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THE INHIBITION OF FOAM CELL FORMATION BY SODIUM DIETHYLDITHIOCARBAMATE

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A prominent feature of human atherosclerosis is the lipid-laden foamy macrophage, which often also contains the insoluble pigment, ceroid. The culture of macrophage-like cells, P388D$_1$s, with artificial lipoproteins composed of cholesteryl linoleate (CL) and bovine serum albumin (BSA) results in foam cell formation with lipoprotein uptake and the intracellular accumulation of ceroid. Ceroid accumulation is accompanied by the oxidation of the cholesterol ester as monitored by gas chromatography. The sodium salt of diethyldithiocarbamic acid (DDC) at 1–5 μM effectively inhibited lipoprotein uptake, cholesteryl linoleate oxidation and ceroid accumulation in cultures of P388D$_1$. Further studies showed that intracellular ceroid accumulation appeared to require the presence of cystine in the medium. Lipoprotein oxidation by this macrophage-like cell therefore appears to involve a mechanism dependent on cystine metabolism which is consistent with previous reports of macrophage-mediated lipoprotein oxidation. Studies on CL/BSA-induced ceroid accumulation in human monocytes also showed that DDC behaved in much the same manner. This inhibitory effect of DDC on foam cell formation, often considered a primary event of atherosclerosis, at concentrations as low as 1 μM, suggests the need for further, more comprehensive, studies on this compound's activities.

KEY WORDS: Lipoprotein oxidation; macrophages; diethyldithiocarbamate; ceroid; foam cells.

INTRODUCTION

The oxidative modification of low density lipoprotein (LDL) has been suggested to be associated with increased atherogenicity, leading to an enhanced uptake by macrophages which results in foam cell formation and the subsequent development of atherosclerotic lesions[1-5]. To date, the oxidation of LDL has been achieved *in vitro* by co-culture with a number of cell types, including both primary cultures of macrophages[6-8] and endothelial cells[9], as well as certain cell lines[10,11].

The obvious choice of substrate for investigation of mechanisms of cell-mediated lipoprotein oxidation is LDL itself. However, LDL from different individuals contains variable proportions of lipids and natural antioxidants. Thus, we have made use of a model system for studying processes relevant to lipoprotein oxidation and subsequent cellular processing, using an artificial lipoprotein and the macrophage-like cell line, P388D$_1$. This cell has been shown to bind and degrade native and modified LDL[12,13], as well as to take up and accumulate ceroid[12], in a manner analogous to that shown in murine peritoneal macrophages[14,15]. The artificial lipoprotein used in these studies consists of a single lipid species, cholesteryl linoleate (CL) which is known to be

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abundant in LDL and less so in the atherosclerotic lesion[3], emulsified with bovine serum albumin (BSA), a well characterised protein. This artificial lipoprotein (CL/BSA), which undergoes less variable oxidation than that of LDL[14], has enabled a number of studies including the investigation of the pro-oxidant nature of ascorbic acid[15], as well as showing different specificities of the antioxidants vitamin E and probucol for the oxidation of the lipid and protein moieties of lipoproteins when oxidised by macrophages[14].

The underlying mechanism by which macrophages and other cells oxidise lipoproteins to a more atherogenic form is as yet unknown, but appears to be dependent on the presence of redox active transition metals[10,16–19]. There is some evidence that transition metals are associated with coronary artery disease[19] and that they are also present in advanced atherosclerotic lesions[20]. Furthermore, the copper-containing protein caeruloplasmin, which is an acute phase plasma protein found to correlate with an increased progression of atherosclerosis in man, can induce the oxidative modification of LDL by macrophages[21], especially when pre-exposed to mildly acid conditions[22]. Several studies in a variety of cell types have also suggested that thiol compounds, generated as a result of cystine metabolism, are also involved in cell-mediated LDL oxidation[10,16,18]. Thiols may maintain transition metals in a reduced state which are known to promote peroxide decomposition and involved in the formation of reactive oxygen species[16,23–26]. We have performed screening experiments on a number of different compounds likely to affect macrophage foam cell formation[14,15]. Since diethyldithiocarbamate is able to scavenge free radical species[27,28] and chelate metals[29] we have investigated the effect of diethyldithiocarbamate on foam cell formation and lipoprotein oxidation by P388D_1 macrophage-like cells.

MATERIALS AND METHODS

All reagents were supplied by Sigma Chemical Co., including diethyldithiocarbamic acid (DDC, sodium salt; D 3506). Statistical analyses were performed using 'Microsoft Excel 4.0' (Apple Macintosh).

Artificial lipoprotein preparation

Lipoproteins were prepared as previously described[14,30]. The molar ratio of lipid to bovine serum albumin (BSA) was 60:1. BSA was used at 10 mg/ml (150 μM) in phosphate-buffered saline (PBS). The cholesterol ester (9 mM) was first dissolved in acetone (BDH-Analar), added to the BSA whilst vortexing and then sonicated for 1 minute. Acetone was then evaporated by gassing with a stream of nitrogen. The resulting emulsion was added to the culture medium at 50 μl/ml medium.

Gas chromatography

Products of cholesteryl linoleate (CL) oxidation in culture medium were measured using gas chromatography (GC). Sample preparation and GC were performed as previously described[28], using a Carlo Erba Mega 5360 gas chromatograph equipped with a 25 m OV-1 Crossbond™ fused silica capillary column of diameter of 0.32 mm and film thickness of 0.1–0.15 mm (Mega, Milan, Italy). Hydrogen was used as the carrier gas. Amounts of compounds were measured relative to internal standards, as previously described[31].

Cell culture

The murine P388D$_1$ macrophage cell line was cultured in 75 cm^2 tissue culture flasks (Falcon) in Dulbecco's modified Eagle's medium without phenol red (Sigma), containing 10% heat-inactivated foetal calf serum (FCS) (Gibco)[12]. Prior to use the cells were harvested by agitation of the flask and counted.

Cells were seeded at 0.6×10^6 cells in 3 ml dishes, using 3 ml of Dulbecco's Modified Eagles Medium (DMEM) with 10% lipoprotein-deficient foetal calf serum (LPDFCS), prepared as previously described[12]. Lipoprotein was then added to the wells and cells were harvested at 24 hours by agitation of the medium in the well, and then transferred into RB tubes (Falcon). The remaining adherent cells were harvested by adding further phosphate-buffered saline (PBS) and pooled. The tubes were centrifuged at 1000 g for 5 minutes at 4°C. The pellet was washed three times with PBS and then suspended in 250 ml fixative (1.5% formaldehyde and 0.1% bovine serum albumin, BSA, in PBS) and stored at 4°C for up to 4 weeks[30] until analysed by flow cytometry.

In human monocyte studies, cells were prepared by density centrifugation followed by removal of non-adherent cells, as previously described[32]. Essentially, human monocytes were isolated from blood (120 ml) obtained from adult volunteers. Blood was adjusted to 1 mg/ml EDTA (ethylenediamine tetraacetic acid) and centrifuged at 1000 g for 10 minutes at 4°C, the plasma was removed and replaced with PBS (phosphate-buffered saline). The resulting cell suspension was then layered onto lymphoprep (Nycomed, Oslo, Norway) at room temperature and centrifuged at 300 g for 30 minutes. Mixed mononuclear cells were removed from the interface, washed with PBS-containing bovine serum albumin (4 mg/ml) and diluted to 3×10^6 cells/ml in Iscoves modified Dulbeccos medium (Gibco, Paisley, UK). Cells were counted by haemocytometer and cultures (3×10^6 cells/well) were performed in 24-well plates (Becton Dickinson, New Jersey, USA) which had been pre-coated with foetal calf serum. Non-adherent cells were washed off with PBS after 1 hour, leaving a monolayer of macrophages which were used 24 hours after culture. At the end of 24 hour hour cultures with lipoproteins, these cells were removed by scrape-harvesting after a 30 minute exposure to 1 mg/ml EDTA, as previously described for murine peritoneal macrophages[14,30].

Ceroid fluorescence estimation by flow cytometry

Fluorescence in cells was analysed using a FACStar Plus (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) flow cytometer equipped with a Spectra Physics 2025 argon ion laser, as previously described[30]. The laser was tuned to emit 300 mW of light in the UV wavelengths 351–363.8 nm. Forward light scatter and side scatter were also recorded.

Intact macrophages were identified by forward light scatter. Selection of intact populations of macrophages was verified on selected live, unfixed samples, using propidium iodide to stain dead cells as previously described[30]. Ceroid fluorescence was monitored using a 430 nm band pass filter in front of the detector. Mean (geometric) fluorescence intensities of the intact macrophage population were calculated using the Lysis II programme (Becton-Dickinson) for each sample. Mean fluorescence intensities were expressed relative to controls in which cells were exposed to medium alone ("no adds"), since this instrument, in common with all fluorimeters, measures light intensities in arbitrary units[30].

TABLE 1
Analysis by gas chromatography: cell-mediated oxidation of CL/BSA.

CELLS	18:2	CHOLESTEROL	CHOLEST-5-EN-3β,7β-DIOL
NA n = 15	1.8 ± 0.96	6.99 ± 0.98	0.45 ± 0.95
CL/BSA n = 15	24 ± 11	115 ± 7.7	4.39 ± 1.37
1μM DDC n = 3	24 ± 8	109 ± 32	3.67 ± 0.84
5μM DDC n = 3	*43 ± 5	126 ± 31	*1.38 ± 1.97
10μM DDC n = 3	*54 ± 6	*172 ± 31	*0.97 ± 1.0

The effect of DDC upon the oxidation of CL/BSA in the presence of cells was monitored. Changes in linoleate (18:2), cholesterol and the cholesterol oxidation product, cholest-5-en-3β,7β-diol, were determined by gas chromatography (GC). Cholesterol and unsaturated fatty acid (linoleate; 18:2) present in CL/BSA prior to experimentation were 230 ± 20 μg/ml and 120 ± 6 μg/ml, respectively. Values (μg/ml medium)±standard deviation (SD) from 3 experiments are shown. Controls of GC-detectable material in medium in which cells were cultured for 24 hours with (**CL/BSA**) and without lipoprotein (**NA**) are shown. In this and all other figures; **DDC**=sodium diethyldithiocarbamate. Values are significantly different from CL/BSA cultured with cells by the Student's t-test; * = P <0.05.

Estimation of cellular uptake of protein

Bovine serum albumin (BSA) was labelled at lysine residues by fluorescein isothiocyanate (FITC), as previously described[14]. Labelling by FITC resulted in less than 4% lysine modification which has little effect on protein uptake[14]. Cellular uptake of BSA (FITC-labelled) within artificial lipoproteins was monitored by flow cytometry as described above, but with the laser tuned to emit 300 mW of light at 488 nm and emission monitored using a 530 nm band pass filter[14]. To determine whether DDC affected lipoprotein uptake independent of any inhibitory effect on lipoprotein oxidation, studies were performed with FITC-BSA and cholesteryl oleate-containing

TABLE 2
Analysis by gas chromatography: autoxidation of CL/BSA in culture medium without cells.

No CELLS	18:2	CHOLESTEROL	CHOLEST-5-EN-3β,7β-DIOL
CL/BSA n = 15	24 ± 6.8	100 ± 20	*2.72 ± 1.0
1μM DDC n = 3	44 ± 7	147 ± 57	2.52 ± 2.0
5μM DDC n = 3	85 ± 25	131 ± 53	*1.16 ± 0.4
10μM DDC n = 3	58 ± 7	104 ± 13	*2.18 ± 0.2

The effect of DDC (in the absence of cells) upon the oxidation of cholesteryl linoleate after 24 hours in culture medium (37°C), monitoring changes in linoleate (18:2), cholesterol and the cholesterol oxidation product, cholest-5-en-3β,7β-diol, was determined by GC. Values (μg/ml medium)±standard deviation (SD) from 3 experiments are shown. Controls of GC-detectable material in medium in which cells were cultured with (**CL/BSA**) lipoprotein are shown. Values are significantly different from studies with cells cultured with CL/BSA by the Student's t-test; * = P <0.05.

lipoproteins. The latter have previously been shown to be non-oxidisable by cells, are avidly taken up by macrophages and unable to induce ceroid accumulation in macrophages[31,32].

RESULTS

Lipoprotein oxidation, lipoprotein uptake and ceroid accumulation

Analysis by gas chromatography of the medium from similar cultures showed consistent trends with those of ceroid accumulation. Table 1 shows that DDC inhibited the disappearance of both cholesterol and 18:2 (linoleate) and the formation of the cholesterol oxidation product, cholest-5-en-3β,7β-diol. Controls of CL/BSA incubated in medium without cells are shown in table 2, the inclusion of DDC made much less difference to CL oxidation. In the absence of DDC, the changes in cholesterol oxidation in the presence of cells consistently exceeded that without cells by about 2-fold.

DDC (1–50 μM) also showed a pronounced inhibitory effect on ceroid accumulation by P388D$_1$ cells from CL/BSA (figure 1a), inhibitory effects being obvious at concentrations as low as 1–5 μM.

Using artificial lipoprotein composed of FITC-BSA and cholesteryl linoleate, when monitored by flow cytometry, DDC effectively inhibited lipoprotein uptake (figure 1b). That DDC had no effect on the cellular machinery of uptake itself is suggested by its inability to affect the uptake of cholesteryl oleate-containing lipoprotein which is known to be resistant to cell-mediated lipid oxidation (figure 1b)[31]. Uptake of CO-containing artificial lipoproteins by macrophages probably involves phagocytic activity[31,33].

A study on the effect of DDC on CL/BSA-induced ceroid accumulation in human monocytes is shown in figure 2; again DDC effectively decreased ceroid accumulation at concentrations as low as 5 μM.

Cystine and ceroid accumulation

In cystine-deficient medium, ceroid accumulation in P388D$_1$ was much less than in cystine-supplemented medium (figure 3a). However, doses of 5μM DDC and above abolished this ceroid accumulation (figure 3a).

Further studies showed that cystine supplementation (200 μM) of cystine-deficient medium returned ceroid accumulation in the cells to levels achieved in cystine-replete medium (figure 3b).

The above observations are in agreement with previous studies[10,16], in both primary macrophage cultures[16] and a cell line with macrophage-like activity[10], that suggest cell-mediated lipoprotein oxidation is dependent upon cystine metabolism.

DISCUSSION

Here we show that DDC inhibits foam cell formation and lipoprotein oxidation. In the absence of cells, the low level of oxidation of CL/BSA was much less affected by DDC (table 2), suggesting that the enhanced CL/BSA oxidation on culture with cells was due to a cell-associated mechanism vulnerable to an inhibitory effect of diethyldithiocarbamic acid (DDC). The failure of DDC to inhibit lipoprotein oxidation in the

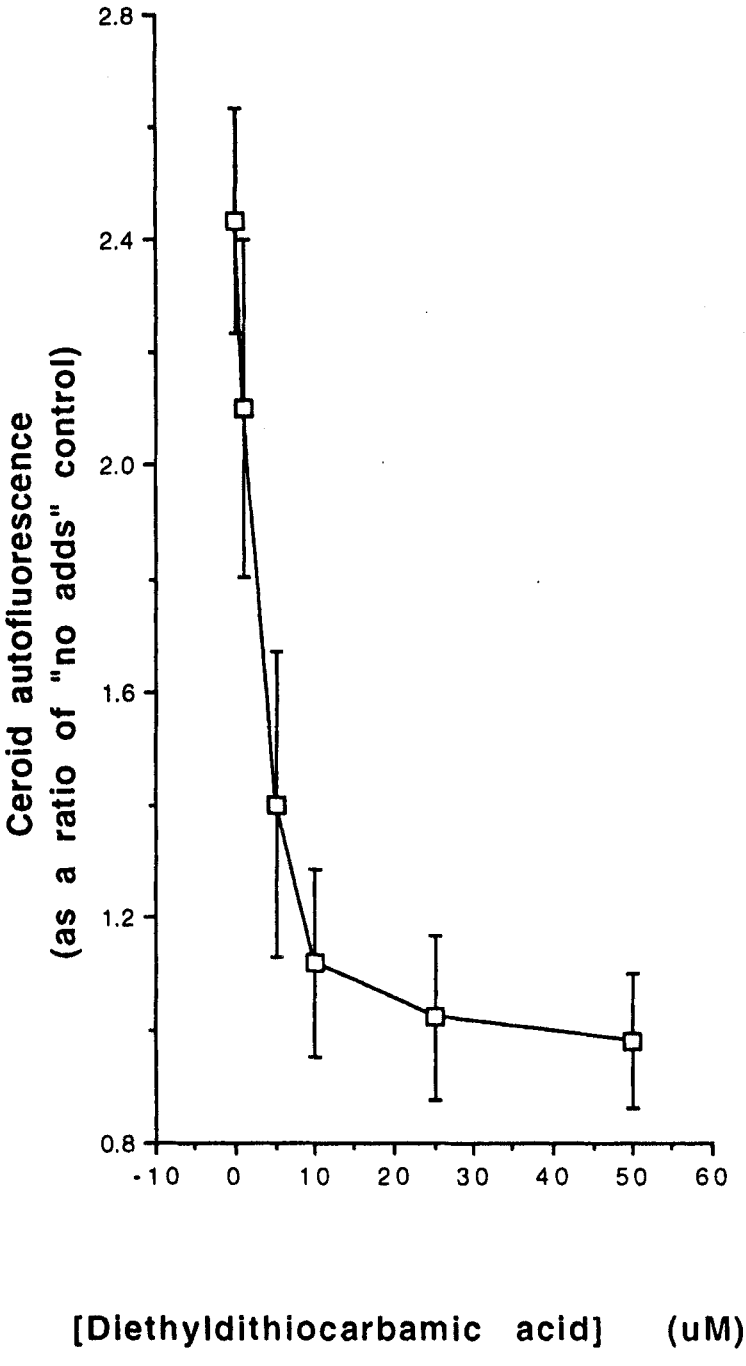

FIGURE 1 The effect of DDC on lipoprotein-mediated ceroid accumulation in P388D$_1$s. **(A)** The effect of DDC (1–50 μM) on ceroid accumulation in P388D$_1$s on culture with CL/BSA over 24 hours is shown.

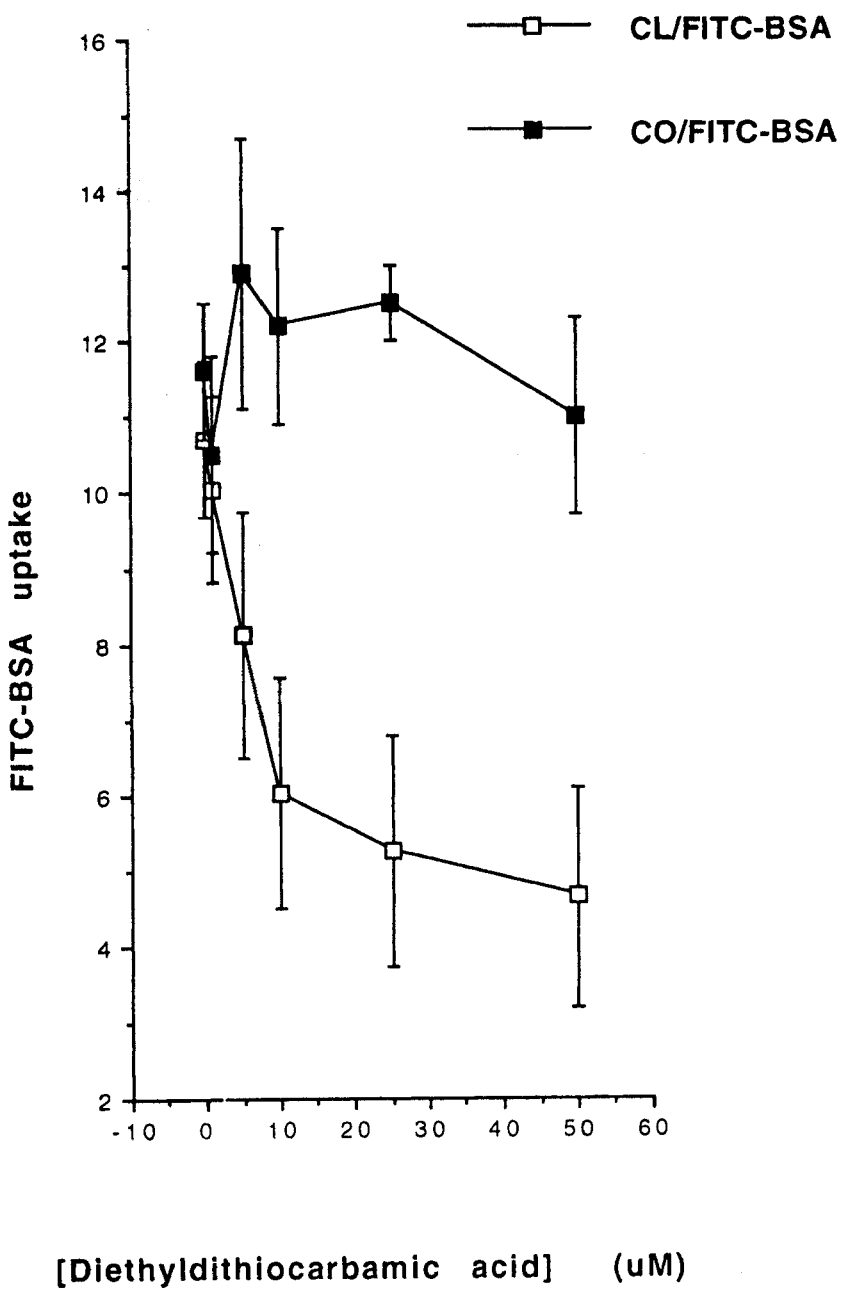

FIGURE 1 *continued* **(B)** The effect of DDC (1–50 μM) on lipoprotein uptake by P388D$_1$s on culture with CL/FITC-BSA over 24 hours is shown. Also shown is the effect of DDC on the uptake of CO/FITC-BSA. Values in this and subsequent figures are expressed as the mean ± standard deviation of 3 experiments. In each experiment cell cultures were performed in quadruplicate.

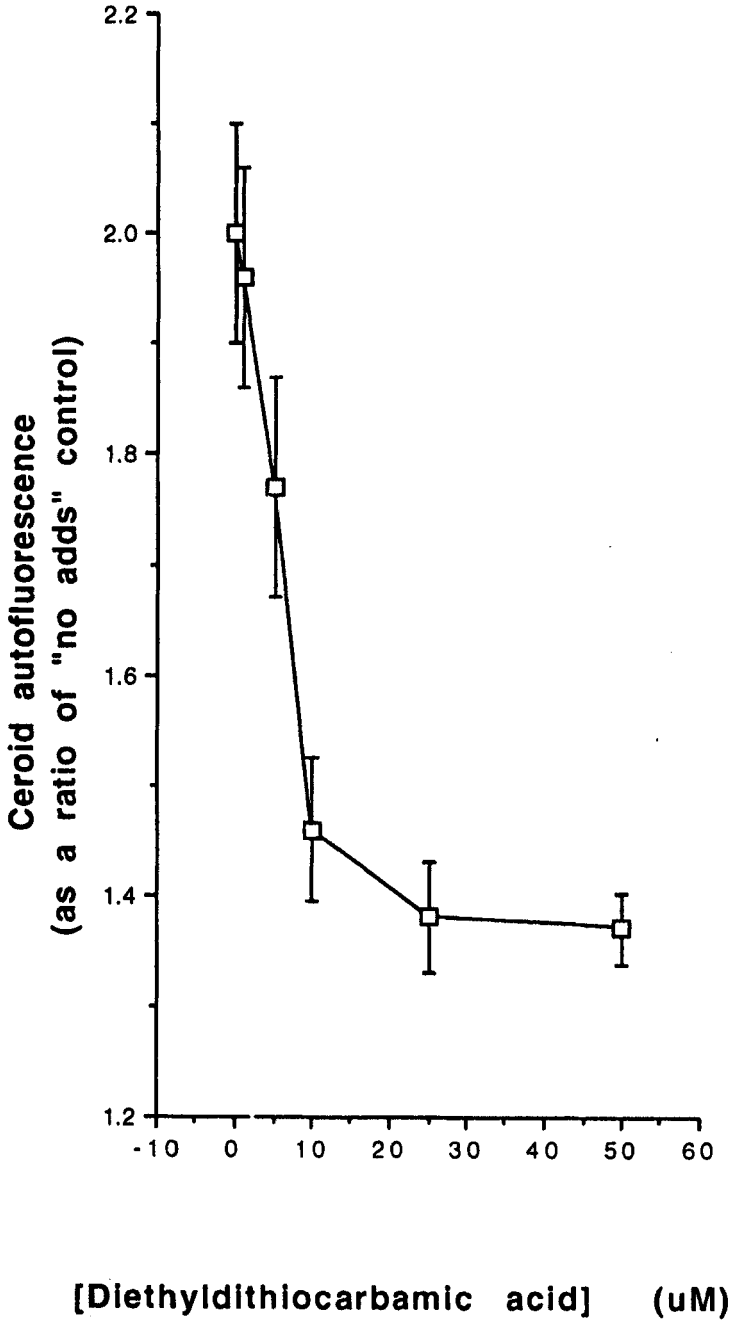

FIGURE 2 The effect of DDC on lipoprotein-mediated ceroid accumulation in human monocyte-macrophages. The effect of DDC (1–50 μM) on ceroid accumulation in human monocytes on culture with CL/BSA over 24 hours is shown.

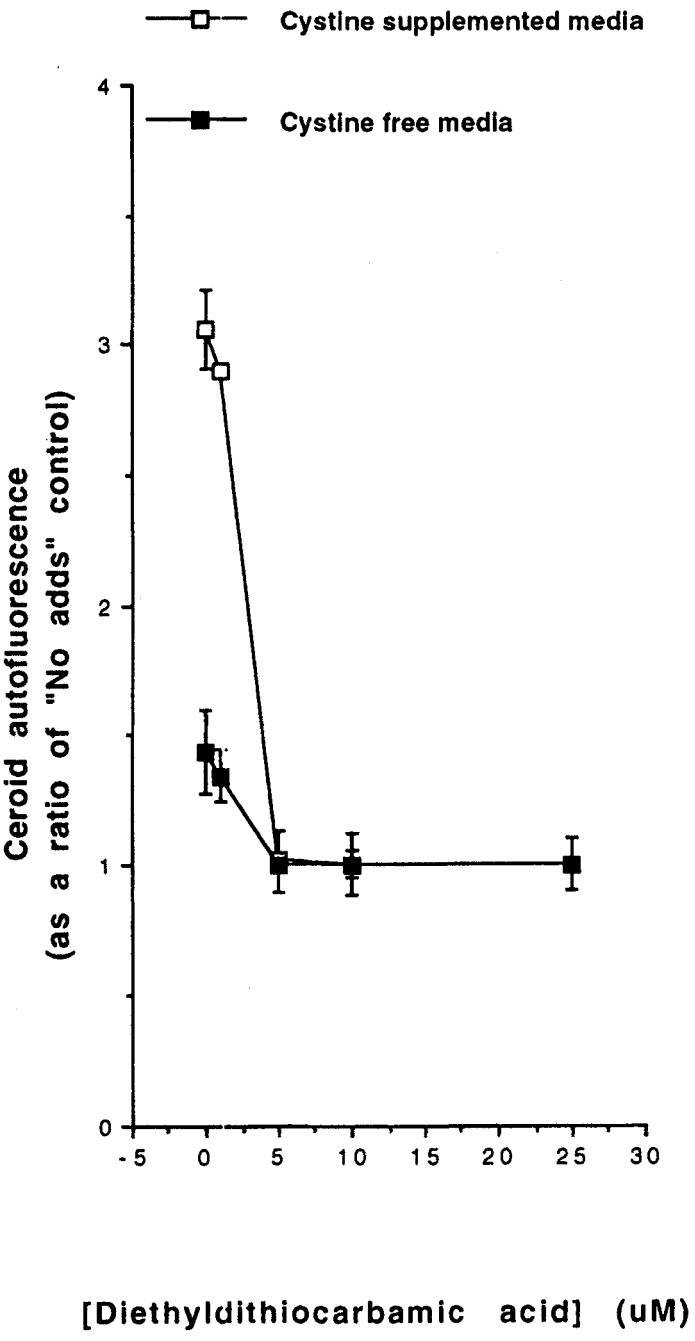

FIGURE 3 The effect of DDC on ceroid accumulation in media with and without cystine. **(A)** The effect of 0–25 μM DDC on ceroid accumulation in P388D$_1$s cultured with CL/BSA for 24 hours in either cystine-deficient or cystine-replete (200 μM cystine) is shown.

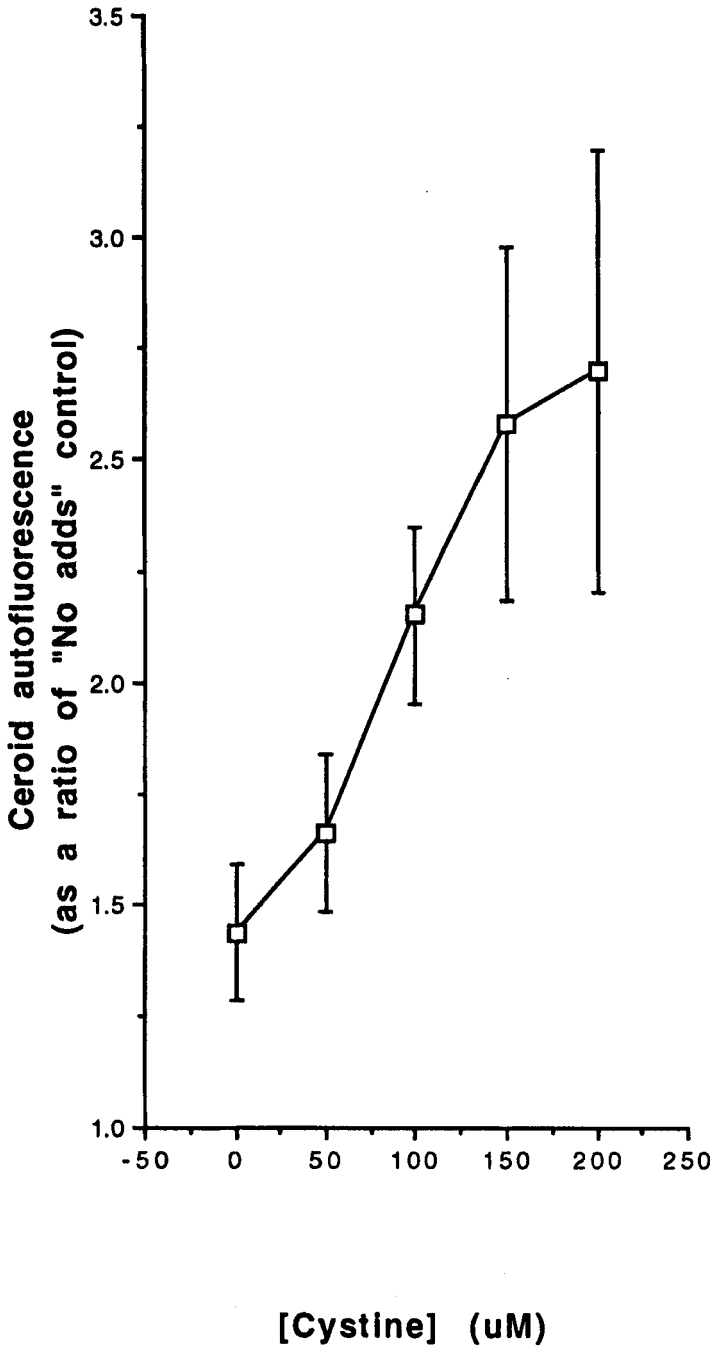

FIGURE 3 *continued* **(B)** The effect of cystine upon ceroid accumulation, produced on culture with CL/BSA over 24 hours was studied by the supplementation of cystine-deficient media (0–200 μM cystine).

absence of cells suggests that the metal-chelating properties of this compound play little role in its activity. Of particular interest is the low concentration (1–5 μM) of DDC required to bring about an effective inhibition of cell-mediated CL/BSA oxidation, ceroid accumulation and FITC-BSA uptake. As previously shown, ceroid accumulation appears to be a good intracellular marker of the presence of oxidised extracellular lipoprotein[12,14,15].

Many activities have been attributed to DDC, including effects on arachidonic acid metabolism[34], SOD activity[35], free radical scavenging (including nitric oxide, superoxide)[27,28], and P_{450} activity[36]. DDC has also been used both in the chelation therapy of heavy metal poisoning[29] and in AIDS therapy[37]. The manner in which DDC inhibits lipoprotein oxidation and subsequent lipoprotein uptake and ceroid accumulation in macrophages remains unknown, and does not appear to involve any metal-chelating activity. Also, the inhibitory activity of DDC is not reversed by adding cystine (figure 3) and appears to be effective in both a cell line and a primary culture of human monocytes.

What is apparent in these studies is that at very low concentrations (less than 10 μM) DDC can inhibit foam cell formation *in vitro*. It is interesting to note that most, if not all, studies using DDC make use of concentrations above 50 μM and even as high as the millimolar range. We suggest that the underlying mechanisms of DDC activity merit further study, at least within the context of foam cell formation and atherosclerosis.

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