 5 Effects of CRP and LDL/CRP on the expression of adipophilin in macrophages

Macrophages were incubated without additions (lane 1), immobilized aggregated CRP (lane 2) and immobilized aggregated CRP/LDL (lane 3) at 37 °C for 12 h before harvesting. Total protein extracts were subjected to Western-blotting analysis using antibodies raised against 50 kDa human adipophilin or 42 kDa human β -actin, as described in the Materials and methods section.

conditions similar to those described in Figure 2, and the formation of [¹⁻¹⁴C]cholesteryl oleate measured at increasing time intervals. The results in Figure 4 show that there was a progressive increment of [¹⁻¹⁴C]cholesteryl oleate in the cells incubated with the aggregated LDL but not with the aggregated CRP only. During 8 h of incubation, there was a 3-fold accumulation of [¹⁻¹⁴C]cholesteryl oleate, indicating that its synthesis in the cell cytosol was stimulated as a result of the LDL uptake.

To determine whether the cytosolic accumulation of cholesteryl esters could lead to the formation of foam cells, we examined the expression of adipophilin in the cultured macrophages before and after incubation with aggregated CRP alone or with LDL bound to the aggregated CRP. Adipophilin is a 50 kDa protein that is a sensitive and specific marker of cellular lipid accumulation [23] and which has been shown to be present in foam cells of human atherosclerotic lesions [24]. Figure 5 shows that the expression of adipophilin in cells incubated with the LDL bound to the immobilized CRP for 12 h was substantially greater than that of cells incubated with CRP alone. In other experiments (results not shown), we observed that the increase in adipophilin expression occurred as early as 6 h after incubation with LDL bound to the immobilized CRP.

Role of a CRP receptor in the macrophage uptake of LDL bound to immobilized CRP aggregates

To determine whether CD32 is involved in the macrophage uptake of CRP/LDL immobilized on a solid phase, we used fluorescence microscopy to examine the cellular distribution of this CRP receptor in macrophages incubated with: (a) no aggregates, (b) aggregated CRP only or (c) with CRP/LDL aggregates. Figure 6(a) shows that, as anticipated, in macrophages incubated in the absence of immobilized CRP or CRP/LDL aggregates CD32 was localized on the surface of the cell. Also as anticipated, in macrophages incubated with aggregated CRP alone (Figure 6b), CD32 was detected in intracellular sites, indicating that this receptor was internalized in the process of CRP uptake by the cells. In contrast, CD32 expression in macrophages incubated with the immobilized, aggregated CRP/LDL (Figure 6c) remained on the surface of the cell, indicating that this receptor was not involved in the internalization of the CRP/LDL complex. These observations were further confirmed by flow-cytometry analysis of macrophages immunostained with anti-CD32 antibodies (Figure 7A) and antibodies to CD14, a control macrophage surface marker (Figure 7B). Control macrophages, i.e. macrophages incubated in microtitre wells containing no aggregates, expressed high levels of CD32 and CD14. The macrophages incubated with CRP aggregates only had a markedly decreased CD32 expression on their surface compared with the expression of CD32 in the control macrophages. In contrast, CD32 expression on the surface of cells incubated with immobilized, aggregated CRP/LDL was similar to that of control macrophages. The expression of CD14 was similar in all three groups (Figure 7B). These results confirm the finding (Figure 6) that whereas CD32 was internalized during the process of CRP uptake by the cells, the internalization of CRP complexed with LDL did not involve CD32.

DISCUSSION

CRP present in atherosclerotic lesions has long been suggested to participate in the generation of foam cells [8,13]. The results of the present investigation show that (a) macrophages readily take up LDL bound to immobilized CRP aggregates and (b) this uptake induces the expression of adipophilin, a specific marker of cholesterol-laden macrophages [24], is consistent with the role for CRP in the formation of foam cells.

The results shown here indicate that for CRP to be involved in the formation of foam cells *in vivo* it is necessary that, in the arterial wall, this protein form aggregates that bind and cluster LDL molecules. The aggregation of CRP molecules occurs by a calcium-dependent process (Figure 1). Each of the five subunits

Figure 6 Cellular localization of CD32

Macrophages were incubated for 4 h in microtitre plates containing no aggregates (**a**), immobilized CRP aggregates (**b**), and immobilized CRP/LDL aggregates (**c**). CD32 was detected by immuno-staining with FITC-conjugated mouse anti-(human CRP) antibody. Original magnification $\times 3500$.

Figure 7 Expression of CD32 on the surface of macrophages

Flow cytometry of macrophages immuno-stained with anti-CD32 (**A**) and anti-CD14 (**B**) antibodies was performed, as described in the Materials and methods section, after the cells had been incubated at 37 °C for 4 h in microtitre wells containing immobilized aggregated CRP (—); aggregated CRP/LDL (---), or neither (....).

of CRP can bind two molecules of calcium, a process known to induce conformational changes in the molecule [11] that, presumably, are responsible for their aggregation. The binding of LDL to aggregated CRP (Figure 2) has long been known to be a calcium-dependent process [11]. In human arteries, the concentration of calcium is known to increase progressively with age, often in parallel with the development of atherosclerotic lesions [25]. Of particular significance, the calcium content of human coronary arteries with fatty streaks (i.e. accumulated foam cells) has been reported to be over 10 times higher than that in normal segments of the arteries [25]. Whereas most of the calcium in the fatty streaks is presumably in the form of insoluble salts, it is likely that the concentration of ionized calcium is also proportionately increased. It is conceivable therefore that in the presence of elevated calcium concentrations in the arterial wall, CRP derived either from filtration from plasma CRP or

synthesized *in situ* by macrophages, may form aggregates. Such aggregates may then bind and cluster LDL particles capable of being phagocytized by sub-endothelial macrophages leading to the formation of foam cells.

The mechanism of macrophage uptake of LDL bound to the immobilized CRP aggregates remains to be elucidated. Our results show, however, that the uptake does not involve the mediation of the receptor CD32. The flow-cytometry results clearly demonstrated that in macrophages incubated with aggregated CRP the CD32 expression on the cell surface was significantly lower than that in control cells indicating that, as anticipated, internalization of CD32 accompanied the uptake of CRP. In contrast, in cells incubated with immobilized CRP/LDL aggregates the CD32 expression on the cell surface was similar to that of the control cells even though CRP, as well as LDL, was internalized by the macrophages indicating that uptake of the

CRP/LDL complex was not mediated by the binding of CRP to CD32. The discrepancy between our findings and those of Zwaka et al. [13], who reported that the uptake of CRP/LDL complexes by macrophages was accompanied by CD32 internalization, can be clearly attributed to the differences in experimental conditions used. Zwaka et al. [13] examined the cellular uptake of CRP/LDL complexes in solution, whereas in the present investigation the CRP/LDL complexes were immobilized on a solid substratum. In the arterial wall foam cells are formed when sub-endothelial macrophages take up LDL aggregates immobilized in the extracellular matrix. Since the conditions used in the present study more closely resemble those that exist in the arterial wall *in vivo*, it is more likely that CRP present in the arterial wall may contribute to the formation of foam cells primarily by its long-known [22] ability to aggregate LDL molecules in the presence of calcium, rather than by opsonizing LDL particles, as proposed by Zwaka et al. [13]. Whether our model of foam-cell formation, which is based on experiments performed *in vitro*, is relevant to atherogenesis *in vivo* remains to be determined.

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