The oxidative modification of low-density lipoproteins by macrophages

David S. LEAKE* and Sara M. RANKIN†

Pharmacology Group, Division of Biomedical Sciences, King's College London (University of London), Strand, London WC2R 2LS, U.K.

1. Mouse resident peritoneal macrophages in culture modified human ^{125}I -labelled low-density lipoprotein (LDL) to a form that other macrophages took up about 10 times as fast as unmodified LDL. The modified LDL was toxic to macrophages in the absence of serum. 2. There was a lag phase of about 4–6 h before the LDL was modified so that macrophages took it up faster. A similar time lag was observed when LDL was oxidized by $5\text{ }\mu\text{M}$ - CuSO_4 in the absence of cells. 3. LDL modification was maximal when about 1.5×10^6 peritoneal cells were plated per 22.6 mm-diam. well. 4. Re-isolated macrophage-modified LDL was also taken up much faster by macrophages, indicating that the increased uptake was due to a change in the LDL particle itself. 5. Micromolar concentrations of iron were required for the modification of LDL by macrophages to take place. The nature of the other components in the culture medium was also important. Macrophages would modify LDL in Ham's F-10 medium but not in Dulbecco's modified Eagle's medium, even when iron was added to it. 6. The macrophage-modified LDL appeared to be taken up almost entirely via the acetyl-LDL receptor. 7. LDL modification by macrophages was inhibited partially by EDTA and desferrioxamine and completely by the general free radical scavengers butylated hydroxytoluene, vitamin E and nordihydroguaiaretic acid. It was also inhibited completely by low concentrations of foetal calf serum and by the anti-atherosclerotic drug probucol. It was not inhibited by the cyclo-oxygenase inhibitors acetylsalicylic acid and indomethacin. 8. Macrophages are a major cellular component of atherosclerotic lesions and the local oxidation of LDL by these cells may contribute to their conversion into cholesterol-laden foam cells in the arterial wall.

INTRODUCTION

Low-density lipoprotein (LDL) has been heavily implicated in the pathogenesis of atherosclerosis, the underlying cause of heart attacks and strokes. It was shown by Henriksen *et al.* (1981) that LDL could be modified by arterial endothelial cells in culture so that it was taken up faster by macrophages. As macrophages accumulate much of the cholesterol and cholesteryl esters that are deposited in atherosclerotic lesions (Leary, 1941; Poole & Florey, 1958; Fowler *et al.*, 1979), this observation may help to explain why these cells are converted into lipid-laden foam cells in this disease. Endothelial-cell-modified LDL is taken up rapidly by receptors on macrophages which are known as scavenger receptors or acetyl-LDL receptors. Scavenger receptors have recently been cloned and sequenced (Kodama *et al.*, 1990; Rohrer *et al.*, 1990), but the exact number and nature of distinct receptors that mediate the uptake of cell-modified LDL is still unclear (Sparrow *et al.*, 1989; Arai *et al.*, 1989). Native (i.e. unmodified) LDL is not recognized by these receptors and is taken up only slowly by a limited number of LDL receptors on macrophages. Modified LDL, but not native LDL, therefore induces large-scale cholesteryl ester accumulation in macrophages.

Arterial smooth muscle cells (Henriksen *et al.*, 1983; Heinecke *et al.*, 1984) and mouse peritoneal macrophages (Parthasarathy *et al.*, 1986a; Rankin & Leake, 1987, 1988; Naruszewicz *et al.*, 1989; Jessup *et al.*, 1990) can also modify LDL so that macrophages will take it up faster. The mechanism of LDL modification by cells is not entirely clear but involves lipid peroxidation (Steinbrecher *et al.*, 1984, 1989; Heinecke *et al.*, 1984, 1986, 1987; van Hinsbergh *et al.*, 1986; Steinbrecher, 1987, 1988;

Esterbauer *et al.*, 1987; Hiramatsu *et al.*, 1987; Parthasarathy *et al.*, 1989; Cathcart *et al.*, 1989). LDL has been shown to be oxidized by human monocytes (Cathcart *et al.*, 1985, 1988, 1989), monocyte-derived macrophages (Chisolm *et al.*, 1983), myelomonocytic cell lines U937 and HL60 (Cathcart *et al.*, 1988) and neutrophils (Cathcart *et al.*, 1985), but its subsequent uptake by macrophages was not reported. Human blood mononuclear cells were shown by Hiramatsu *et al.* (1987) to oxidize LDL, but the oxidized LDL was apparently not taken up faster than unmodified LDL by J774 macrophages. In one study (Montgomery *et al.*, 1986), no oxidation of LDL by human mononuclear cells was found. Gorog & Kakkar (1987) have shown that human monocytes can modify LDL so that arterial endothelial cells *in vivo* will take it up faster.

There is increasing evidence that oxidized LDL is present in atherosclerotic lesions (Shaikh *et al.*, 1988; Haberland *et al.*, 1988; Trakht *et al.*, 1988; Daugherty *et al.*, 1988; Mowri *et al.*, 1988; Palinski *et al.*, 1989; Steinberg *et al.*, 1989). It appears, however, that oxidized LDL is not present in the normal arterial wall (Haberland *et al.*, 1988; Trakht *et al.*, 1988; Palinski *et al.*, 1989; Yla-Herttuala *et al.*, 1989). The normal arterial wall contains endothelial cells and smooth muscle cells, whereas atherosclerotic lesions also contain macrophages and T-lymphocytes (Jonasson *et al.*, 1986). This suggests either that the endothelial or smooth muscle cells become stimulated to oxidize LDL when atherosclerotic lesions develop, or that it is the macrophage that is mainly responsible for modifying LDL in the lesions [lymphocytes do not appear to oxidize LDL (Cathcart *et al.*, 1988)]. It is therefore of importance to fully characterize the modification of LDL by macrophages. This paper describes additional information about this subject.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; LDL, low-density lipoprotein.

* To whom correspondence and reprint requests should be sent, at Department of Biochemistry and Physiology, University of Reading, Whiteknights, P.O. Box 228, Reading, Berks. RG6 2AJ, U.K.

† Present address: Division of Endocrinology and Metabolism, Department of Medicine, University of California, San Diego, CA 92093, U.S.A.

MATERIALS AND METHODS

Isolation of mouse peritoneal macrophages

Resident peritoneal macrophages were isolated and cultured from female Swiss T.O. mice weighing about 20–30 g (obtained from A. Tuck & Sons, Battlesbridge, Essex, U.K.) by the method of Cohn & Benson (1965), as described previously (Jessup *et al.*, 1990). They were plated in 22.6 mm-diam. wells in 12-well cluster plates (Costar; supplied by Northumbria Biologicals Ltd., Cramlington, Northumbria, U.K.), at 2×10^6 peritoneal cells per well for the macrophages that were to modify LDL, and at 1×10^6 /well for the macrophages that were to receive the modified LDL.

Isolation and radioiodination of LDL

LDL (1.019–1.063 g/ml) was isolated from normal human blood, in the presence of EDTA to slow down auto-oxidation, by sequential density ultracentrifugation, as described previously (Rankin *et al.*, 1989). It was labelled with ^{125}I using ICI (Rankin *et al.*, 1989). It was found that the labelling efficiency was much higher if 2 mg of LDL (in terms of protein) was labelled (and then diluted with non-labelled LDL) than if 10–20 mg of LDL was labelled at once. Sterile ^{125}I -labelled LDL was stored at 4 °C in 154 mM-NaCl, 16.7 mM- NaH_2PO_4 , 21.1 mM- Na_2HPO_4 and 100 μM - Na_2EDTA (pH 7.4). It was discarded after about 1 month from the date on which the blood was drawn.

LDL was acetylated as described by Basu *et al.* (1976), except that a total of 10 μl of acetic anhydride per mg of LDL-protein was used (a 144 molar excess with respect to the total lysyl residues in LDL).

Modification of LDL

To avoid confusion, the macrophages that were used to modify the ^{125}I -labelled LDL are referred to as 'modifying' macrophages and those that were used to measure the increased rate of uptake of the modified LDL are termed 'target' macrophages.

^{125}I -labelled LDL (100 μg of protein/ml) was incubated with modifying macrophages (macrophage-modified LDL) or cell-free wells (control LDL) in 0.5 ml of Ham's F-10 medium (catalogue no. 12-412; Flow Laboratories Ltd., High Wycombe, Bucks., U.K.) containing 50 μg of gentamicin/ml, to which 3 μM freshly dissolved FeSO_4 had been added. After incubation (usually for 24 h), the medium was centrifuged (1500 g for 10 min at 4 °C), to remove any detached cells.

In some experiments, ^{125}I -labelled LDL was modified by incubating it at 37 °C under sterile conditions for up to 24 h at 100 μg of protein/ml in F-10 medium containing gentamicin (50 μg /ml) and freshly dissolved CuSO_4 (5 or 100 μM).

Determination of modified LDL degradation by target macrophages

Modified or control ^{125}I -labelled LDL was diluted to 10 μg of LDL-protein/ml with Dulbecco's modified Eagle's medium (DMEM) containing foetal calf serum (10%, v/v) and gentamicin (50 μg /ml) (a medium in which macrophages cannot modify LDL, as described in the Results section). It was then incubated for 24 h with target macrophages or cell-free wells (0.5 ml per well) and its rate of uptake was estimated by measuring the radioactive non-iodide trichloroacetic acid-soluble degradation products released into the medium after the lysosomal degradation of the LDL (Drevon *et al.*, 1981). At the end of the incubation, 0.4 ml of medium was taken from each well, made up to 1 ml with water and processed as described previously (de Whalley *et al.*, 1990). The degradation products in the cell-free wells were subtracted from those in the medium from the corresponding target macrophages.

After the medium had been removed, the macrophages were washed several times with ice-cold Dulbecco's phosphate-buffered saline containing Ca^{2+} and Mg^{2+} and solubilized in 600 μl of 200 mM-NaOH for 20 min at room temperature. After shaking, 500 μl was taken for the determination of protein by a modified Lowry procedure (Schacterle & Pollak, 1973).

Re-isolation of modified LDL

After incubation with modifying macrophages or cell-free wells, ^{125}I -labelled LDL was re-isolated by adjusting the density of the medium to 1.1 g/ml with solid KBr (Radding & Steinberg, 1960). It was then centrifuged at 108 000 g (r_{av} 64.3 mm) for 18 h at 10 °C in an MSE 18 $\times$ 6.5 ml titanium rotor (Fisons, Croydon, Surrey, U.K.). The tubes were sliced and the top layer containing the LDL was dialysed against the NaCl/ NaH_2PO_4 / Na_2HPO_4 / Na_2EDTA buffer described above. The protein content (Schacterle & Pollak, 1973), radioactivity and rate of degradation by target macrophages of LDL were then determined.

Materials

Materials not mentioned above were obtained from the following sources: Ham's F-10 medium (powder) deficient in heavy metal ions (FeSO_4 , CuSO_4 , ZnSO_4) was specially made up by Irvine Scientific, Santa Ana, CA, U.S.A.; vitamin E, acetylsalicylic acid, indomethacin, nordihydroguaiaretic acid and 5'-polyinosinic acid (potassium salt) were from Sigma; and probucol was a gift from Merrel Dow Pharmaceuticals Ltd., Staines, Middlesex, U.K.

RESULTS

Cytotoxicity, lag phase, effect of cell number and re-isolated modified LDL

Mouse resident peritoneal macrophages modified ^{125}I -labelled LDL so that target macrophages degraded it much faster than LDL pre-incubated in the absence of cells (Fig. 1a). The macrophages that were used to modify the LDL were obviously damaged, in that they became rounded and some detached from the wells. The target macrophages, in which the uptake of the modified LDL was measured, did not appear to be damaged, however, at least as seen under the phase contrast microscope.

The modification of ^{125}I -labelled LDL by macrophages exhibited a lag phase of several hours before a form of LDL was generated that was degraded faster by the target macrophages (Fig. 1a). In this experiment, the modification plateaued at about 12 h but in another time-course experiment (results not shown) it continued to increase between 12 and 24 h. There was a similar lag phase when ^{125}I -labelled LDL was modified by 5 μM - CuSO_4 in the absence of cells (Fig. 1b). This lag phase was shortened when the CuSO_4 concentration was increased (Jessup *et al.*, 1990).

Fig. 2 shows the effect of varying the number of peritoneal cells in each well on the modification of ^{125}I -labelled LDL. It should be noted that only about half of the peritoneal cells are macrophages (Cohn & Benson, 1965); the remaining cells, mainly lymphocytes, are non-adherent and are removed by the washing procedure. There was an increase in the modification of LDL as the number of cells was increased up to 1.5×10^6 peritoneal cells per well, and then it plateaued. Future experiments therefore used 2×10^6 peritoneal cells per well.

When macrophage-modified ^{125}I -labelled LDL was re-isolated by ultracentrifugation and added to cultured macrophages, it was degraded much faster than native LDL or re-isolated control

Fig. 1. Time course of LDL modification by macrophages or Cu^{2+} ions

(a) ¹²⁵I-labelled LDL was incubated at 37 °C for various times up to 24 h with modifying macrophages (●) or in cell-free wells (○) in Ham's F-10 medium supplemented with freshly added 3 μM - FeSO_4 . The LDL was then incubated with target macrophages or in cell-free wells and its degradation was determined. The degradation of native (not pre-incubated) ¹²⁵I-labelled LDL is also shown (Δ). Each point shows the mean $\pm$ S.E.M. of three wells of cells, except where the error bars are smaller than the symbols. (b) ¹²⁵I-labelled LDL was incubated at 37 °C for up to 18 h with F-10 medium with (■) or without (○) 5 μM - CuSO_4 in the absence of cells. It was then incubated with macrophages or cell-free wells and its degradation was measured. Each point shows the mean $\pm$ S.E.M. of three wells of cells, except where the error bars are smaller than the symbols.

LDL (Table 1). The specific activity of the re-isolated ¹²⁵I-labelled LDL was not significantly affected by its modification by macrophages.

Dependence of modification on iron

As shown in Fig. 3, little or no modification of LDL took place when it was incubated with macrophages in Ham's F-10 medium specially formulated to be deficient in heavy metal ions (FeSO_4 , CuSO_4 , ZnSO_4). Adding 3 or 5 μM freshly dissolved FeSO_4 increased the modification of LDL by macrophages above the control levels. (F-10 is normally formulated to contain 3 μM - FeSO_4 .) Increasing the concentration further increased the degree of modification, but the control LDL was also modified at 10 μM - FeSO_4 or above so that it was degraded at a similar rate to the macrophage-modified LDL.

Macrophages modified LDL well when incubated in normal Ham's F-10 medium containing heavy metal ions (Table 2). Sometimes adding additional FeSO_4 (3 μM) did not affect the modification (as shown in Table 2) but sometimes it increased it (results not shown). Macrophages were unable to modify LDL in DMEM, even when 3 μM - FeSO_4 was added to it. CuSO_4 (5 μM) modified LDL effectively when it was added to F-10 medium but not to DMEM.

Fig. 2. Effect of cell number on LDL modification

Various numbers of mouse peritoneal cells were added to culture wells. The macrophages were allowed to adhere and the other cells were washed away. ¹²⁵I-labelled LDL was incubated for 24 h with these modifying macrophages or in cell-free wells. It was then incubated for 24 h with target macrophages (1×10^6 peritoneal cells/well) or in cell-free wells and its degradation was measured. The broken line shows the degradation of native (i.e. not pre-incubated) ¹²⁵I-labelled LDL. Each point represents the mean $\pm$ S.E.M. of four wells of cells.

Table 1. Degradation of re-isolated macrophage-modified LDL by macrophages

¹²⁵I-labelled LDL (100 μg of protein/ml) was incubated for 24 h with macrophages or cell-free wells in F-10 medium. The density of the medium, and also of native ¹²⁵I-labelled LDL, was increased to 1.1 g/ml with solid KBr and the LDL was re-isolated by ultracentrifugation. It was then dialysed and its protein content and radioactivity were determined. It was diluted to 10 μg of LDL-protein/ml with serum-containing medium and added to target macrophages or cell-free wells, and its degradation was measured after 24 h. The degradation of native ¹²⁵I-labelled LDL that had not been re-isolated was also determined. The means $\pm$ S.E.M. for three wells of cells are given.

Type of ¹²⁵ I-labelled LDL	Degradation by macrophages (μg of protein/24 h per mg of cell protein)
Native	0.79 ± 0.09
Re-isolated native	0.74 ± 0.05
Re-isolated control	0.71 ± 0.08
Re-isolated macrophage-modified	7.88 ± 0.37

Nature of the binding site

The degradation of macrophage-modified LDL by target macrophages showed evidence of saturation as its concentration was increased (Fig. 4). In this particular experiment, the control LDL (which had been pre-incubated in iron-containing Ham's F-10 medium in cell-free wells) was degraded somewhat faster than native LDL (which had not been pre-incubated). This was sometimes observed in other experiments but often the two types of LDL were degraded at similar rates.

The nature of the binding site for macrophage-modified LDL was determined by competition experiments. The degradation by macrophages of macrophage-modified ¹²⁵I-labelled LDL (10 μg of protein/ml) was inhibited by about 95% by adding non-

Fig. 3. Effect of iron concentration on the modification of LDL by macrophages

^{125}I -labelled LDL was incubated for 24 h with modifying macrophages (●) or in cell-free wells (○) in Ham's F-10 medium specially formulated to be deficient in heavy metal ions and to which freshly dissolved FeSO_4 had been added at the indicated concentrations. The LDL was then incubated with target macrophages or in cell-free wells and its rate of degradation was measured. Each point shows the mean $\pm$ S.E.M. for three wells of cells, except where the error bars are smaller than the symbols. The degradation of native (i.e. not pre-incubated) ^{125}I -labelled LDL was 0.48 ± 0.05 μg of protein/24 h per mg of cell protein.

Table 2. Modification of LDL in different media

^{125}I -labelled LDL (100 μg of protein/ml) was incubated for 24 h in various media with macrophages (macrophage-modified LDL) or cell-free wells (control LDL) (Expt. 1) or with 5 μM freshly dissolved CuSO_4 (Expt. 2). It was then diluted to 10 μg of protein/ml in DMEM containing 10% (v/v) foetal calf serum and gentamicin (50 $\mu\text{g}/\text{ml}$) and incubated for 24 h with target macrophages or cell-free wells and its degradation determined. Each value is the mean ($\pm$ S.E.M.) for three wells of cells.

Medium used for first incubation (Expt. 1)	Degradation of ^{125}I -labelled LDL by the target macrophages (μg of protein/24 h per mg of cell protein)	
	Control LDL	Macrophage-modified LDL
Native LDL (not incubated)		(0.73 ± 0.05)
Ham's F-10	0.84	8.10 ± 0.23
Ham's F-10 plus 3 μM - FeSO_4	0.97	8.12 ± 0.20
DMEM	0.47	0.27 ± 0.06
DMEM plus 3 μM - FeSO_4	0.28	0.12 ± 0.01
<hr/>		
(Expt. 2)	Copper-oxidized LDL	
Native LDL (not incubated)		0.41 ± 0.04
Ham's F-10 plus 5 μM - CuSO_4		12.6 ± 0.74
DMEM plus 5 μM - CuSO_4		0.33 ± 0.04

Fig. 4. Effect of concentration of macrophage-modified LDL on its degradation by macrophages

Macrophages or cell-free wells were incubated with the indicated concentrations of macrophage-modified (●), control (○) or native (Δ) ^{125}I -labelled LDL. The degradation of the lipoproteins was then measured. Each point shows the mean $\pm$ S.E.M. for three wells of cells, except where the error bars are smaller than the symbols.

Fig. 5. Competition between macrophage-modified or copper-oxidized ^{125}I -labelled LDL and non-labelled native LDL or acetyl-LDL for degradation by macrophages

Macrophages were incubated with macrophage-modified (●, ○, Δ) or copper-oxidized (■, □) ^{125}I -labelled LDL (10 μg of protein/ml for each) in the presence of various concentrations of non-labelled native LDL (○, □), acetyl-LDL (●, ■) or 5'-polyinosinic acid, potassium salt (Δ). The radioactive degradation products in the medium were then measured. The macrophage-modified ^{125}I -labelled LDL was formed by incubating labelled LDL (100 μg of protein/ml) for 24 h with macrophages in Ham's F-10 medium supplemented with freshly dissolved 3 μM - FeSO_4 , and copper-oxidized ^{125}I -labelled LDL was produced by incubating ^{125}I -labelled LDL (100 μg of protein/ml) for 24 h with 100 μM - CuSO_4 in Ham's F-10 medium. Each point shows the mean $\pm$ S.E.M. for three wells of cells, except where the error bars are smaller than the symbols. The degradation of macrophage-modified ^{125}I -labelled LDL in the absence of any competing ligand was about 15 times greater than that of control ^{125}I -labelled LDL.

labelled acetyl-LDL at a concentration of 50 or 100 μg of protein/ml (Fig. 5). In a similar experiment, about 85–90% inhibition was observed. 5'-Polyinosinic acid also inhibited the degradation of macrophage-modified ^{125}I -labelled LDL (85% or more inhibition at 10 $\mu\text{g}/\text{ml}$ or above) (Fig. 5). In contrast, there was much less inhibition (Fig. 5) or no inhibition (results not shown) of macrophage-modified ^{125}I -labelled LDL degradation by non-labelled native LDL. The degradation of copper-oxidized ^{125}I -labelled LDL was inhibited strongly by non-labelled acetyl-LDL (about 95% inhibition at 100 μg of acetyl-LDL protein/ml), but not by non-labelled native LDL (Fig. 5).

Inhibition of LDL modification

Modification of ^{125}I -labelled LDL by macrophages was inhibited by Na_2EDTA and by the selective iron chelator desferrioxamine B in a concentration-dependent manner, but relatively high concentrations were required (Fig. 6). The lipophilic antioxidants butylated hydroxytoluene, vitamin E and nordihydroguaiaretic acid were more effective inhibitors (Table 3). The concentrations of Na_2EDTA , desferrioxamine and antioxidants that would have been carried over to the target macrophages (one-tenth of the concentration to which the modifying macrophages were exposed) did not affect their degradation of macrophage-modified LDL (results not shown). The cyclo-oxygenase inhibitors acetylsalicylic acid and indomethacin did not inhibit the modification of LDL (Table 3). As shown in Fig. 7, the anti-atherosclerotic drug probucol was an effective inhibitor of LDL modification by macrophages, the concentration required to inhibit the modification by 50% being less than 1 μM , with 3 μM producing complete inhibition. Probucol, even at a concentration of 10 μM , did not affect the degradation of macrophage-modified LDL by target macrophages.

Fig. 6. Inhibition by EDTA and desferrioxamine of LDL modification by macrophages

Macrophages or cell-free wells were incubated with ^{125}I -labelled LDL in the presence of the indicated concentrations of Na_2EDTA or desferrioxamine B. The LDL was then incubated with target macrophages or in cell-free wells and its degradation was measured. The bars drawn in continuous lines show the degradation of macrophage-modified LDL, whereas the dashed lines show the degradation of control LDL. The degradation of native (i.e. not pre-incubated) ^{125}I -labelled LDL by the target macrophages is also shown. Each point is the mean $\pm$ S.E.M. of three wells of cells. The differences between the modification of LDL in the presence and absence of an inhibitor were assessed by Student's *t* test: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Table 3. Effect of lipophilic antioxidants and eicosanoid synthesis inhibitors on LDL modification by macrophages

Macrophages or cell-free wells were incubated for 24 h with ^{125}I -labelled LDL (100 μg of protein/ml) in Ham's F-10 medium supplemented with freshly dissolved 3 μM - FeSO_4 in the presence of the indicated compounds. After being diluted to 10 μg of LDL-protein/ml with serum-containing medium, the LDL was incubated for 24 h with target macrophages or cell-free wells and its degradation was measured. The degradation of native (i.e. not pre-incubated) ^{125}I -labelled LDL by the recipient macrophages was also determined. The mean ($\pm$ S.E.M.) of three wells of cells is shown. Butylated hydroxytoluene, vitamin E and nordihydroguaiaretic acid were dissolved in ethanol at 1 mM before being added to the modifying macrophages.

Agent added to the modifying macrophages	Degradation of ^{125}I -labelled LDL by the target macrophages (μg of protein/24 h per mg of cell protein)	
	Control LDL	Macrophage-modified LDL
Expt. 1:		
Native LDL (not incubated)		(0.67 $\pm$ 0.32)
None	0.02	9.89 $\pm$ 0.86
Butylated hydroxytoluene (20 μM)	0.26	0.43 $\pm$ 0.05
Vitamin E (20 μM)	0.74	0.78 $\pm$ 0.12
Expt. 2:		
Native LDL (not incubated)		(0.38 $\pm$ 0.10)
None	0.94	4.38 $\pm$ 0.29
Acetylsalicylic acid (50 μM)	1.00	4.69 $\pm$ 0.19
Indomethacin (50 μM)	0.78	4.18 $\pm$ 0.30
Nordihydroguaiaretic acid (10 μM)	1.04	1.06 $\pm$ 0.03

Fig. 7. Inhibition by probucol of LDL modification by macrophages

^{125}I -labelled LDL (100 μg of protein/ml) was incubated for 24 h with modifying macrophages or in cell-free wells in the presence of the indicated concentrations of probucol. The LDL was then incubated with target macrophages or in cell-free wells and its degradation was determined. The bars drawn in continuous lines show the degradation of macrophage-modified LDL, and the dashed lines show the degradation of control LDL. The degradation of native (i.e. not pre-incubated) ^{125}I -labelled LDL by the recipient macrophages is also shown. Each point is the mean $\pm$ S.E.M. for three wells of cells.

Fig. 8. Inhibition by serum of LDL modification by macrophages

^{125}I -labelled LDL was incubated for 24 h with modifying macrophages (●) or in cell-free wells (○) in the presence of the indicated amounts of foetal calf serum (not heat-activated). The LDL was then incubated with target macrophages or in cell-free wells and its degradation was measured. The degradation of native (not pre-incubated) ^{125}I -labelled LDL by the recipient macrophages is shown (△). Each point shows the mean $\pm$ S.E.M. of three wells of cells, except where the error bars are smaller than the symbols.

The modification of ^{125}I -labelled LDL by macrophages could be inhibited completely by foetal calf serum (Fig. 8). A serum concentration of 1% (v/v) gave about 50% inhibition. Lipoprotein-deficient foetal calf serum (which was prepared by adjusting its density to 1.25 g/ml with KBr followed by ultracentrifugation and dialysis) also inhibited LDL modification (results not shown). In contrast to its effects on the modification of LDL by macrophages, whole foetal calf serum (10%, v/v) had little effect on the modification of LDL by $5\text{ }\mu\text{M}$ -CuSO₄ in Ham's F-10 medium (results not shown).

DISCUSSION

Mouse resident peritoneal macrophages have been shown to modify ^{125}I -labelled LDL so that other macrophages will take it up about 10 times as fast as unmodified LDL (Rankin & Leake, 1987, 1988). Similar observations have been made by Parthasarathy *et al.* (1986a) and Naruszewicz *et al.* (1989). This raises the possibility that macrophages may be responsible for modifying much of the oxidized LDL that appears to be formed in atherosclerotic lesions.

Although the ^{125}I -labelled LDL was oxidized by the modifying macrophages, it would have been unlikely to have been further oxidized in these experiments by the target macrophages in which its uptake was measured. This is because the modified LDL was diluted by 1:10 in DMEM containing 10% (v/v) foetal calf serum before it was added to the target macrophages. As shown in this study, macrophages cannot oxidize LDL in DMEM or in the presence of low concentrations of foetal calf serum.

The macrophages that modified the LDL were damaged, probably due to the cytotoxic effects of oxidized LDL (Morel *et al.*, 1983, 1984; Chisolm *et al.*, 1983; Hessler *et al.*, 1983; Cathcart *et al.*, 1985, 1988, 1989). The target macrophages to which the oxidized LDL was added, however, did not appear by phase contrast microscopy to be damaged by the LDL. This was probably because serum was present during the incubation of the

LDL with the target macrophages (but not during the incubation with the modifying macrophages), and this has been shown to protect cells against the cytotoxic effects of oxidized LDL (Henriksen *et al.*, 1979). Also, the LDL was added to the target macrophages at only one-tenth of the concentration at which it was added to the modifying macrophages.

The modification of LDL by macrophages or by $5\text{ }\mu\text{M}$ -CuSO₄ exhibited a time lag of several hours before the uptake of the modified LDL by macrophages was increased. A time lag for the oxidative modification of LDL by endothelial cells (Henriksen *et al.*, 1983), smooth muscle cells (Heinecke *et al.*, 1984), monocytes (Cathcart *et al.*, 1989) and auto-oxidation (Esterbauer *et al.*, 1987) has been shown previously. This lag phase is due, at least in part, to the time taken for the endogenous antioxidants, such as vitamin E, to be consumed in LDL, after which there is a rapid rise in the levels of hydroperoxides in LDL (Esterbauer *et al.*, 1987; Jessup *et al.*, 1990).

When LDL was incubated with macrophages and re-isolated by ultracentrifugation, it was taken up much faster by the target macrophages than was native LDL or re-isolated control LDL. Thus the increased uptake of the macrophage-modified LDL was due to a change in the LDL itself and not to some factor released from the macrophages that increased the uptake of unmodified LDL.

The modification of LDL by the macrophages was dependent on micromolar amounts of iron. Little or no modification was observed in Ham's F-10 medium which had been specially formulated to be deficient in heavy metal ions, but adding FeSO₄ to the medium restored the ability of the macrophages to modify LDL. The oxidation of LDL by human mononuclear cells (Hiramatsu *et al.*, 1987) or smooth muscle cells (Heinecke *et al.*, 1984) has also been shown to be increased by iron. If too much FeSO₄ was added to our system, however, the LDL was also modified in the cell-free wells. Thus a difference in the modification of LDL by macrophages and cell-free wells was only seen over a rather narrow range of iron concentration (below $10\text{ }\mu\text{M}$).

Iron could promote the modification of LDL by initiating lipid peroxidation or by promoting the breakdown of lipid hydroperoxides (Aust *et al.*, 1985; Halliwell & Gutteridge, 1989). The mechanisms by which iron initiates lipid peroxidation are far from clear. It could catalyse the formation of hydroxyl radicals, or possibly ferryl radicals (FeOH³⁺ or FeO²⁺), by a Haber-Weiss reaction, or it could possibly form perferryl ions (Fe²⁺-O₂) or a ferrous-dioxygen-ferric complex. These species could then initiate lipid peroxidation. Both Fe²⁺ and soluble Fe³⁺ complexes promote the conversion of lipid hydroperoxides to free radicals (lipid alkoxyl and peroxy radicals respectively). These radicals can stimulate the chain reaction of lipid peroxidation or can fragment by complex reactions (Frankel, 1982) to form a variety of compounds, including aldehydes, which may react with apolipoprotein B-100 (Steinbrecher, 1987; Steinbrecher *et al.*, 1989). Iron has been shown to bind to phospholipids in liposomes and microsomes (Vile & Winterbourn, 1987) and this raises the interesting possibility that iron-mediated free-radical generation may actually occur on the surfaces of the LDL particles.

Macrophages were able to modify LDL in Ham's F-10 medium containing micromolar amounts of iron, but not in DMEM, even when $3\text{ }\mu\text{M}$ -FeSO₄ was added to it. Endothelial cells (Steinbrecher *et al.*, 1984), smooth muscle cells (Heinecke *et al.*, 1984) and monocytes (Cathcart *et al.*, 1988) also cannot oxidize LDL readily in DMEM. LDL was modified well by $5\text{ }\mu\text{M}$ -CuSO₄ when it was added to F-10, but not when it was added to DMEM, and so there is probably an antioxidant factor of some kind present in DMEM.

The macrophage-modified ^{125}I -labelled LDL was taken up by the mouse peritoneal macrophages apparently almost entirely

by the acetyl-LDL receptor, as its degradation was inhibited by about 85–95 % by excess non-labelled acetyl-LDL but much less, or not at all, by non-labelled native LDL. The degradation of copper-oxidized LDL was also inhibited by about 95 % by excess non-labelled acetyl-LDL. In contrast, the degradation of endothelial cell-modified or copper-oxidized ^{125}I -labelled LDL by mouse peritoneal macrophages was inhibited by only about 50 % by excess non-labelled acetyl-LDL in a study by Sparrow *et al.* (1989), who concluded that LDL oxidized by endothelial cells or by copper was taken up by the acetyl-LDL receptor and at least one other receptor. The reason for this difference in our results is unknown.

EDTA partially inhibited the modification of LDL by macrophages, as did the selective Fe^{3+} chelator desferrioxamine B, but relatively large amounts were required. Desferrioxamine binds Fe^{3+} (but not Fe^{2+} significantly) so that it is not available for reaction. FeSO_4 was added to the Ham's F-10 medium, but autooxidation of Fe^{2+} may have occurred, especially as this is promoted by desferrioxamine (Klebanoff *et al.*, 1989), and there may have been redox recycling of Fe^{2+} and Fe^{3+} during lipid peroxidation. The iron should therefore in theory have been available for binding to the desferrioxamine. As relatively large amounts of desferrioxamine were required, however, this raises the possibility that it may also have been acting as a scavenger for superoxide anions (Sinaceur *et al.*, 1984) or lipid peroxy radicals (Darley-Usmar *et al.*, 1989).

The chain-breaking antioxidants butylated hydroxytoluene and vitamin E totally prevented the modification of LDL, as did the lipid-lowering drug probucol. Similar effects of these compounds have been reported for LDL oxidation by endothelial cells (Steinbrecher *et al.*, 1984; Morel *et al.*, 1984; Parthasarathy *et al.*, 1986b; van Hinsbergh *et al.*, 1986), smooth muscle cells (Morel *et al.*, 1984; Heinecke *et al.*, 1986), monocytes (Cathcart *et al.*, 1985, 1989; Hiramatsu *et al.*, 1987), macrophages (Parthasarathy *et al.*, 1986a) and neutrophils (Morel *et al.*, 1983). A concentration of probucol of $3\text{ }\mu\text{M}$ completely inhibited LDL modification by the macrophages which agrees with the observation of Parthasarathy *et al.* (1986b) using endothelial cells. The concentration of probucol in the plasma of hypercholesterolaemic patients treated with this drug is about $100\text{ }\mu\text{M}$ (Parthasarathy *et al.*, 1986b), and the compound is carried within the plasma lipoproteins, including LDL (Marshall, 1982). ProbucoL inhibits spontaneous atherosclerosis in the LDL-receptor-deficient WHHL strain of rabbits by an effect that is independent of its plasma-cholesterol-lowering effect, presumably by inhibiting the oxidative modification of LDL within the arterial wall (Carew *et al.*, 1987; Kita *et al.*, 1987). The structure of probucol is similar to that of butylated hydroxytoluene, and it may be acting as a chain-breaking antioxidant in the LDL particles.

The cyclo-oxygenase inhibitors acetylsalicylic acid and indomethacin, at concentrations that would have completely inhibited the enzyme, did not inhibit the modification of LDL by macrophages. This indicates that free radicals formed as a by-product of this enzymic activity were not essential for the oxidation of LDL. Similar results have been reported by van Hinsbergh *et al.* (1986) and Parthasarathy *et al.* (1989) for the modification of LDL by endothelial cells. The non-selective lipooxygenase inhibitor nordihydroguaiaretic acid inhibited LDL modification by the macrophages, but this does not necessarily prove that lipooxygenase is involved in LDL oxidation by these cells, as this inhibitor is also a general lipid-soluble antioxidant. Parthasarathy *et al.* (1989) have presented evidence that lipooxygenase is involved in LDL modification by endothelial cells, but more work will be needed to see if this enzyme is involved in LDL modification by macrophages.

LDL modification by macrophages was also inhibited com-

pletely by low concentrations of foetal calf serum [1 % (v/v) serum giving about 50 % inhibition]. The reasons for this are unknown, but it was not due (or at least not due entirely) to the presence of serum lipoproteins or of a dialysable factor, as lipoprotein-deficient foetal calf serum, which had been subjected to ultracentrifugation at a density of 1.25 g/ml and then dialysed, also inhibited the modification. Among the many candidates for the inhibitory factors in whole serum are transferrin (a plasma protein that binds Fe^{3+} tightly and is not normally saturated with Fe^{3+}), caeruloplasmin [a plasma protein which catalyses the oxidation of Fe^{2+} to Fe^{3+} and can inhibit iron-dependent lipid peroxidation (Gutteridge, 1983)], extracellular superoxide dismutase (Karlsson & Marklund, 1988), high-density lipoproteins [which have been reported to inhibit certain aspects of LDL modification (van Hinsbergh *et al.*, 1986; Klimov *et al.*, 1987)], the serum proteins in general (which may have undergone 'sacrificial' oxidation in preference to the LDL) and the antioxidants vitamin E, ascorbic acid, uric acid or bilirubin (Wayner *et al.*, 1987; Frei *et al.*, 1989). Whatever the nature of the inhibitory factors present in serum, the results suggest that LDL modification may not occur to any significant extent in the circulating blood but may only occur in localized environments, such as in the arterial wall, where the ratio of the oxidant stress to antioxidants may be high.

We are grateful to the Wellcome Trust, the Medical Research Council and the British Heart Foundation for financial support. S.M.R. was supported by a Collaborative Award in Science and Engineering from the Science and Engineering Research Council in collaboration with Smith Kline & French Research Ltd. We thank Ms. Marion E. Knowles for dedicated technical assistance, Ms. Sylvia F. Dalton for carefully typing the manuscript and Ms. Jane Collard for skilfully preparing the Figures.

REFERENCES

- Arai, H., Kita, T., Yokode, M., Narumiya, S. & Kawai, C. (1989) *Biochem. Biophys. Res. Commun.* **159**, 1375–1382
- Aust, S. D., Morehouse, L. A. & Thomas, C. E. (1985) *J. Free Radicals Biol. Med.* **1**, 3–25
- Basu, S. K., Goldstein, J. L., Anderson, R. G. W. & Brown, M. S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3178–3182
- Carew, T. E., Schwenke, D. C. & Steinberg, D. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7725–7729
- Cathcart, M. K., Morel, D. W. & Chisolm, G. M., III (1985) *J. Leukocyte Biol.* **38**, 341–350
- Cathcart, M. K., Chisolm, G. M., III, McNally, A. K. & Morel, D. W. (1988) *In Vitro Cell. Dev. Biol.* **24**, 1001–1008
- Cathcart, M. K., McNally, A. K., Morel, D. W. & Chisolm, G. M., III (1989) *J. Immunol.* **142**, 1963–1969
- Chisolm, G. M., Morel, D. W. & Cathcart, M. K. (1983) *Arteriosclerosis* **3**, 472a
- Cohn, Z. A. & Benson, B. (1965) *J. Exp. Med.* **121**, 153–170
- Darley-Usmar, V. M., Hersey, A. & Garland, L. G. (1989) *Biochem. Pharmacol.* **38**, 1465–1469
- Daugherty, A., Zweifel, B. S., Sobel, B. E. & Schonfeld, G. (1988) *Arteriosclerosis* **8**, 768–777
- de Whalley, C. V., Rankin, S. M., Hoult, J. R. S., Jessup, W. & Leake, D. S. (1990) *Biochem. Pharmacol.* **39**, 1743–1750
- Drevon, C. A., Attie, A. D., Pangburn, S. H. & Steinberg, D. (1981) *J. Lipid Res.* **22**, 37–46
- Esterbauer, H., Jurgens, G., Quehenberger, O. & Koller, E. (1987) *J. Lipid Res.* **28**, 495–509
- Fowler, S., Shio, H. & Haley, N. J. (1979) *Lab. Invest.* **41**, 372–378
- Frankel, E. N. (1982) *Prog. Lipid Res.* **22**, 1–33
- Frei, B., England, L. & Ames, B. N. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6377–6381
- Gorog, P. & Kakkar, V. V. (1987) *Atherosclerosis* **65**, 99–107
- Gutteridge, J. M. C. (1983) *FEBS Lett.* **157**, 37–40
- Haberland, M. E., Fong, D. & Cheng, L. (1988) *Science* **241**, 215–218

- Halliwell, B. & Gutteridge, J. M. C. (1989) *Free Radicals in Biology and Medicine*, 2nd edn., Clarendon Press, Oxford
- Heinecke, J. W., Rosen, H. & Chait, A. (1984) *J. Clin. Invest.* **74**, 1890–1894
- Heinecke, J. W., Baker, L., Rosen, H. & Chait, A. (1986) *J. Clin. Invest.* **77**, 757–761
- Heinecke, J. W., Rosen, H., Suzuki, L. A. & Chait, A. (1987) *J. Biol. Chem.* **262**, 10098–10103
- Henriksen, T., Evensen, S. A. & Carlander, B. (1979) *Scand. J. Clin. Lab. Invest.* **39**, 361–368
- Henriksen, T., Mahoney, E. M. & Steinberg, D. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6499–6503
- Henriksen, T., Mahoney, E. M. & Steinberg, D. (1983) *Arteriosclerosis* **3**, 149–159
- Hessler, J. R., Morel, D. W., Lewis, L. J. & Chisolm, G. M. (1983) *Arteriosclerosis* **3**, 215–222
- Hiramatsu, K., Rosen, H., Heinecke, J. W., Wolfbauer, G. & Chait, A. (1987) *Arteriosclerosis* **7**, 55–60
- Jessup, W., Rankin, S. M., de Whalley, C. V., Hoult, J. R. S., Scott, J. & Leake, D. S. (1990) *Biochem. J.* **265**, 399–405
- Jonasson, L., Holm, J., Skalli, O., Bondjers, G. & Hansson, G. K. (1986) *Arteriosclerosis* **6**, 131–138
- Karlsson, K. & Marklund, S. L. (1988) *Biochem. J.* **255**, 223–228
- Kita, T., Nagano, Y., Yokode, M., Ishii, K., Kume, N., Ooshima, A., Yoshida, H. & Kawai, C. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5928–5931
- Klebanoff, S. J., Waltersdorff, A. M., Michel, B. R. & Rosen, H. (1989) *J. Biol. Chem.* **264**, 19765–19771
- Klimov, A. N., Kozhemyakin, L. A., Pleskov, V. M. & Andreeva, L. I. (1987) *Bull. Exp. Biol. Med.* **103**, 622–625
- Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P. & Krieger, M. (1990) *Nature (London)* **343**, 531–535
- Leary, T. (1941) *Arch. Pathol.* **32**, 507–555
- Marshall, F. N. (1982) *Artery* **10**, 7–21
- Montgomery, R. R., Nathan, C. F. & Cohn, Z. A. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6631–6635
- Morel, D. W., Hessler, J. R. & Chisolm, G. M. (1983) *J. Lipid Res.* **24**, 1070–1076
- Morel, D. W., DiCorleto, P. E. & Chisolm, G. M. (1984) *Arteriosclerosis* **4**, 357–364
- Mowri, H.-O., Ohkuma, S. & Takano, T. (1988) *Biochim. Biophys. Acta* **963**, 208–214
- Naruszewicz, M., Mirkiewicz, E. & Klosiewicz-Latoszek, L. (1989) *Atherosclerosis* **79**, 261–266
- Palinski, W., Rosenfeld, M. E., Yla-Herttuala, S., Gurtner, G. C., Socher, S. S., Butler, S. W., Parthasarathy, S., Carew, T. E., Steinberg, D. & Witztum, J. L. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1372–1376
- Parthasarathy, S., Printz, D. J., Boyd, D., Joy, L. & Steinberg, D. (1986a) *Arteriosclerosis* **6**, 505–510
- Parthasarathy, S., Young, S. G., Witztum, J. L., Pittman, R. C. & Steinberg, D. (1986b) *J. Clin. Invest.* **77**, 641–644
- Parthasarathy, S., Wieland, E. & Steinberg, D. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1046–1050
- Poole, J. C. F. & Florey, H. W. (1958) *J. Pathol. Bacteriol.* **75**, 245–252
- Radding, C. M. & Steinberg, D. (1960) *J. Clin. Invest.* **39**, 1560–1569
- Rankin, S. M. & Leake, D. S. (1987) *Biochem. Soc. Trans.* **15**, 485–486
- Rankin, S. M. & Leake, D. S. (1988) *Agents Actions Suppl.* **26**, 233–239
- Rankin, S. M., Knowles, M. E. & Leake, D. S. (1989) *Atherosclerosis* **79**, 71–78
- Rohrer, L., Freeman, M., Kodama, T., Penman, M. & Krieger, M. (1990) *Nature (London)* **343**, 570–572
- Schacterle, G. R. & Pollak, R. L. (1973) *Anal. Biochem.* **51**, 654–655
- Shaikh, M., Martini, S., Quiney, J. R., Baskerville, P., La Ville, A. E., Browne, N. L., Duffield, R., Turner, P. R. & Lewis, B. (1988) *Atherosclerosis* **69**, 165–172
- Sinaceur, J., Ribiere, C., Nordmann, J. & Nordmann, R. (1984) *Biochem. Pharmacol.* **33**, 1693–1694
- Sparrow, C. P., Parthasarathy, S. & Steinberg, D. (1989) *J. Biol. Chem.* **264**, 2599–2604
- Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. & Witztum, J. L. (1989) *N. Engl. J. Med.* **320**, 915–924
- Steinbrecher, U. P. (1987) *J. Biol. Chem.* **262**, 3603–3608
- Steinbrecher, U. P. (1988) *Biochim. Biophys. Acta* **959**, 20–30
- Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witztum, J. L. & Steinberg, D. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3883–3887
- Steinbrecher, U. P., Loughheed, M., Kwan, W.-C. & Dirk, S. M. (1989) *J. Biol. Chem.* **264**, 15216–15223
- Trakht, I. N., Kovaleva, K. A., Janushevskaya, E. V., Balmukhanov, T. S., Printseva, O. Y., Preobrazhensky, S. N., Peklo, M. M., Tsibulsky, V. P. & Pokrovsky, S. N. (1988) *Atheroscler. Rev.* **17**, 51–66
- van Hinsbergh, V. W. M., Scheffer, M., Havekes, L. & Kempen, H. J. M. (1986) *Biochim. Biophys. Acta* **878**, 49–64
- Vile, G. F. & Winterbourn, C. C. (1987) *FEBS Lett.* **215**, 151–154
- Wayner, D. D. M., Burton, G. W., Ingold, K. U., Barclay, L. R. C. & Locke, S. J. (1987) *Biochim. Biophys. Acta* **924**, 408–419
- Yla-Herttuala, S., Palinski, W., Rosenfeld, M. E., Parthasarathy, S., Carew, T. E., Butler, S., Witztum, J. L. & Steinberg, D. (1989) *J. Clin. Invest.* **84**, 1086–1095

Received 1 February 1990/6 April 1990; accepted 31 May 1990