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Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis

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Murine thioglycollate-elicited peritoneal macrophages were cultured in the presence of a variety of fatty acids added as complexes with bovine serum albumin. All fatty acids tested were taken up readily by the cells and both neutral and phospholipid fractions were enriched with the fatty acid provided in the medium. This generated a range of cells enriched in saturated, monounsaturated or polyunsaturated fatty acids, including $n-3$ acids of fish oil origin. Saturated fatty acid enrichment enhanced macrophage adhesion to both tissue culture plastic and bacterial plastic compared with enrichment with polyunsaturated fatty acids. Macrophages enriched with the saturated fatty acids myristate or palmitate showed decreases of 28% and 21% respectively in their ability to phagocytose unopsonized zymosan particles. Those enriched with polyunsaturated fatty acids showed 25–55% enhancement of phagocytic capacity. The greatest rate of uptake was with arachidonate-enriched cells. Phagocytic rate was highly correlated with the saturated/unsaturated fatty acid ratio, percentage of polyunsaturated fatty acid and index of unsaturation, except for macrophages enriched with fish-oil-derived fatty acids; they showed lower phagocytic activity than expected on the basis of their degree of unsaturation. These results suggest that membrane fluidity is important in determining macrophage adhesion and phagocytic activity. However, in the case of phagocytosis, this effect may be partially overcome if the cells are enriched with fish-oil-derived fatty acids. Thus it may be possible to modulate the activity of cells of the immune system, and so an immune response, by dietary lipid manipulation.

INTRODUCTION

In recent years interest has been aroused in the use of $n-3$ polyunsaturated fatty-acid-rich fish oils for protection against, and treatment of, a number of disorders, including atherosclerosis [1–3], inflammation [4–6] and autoimmune diseases [7]. Monocytes and macrophages are involved in the development of atherosclerotic plaques [8,9] and in inflammatory and immune responses [10]. Although monocytes/macrophages are not the 'target' of fish oil treatment, it is reasonable to assume that if the dietary polyunsaturated fatty acids lead to alteration of plasma lipid profiles, as is proposed [11], then the lipid content and composition of macrophages may be affected. Macrophage functions such as secretion, phagocytosis and interaction with other cell types involve intracellular and/or plasma membranes and thus, if macrophage membrane structure is altered, cellular function may also be affected. Therefore it seemed to be important to determine if altered fatty acid composition of macrophages could cause changes in their function.

The fatty acid composition of a variety of cell types can be modified by exposing them to fatty acids complexed to BSA [12,13], and this approach has been used previously with macrophages [14,18]. However, these studies have not used a wide range of fatty acids; in particular, the effects of long-chain polyunsaturated fatty acids have not been investigated.

The fatty acid composition of murine peritoneal macrophages cultured in foetal calf serum (FCS) [18,19] or in serum-free conditions [16–18] has been determined previously, as has the composition of the phospholipid fraction [14]. However, detailed analyses covering more than the major fatty acids are rare.

Reports of the effect of added fatty acids upon the fatty acid composition of murine macrophages have been infrequent, despite the importance of the plasma membrane to the function of these cells. Mahoney *et al.* [14] showed enrichment of the phospholipid fraction of macrophages incubated with BSA complexes of elaidate (*trans*-$C_{18:1,n-9}$) or nonadecanoate ($C_{19:0}$). The effect of culture with palmitate ($C_{16:0}$) or oleate ($C_{18:1,n-9}$), added as BSA complexes, upon the macrophage total lipid fatty acid profile has also been reported [16]; the cellular lipid was greatly enriched in the fatty acid supplied in the external medium. We have previously shown similar enrichment of the total lipid of macrophages cultured in the presence of 0.3 mM-palmitate or -oleate [18]. Recently, Lokesh *et al.* [17] reported the fatty acid composition of the total lipid extracted from macrophages which had been cultured in the presence of eicosapentaenoate ($C_{20:5,n-3}$) or docosahexaenoate ($C_{22:6,n-3}$), added as BSA complexes; the major change to the composition was replacement of arachidonate ($C_{20:4,n-6}$) by the $n-3$ fatty acids. In other studies, the total lipid fatty acid composition of murine macrophages has been modified by adding palmitate, oleate or elaidate directly to cells cultured in delipidated serum, rather than adding the fatty acids as preformed complexes with albumin [19], and the fatty acid composition of the phospholipid fraction was altered by palmitoleic ($C_{16:1,n-9}$), oleic, elaidic, linoleic ($C_{18:2,n-6}$), linolenic ($C_{18:3,n-3}$) or arachidonic acids added in dimethyl sulphoxide [20]. We have previously reported the fatty acid composition of the macrophage neutral lipid fraction [18], but there are no reports of modification of the composition of this lipid fraction.

In this study, the ability of macrophages to take up and incorporate a wide range of fatty acids and the effects of

Abbreviations used: BSTFA, *N,O*-bis-(trimethylsilyl)trifluoroacetamide; FCS, foetal calf serum; MEM, minimal essential medium; PBS, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, pH 7.2; g.c./m.s., gas chromatography/mass spectrometry.

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incorporation of these acids upon adhesion to surfaces and upon phagocytic capacity were investigated. Fatty acids used included myristate ($C_{14:0}$), palmitate ($C_{16:0}$), stearate ($C_{18:0}$), oleate ($C_{18:1,n-9}$), linoleate ($C_{18:2,n-6}$), linolenate ($C_{18:3,n-3}$), arachidonate($C_{20:4,n-6}$), eicosapentaenoate ($C_{20:5,n-3}$) and docosahexanoate ($C_{22:6,n-3}$). To our knowledge, this is the first time that the effects of such a wide range of fatty acids upon macrophage function have been studied.

MATERIALS AND METHODS

Animals

Male PO mice (25 g), bred and housed in the Sir William Dunn School of Pathology animal house, were used.

Chemicals

BSA (fatty-acid free), fatty acids, ATP, Triton X-100, Nonidet P40, zymosan (boiled yeast cell walls), 4-aminoantipyrine, 2,4-dibromophenol, *N*-ethylmaleimide, sodium cholate, cholesterol oxidase, trypsin and *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. FCS and minimum essential medium (MEM) (glutamine-free) were obtained from Gibco, Paisley, Scotland, U.K. Serum-free culture medium supplement (Nutridoma-SP), CoA, acyl-CoA synthetase, acyl-CoA oxidase, glycerophosphate oxidase, glycerokinase, lipase and peroxidase were obtained from Boehringer Corp. Ltd., Lewes, Sussex, U.K. Silicic acid was obtained from BDH, Poole, Dorset, U.K. Solvents were from Fisons Scientific Apparatus, Loughborough, Leics., U.K., and were redistilled before use. An ethereal solution of diazomethane was prepared from Diazald (Aldrich Chemical Co., Gillingham, Dorset, U.K.).

Fatty acid complexes

Fatty acids were complexed with fatty-acid-free BSA in 1:1 molar ratios according to the method of Mahoney *et al.* [14]. Fatty acid (16 μmol) was heated at 70 °C for 60 min in an N_2 atmosphere with a slight excess of 0.015 M-KOH. A solution of defatted BSA (1.15 g) dissolved in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, pH 7.2 (PBS) was added to the K^+ salt of the fatty acid, and the tube was flushed with N_2, capped and placed on a shaker at 37 °C for 48 h. After the salt was entirely dissolved, the pH of the solution was adjusted to 7.2 with KOH and the volume brought to 5.0 ml with PBS. This gave a 3.3 mM stock fatty acid solution which was further diluted with PBS to give a 0.33 mM stock solution. The fatty acid–BSA stock solutions were membrane-filtered and then added to the culture media to give a fatty acid concentration of 33 μM.

Cell culture

Macrophages were obtained from the peritoneal cavity of mice 4 days after intraperitoneal injection of 1.0 ml of thioglycollate broth, and were purified by adherence to plastic Petri dishes treated for tissue culture [21]. The peritoneal exudate cells were resuspended in MEM and cultured in MEM supplemented with 5% (v/v) FCS and 2 mM-glutamine at 37 °C in an air/CO_2 (19:1) atmosphere at a density of 2×10^7 cells per 100 mm-diam. cell culture plate. After 4 h of incubation the tissue culture dishes were washed three times with PBS to remove non-adherent cells. Serum-free tissue culture medium [MEM supplemented with 1% (v/v) Nutridoma-SP and 2 mM-glutamine] was added and the cells were incubated for 16 h at 37 °C in air/CO_2 (19:1). The dishes were washed three times with PBS and fresh tissue culture medium [MEM supplemented with 2 mM-glutamine and 5% (v/v) FCS or 1% (v/v) Nutridoma-SP or 1% (v/v) Nutridoma-SP plus BSA–fatty-acid complexes] added. The final concentration of fatty acid used was 33 μM. After a period of 48 h, the dishes were washed three times with PBS and the adherent cells were removed from the plates using a plastic tissue culture plate scraper. After centrifugation, the harvested cells were resuspended in a small volume of PBS and sonicated (3×15 s at an amplitude of 12 μm in an MSE sonicator), before determination of cellular protein and lipid content or lipid extraction and fatty acid analysis.

Determination of non-esterified fatty acids

Fatty acid concentrations in cell culture media were determined using the acyl-CoA synthetase/acyl-CoA oxidase/peroxidase-coupled reaction procedure of Okabe *et al.* [22].

Cellular lipid and protein content determination

Cellular triacylglycerol and cholesterol contents were determined using the peroxidase-coupled reaction procedures of McGowan *et al.* [23] and Allain *et al.* [24] respectively. To determine the cellular phospholipid content, a portion of the sonicated cell suspension was extracted with chloroform/methanol (2:1, v/v); the lipid extract was dried, hydrolysed at 70 °C in the presence of 0.5 M-HCl, and the inorganic phosphate concentration was measured using the method of Fiske & Subbarow [25]. Protein was solubilized by addition of 0.5 M-triethanolamine/0.2 M-KOH to the sonicated cell extract. Protein concentrations were determined by the method of Bradford [26].

Lipid extraction and fatty acid analysis

Total lipid was extracted using chloroform/methanol (2:1, v/v) as described by Folch *et al.* [27]. Neutral lipids were separated from phospholipids by chromatography through a column of activated silicic acid prewashed with chloroform. The neutral and polar lipids were eluted from the column with chloroform and chloroform/methanol (1:1, v/v) respectively. Fatty acids were prepared by saponification with methanolic 0.5 M-KOH for 60 min at 70 °C and were extracted into ethyl acetate. Fatty acid methyl esters were prepared by reaction with an excess of diazomethane in ether, before separation by g.l.c.

G.l.c. data were recorded with a Hewlett–Packard 5890A gas chromatograph fitted with a 50 m × 0.3 mm bonded phase OV-1 fused silica capillary column, film thickness 0.52 μm. Helium at 2.0 ml/min was used as the carrier gas and the split/splitless injector was used in the split mode with a split ratio of 15:1. Injector and detector temperatures were both 300 °C and the column oven temperature was programmed from 130 °C to 380 °C at 2 °C/min. Results were recorded with a Servoscribe chart recorder and quantitative data were recorded with a Hewlett–Packard 3390A recording integrator. Fatty acid methyl esters were identified by comparison with standards run previously or by g.c./m.s. of the fatty acid trimethylsilyl esters.

Trimethylsilyl esters of fatty acids were prepared by heating with BSTFA for 10 min at 60 °C. Samples of 1 μl were taken for analysis by g.c./m.s. Data were recorded with a VG 70/70F mass spectrometer interfaced to a Varian 2440 gas chromatograph fitted with a SGE split/splitless injection system operated in the split mode with a split ratio of 10:1. The column was a 25 m × 0.2 mm OV-1 bonded-phase fused-silica capillary, film thickness 0.33 μm, terminating 10 mm inside the mass spectrometer ion source. Helium at 1 ml/min (measured in the absence of the mass spectrometer vacuum) was used as the carrier gas. Operating conditions were: injector, transfer line and ion source temperatures, 300, 300 and 280 °C respectively; column oven temperature programmed from 180 to 350 °C at 2 °C/min; accelerating voltage, 4 kV; electron energy, 70 eV; trap current, 1 mA; scan speed, 1 s/decade. Spectra were recorded with a VG 11/250 data system.

Macrophage adhesion assay

Macrophage adhesion was assayed according to the procedure of Rosen & Gordon [28]. Macrophages were prepared and cultured in serum, serum-free medium, or serum-free medium plus fatty acid as described above, at a density of 1×10^5 cells/well in 96-well flat-bottomed tissue-culture-treated plastic or bacterial plastic plates. After incubation for 48 h, plates were washed three times with PBS and adherent cells were fixed with methanol. After staining with 10% Giemsa solution for 10 min, plates were washed with tap water, dried and the retained dye was solubilized in methanol. Stain was quantified by measuring absorbance at 460 nm in an automatic plate reader (Dynatech Laboratories Inc., Alexandra, VA, U.S.A.). The assay is linear between 5×10^3 and 10^5 adherent cells/well [28].

Phagocytosis measurement

Macrophages were prepared and cultured in serum, serum-free medium or serum-free medium plus fatty acid, as described above, at a density of 3×10^5 cells/well in 24-well tissue culture plates. At the end of the culture period the medium was replaced with fresh medium, which differed from that in which the cells had been cultured only by the addition of 50 μg of zymosan/ml. The plates were gently shaken for 30 s on a plate shaker to ensure even dispersal of zymosan, and then centrifuged (1000 *g*, 10 min) to ensure contact between the cells and zymosan. The plates were incubated for 2 h at 37 °C. Unphagocytosed zymosan was removed by washing three times with PBS followed by mild trypsin treatment [2.5% (w/v) trypsin, 15 min]. After a further three washes with PBS, cell-associated zymosan was released by addition of detergent [1% (v/v) Nonidet P40] and counted using a Coulter counter.

RESULTS

Fatty acid uptake, lipid content and cell viability

The uptake of fatty acids by macrophages during a period of 48 h in culture is shown in Table 1; the rate at which the different fatty acids were taken up varied. The lowest rate was observed with arachidonate and the highest with linolenate. Depending upon the fatty acid, between 20% and 48% of the available fatty acid was taken up by the cells over 48 h. There was no indication that the disappearance of fatty acid from the culture medium was due to binding of the fatty acid or fatty-acid–BSA complexes to the cell culture dishes; the fatty acid concentration in the medium was unchanged when medium was incubated for 48 h in cell culture dishes in the absence of cells.

The contents of cholesterol, triacylglycerol and phospholipid in macrophages cultured in different media are shown in Table 1. Culture in serum-free conditions, without added fatty acid, did not significantly alter the cellular content of these lipids. Culture of macrophages in the presence of fatty acids did not alter either the phospholipid or the cholesterol content of the cells. Fatty acids did however lead to an increase in the content of triacylglycerol. The triacylglycerol level of macrophages cultured in the presence of each of the fatty acids was greater than that of cells cultured in the presence of serum or in serum-free medium in the absence of added fatty acids. The increase in triacylglycerol content was most marked for cells cultured in the presence of saturated fatty acids (myristate, palmitate, stearate) or oleate (Table 1).

Culture of macrophages in the presence of fatty acids at a concentration of 33 μM did not cause damage to the cells. Viability of such cells, as measured by exclusion of Trypan Blue, was found to be greater than 90% in the presence of each of the fatty acids used, and culture for 48 h did not change significantly the amount of protein which was adherent to the culture dishes, or the number of adherent cells.

Fatty acid composition

Fatty acid was incorporated into both neutral lipid (Table 2) and phospholipid (Table 3) fractions. This procedure produced, therefore, a range of cells enriched in saturated, monounsaturated and polyunsaturated fatty acids, including those of fish oil origin.

The major fatty acids present in the neutral lipid fraction of macrophages cultured in serum were palmitate and stearate; they comprised 40% and 15% respectively of the total fatty acids (Table 2). The fatty acid composition of the neutral lipid was little changed by replacement of serum with a serum-free culture medium. The neutral lipid of macrophages cultured in either the presence or the absence of serum contained approximately twice as much saturated fatty acid as unsaturated fatty acid; the major

Table 1. Fatty acid uptake and lipid content of murine macrophages in culture

Mouse thioglycollate-elicted macrophages were cultured as described in the Materials and methods section in medium supplemented with 33 μM-fatty acid (added as a BSA–fatty-acid complex; the BSA/fatty acid ratio was 1:1). Medium was removed at the start of the incubation and after culture for 48 h, and fatty acid concentrations were measured [22]. After 48 h the cells were harvested (see the Materials and methods section) and the cellular protein, cholesterol, triacylglycerol and phospholipid contents were determined [23–26]. Phospholipid content is expressed as nmol of lipid phosphorus/mg of protein. Data are means ± S.E.M. from six separate macrophage preparations. Statistical significance (Student's *t* test) of lipid content versus BSA-grown cells is indicated by $*P < 0.05$, $\dagger P < 0.02$, $\ddagger P 0.01$, $\S P < 0.001$.

Supplement	Fatty acid uptake (nmol/48 h per mg of protein)	Cellular lipid content (nmol/mg of protein)		
		Cholesterol	Triacylglycerol	Phospholipid
FCS	–	132±6	51±6	177±14
Nutridoma	–	119±8	49±6	150±12
Nutridoma + BSA	–	115±6	54±6	146±13
+ Myristate	110±9	117±7	91±5§	148±11
+ Palmitate	93±6	125±5	92±7‡	148±7
+ Stearate	126±13	104±9	76±6*	143±12
+ Oleate	101±8	101±5	80±7†	131±15
+ Linoleate	164±10	124±5	70±5	148±8
+ Linolenate	190±9	125±6	67±6	144±9
+ Arachidonate	79±8	129±4	62±5	138±14
+ Eicosapentaenoate	105±7	125±4	69±6	139±11
+ Docosahexaenoate	95±5	127±5	67±8	137±14

Table 2. Fatty acid composition (mol %) of the neutral lipid fraction of mouse peritoneal macrophages

Macrophages were cultured as described in the Materials and methods section. The medium was supplemented with FCS, albumin or fatty acids (33 μM) complexed to BSA. At the end of the culture period (48 h) the cells were washed and harvested and the lipid was extracted [27]. Neutral lipids and phospholipds were separated by silicic acid column chromatography. Fatty acids were prepared by saponification and methylated. The methyl esters were separated and analysed by g.c./m.s. Data are the averages of two to five separate samples; n.d. indicates that a fatty acid was not detected. Only the major fatty acids are shown.

	Fatty acid (mol %)														
Addition to medium	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1,n-9}$	$C_{18:0}$	$C_{18:1,n-6+n-9}$	$C_{18:2,n-6}$	$C_{18:3,n-3}$	$C_{20:4,n-6}$	$C_{20:5,n-3}$	$C_{22:5,n-3}$	$C_{22:6,n-3}$	S/U†	PUFA‡	Index§
Serum*	8	5	40	3	15	14	6	n.d.	4	n.d.	n.d.	n.d.	2.1	10	0.5
BSA	7	6	36	5	16	15	7	n.d.	3	n.d.	n.d.	n.d.	2.1	10	0.5
BSA + $C_{14:0}$	3	14	34	4	18	12	6	n.d.	3	n.d.	n.d.	n.d.	2.2	9	0.4
BSA + $C_{16:0}$	3	4	54	3	14	11	5	n.d.	3	n.d.	n.d.	n.d.	3.0	8	0.4
BSA + $C_{18:0}$	3	5	31	3	38	9	4	n.d.	3	n.d.	n.d.	n.d.	3.3	7	0.4
BSA + $C_{18:1,n-9}$	2	2	29	3	10	37	4	n.d.	3	n.d.	n.d.	n.d.	0.8	7	0.7
BSA + $C_{18:2,n-6}$	4	3	33	3	14	10	26	n.d.	4	n.d.	n.d.	n.d.	1.2	30	0.8
BSA + $C_{18:3,n-3}$	6	3	35	3	12	9	7	12	4	n.d.	n.d.	n.d.	1.3	23	0.8
BSA + $C_{20:4,n-6}$	5	4	34	3	11	11	6	n.d.	14	n.d.	n.d.	n.d.	1.2	20	0.5
BSA + $C_{20:5,n-3}$	6	3	35	2	15	11	4	n.d.	3	6	1	1	1.4	15	0.7
BSA + $C_{22:6,n-3}$	5	4	33	4	12	11	5	n.d.	3	n.d.	n.d.	5	1.2	13	0.7

* Data taken from [18]
† Saturated/unsaturated fatty acid ratio.
‡ Percentage of polyunsaturated fatty acid.
§ Index of unsaturation value = Σ (% unsaturated fatty acid × no. of double bonds)/100.

Table 3. Fatty acid composition (mol %) of the phospholipid fraction of mouse peritoneal macrophages

Macrophages were cultured as described in the Materials and methods section. The medium was supplemented with FCS, albumin or fatty acids (33 μM) complexed to BSA. At the end of the culture period (48 h) the cells were washed and harvested and the lipid was extracted [27]. Neutral lipids and phospholipids were separated by silicic acid column chromatography. Fatty acids were prepared by saponification and methylated. The methyl esters were separated and analysed by g.c./m.s. Data are the averages of two to five separate samples; n.d. indicates that a fatty acid was not detected. Only the major fatty acids are shown.

	Fatty acid (mol %)															
Addition to medium	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1,n-9}$	$C_{18:0}$	$C_{18:1,n-6+n-9}$	$C_{18:2,n-6}$	$C_{18:3,n-3}$	$C_{20:4,n-6}$	$C_{20:5,n-3}$	$C_{22:1,n-3}$	$C_{22:4,n-3}$	$C_{22:5,n-3}$	$C_{22:6,n-3}$	S/U†	PUFA‡	Index§
Serum*	5	2	23	1	23	14	7	n.d.	18	n.d.	n.d.	n.d.	n.d.	2	1.2	27	1.2
BSA	8	3	21	2	18	15	5	n.d.	14	n.d.	n.d.	n.d.	1	1	1.3	22	1.0
BSA + $C_{14:0}$	3	12	29	2	21	15	6	n.d.	8	n.d.	n.d.	n.d.	n.d.	n.d.	2.0	14	0.6
BSA + $C_{16:0}$	1	4	34	2	20	16	5	n.d.	7	n.d.	n.d.	n.d.	n.d.	n.d.	2.1	12	0.5
BSA + $C_{18:0}$	3	4	24	4	31	17	6	n.d.	8	n.d.	n.d.	n.d.	n.d.	n.d.	1.8	14	0.7
BSA + $C_{18:1,n-9}$	1	1	19	4	19	37	7	n.d.	9	n.d.	n.d.	n.d.	n.d.	n.d.	0.7	16	0.9
BSA + $C_{18:2,n-6}$	1	1	20	4	22	6	38	n.d.	7	n.d.	n.d.	n.d.	n.d.	n.d.	0.8	45	1.1
BSA + $C_{18:3,n-3}$	1	1	24	3	23	7	8	16	11	n.d.	n.d.	n.d.	n.d.	n.d.	1.0	41	1.4
BSA + $C_{20:4,n-6}$	1	1	20	2	24	9	6	n.d.	28	n.d.	n.d.	n.d.	n.d.	n.d.	0.9	34	1.4
BSA + $C_{20:5,n-3}$	1	1	21	2	20	10	4	n.d.	6	12	2	3	2	2	0.8	41	1.9
BSA + $C_{22:6,n-3}$	2	1	21	4	18	10	4	n.d.	10	1	3	4	1	13	0.8	35	1.7

* Data taken from [18]
† Saturated/unsaturated fatty acid ratio.
‡ Percentage of polyunsaturated fatty acid.
§ Index of unsaturation value = Σ (% unsaturated fatty acid × no. of double bonds)/100.

unsaturated fatty acid was oleate and only 10% of the fatty acids were polyunsaturated (Table 2). The proportions of different fatty acids are presented as the saturated/unsaturated fatty acid ratios, the percentage of polyunsaturated fatty acid and the index of unsaturation (Table 2).

The fatty acid composition of the neutral lipid of macrophages was altered after 48 h in culture in medium containing an added fatty acid (Table 2). Generally, the neutral lipid fraction was enriched solely in the fatty acid added to the medium; addition of a saturated fatty acid increased the saturated/unsaturated fatty acid ratio from 2.1 to as much as 3.3 (addition of stearate), and lowered the index of unsaturation (Table 2). Addition of a polyunsaturated fatty acid increased the proportion of that polyunsaturated fatty acid in the neutral lipid fraction; the saturated/unsaturated fatty acid ratio was decreased to 1.2–1.4, and the index of unsaturation was increased (Table 2). The fish oil $n-3$ fatty acids and linolenate were not present in control cells but were incorporated into the neutral lipid after addition of these fatty acids to the medium.

Culture in the presence of fatty acids increased the triacylglycerol content of the macrophages, although this increase was not statistically significant for cells cultured in the presence of polyunsaturated fatty acids (Table 1). Since triacylglycerols are one of the major constituents of the neutral lipid fraction, the alterations observed in the fatty acid composition of this fraction are accompanied by a change in absolute amounts of the fatty acids present. Thus a fatty acid whose proportion is increased in the neutral lipid fraction will also be present in a greater amount in this fraction.

In the phospholipid fraction of macrophages cultured in serum, the major fatty acids were palmitate and stearate; they each comprised over 20% of the total fatty acids (Table 3). The content of polyunsaturated fatty acid was higher in the phospholipid fraction compared with the neutral lipid fraction; it was 27%, and the major portion of this was arachidonate, which comprised 18% of the total fatty acids. This composition was only slightly altered by substitution of serum with albumin (Table 3).

Addition of a fatty acid to the culture medium produced marked variations in the composition of the phospholipids and hence in the saturated/unsaturated fatty acid ratio, the percentage polyunsaturated fatty acid and the index of unsaturation (Table 3).

Addition of a saturated fatty acid decreased the percentage of polyunsaturated fatty acids in the phospholipid fraction (Table 3). For example, addition of palmitate increased the saturated/unsaturated fatty acid ratio from 1.2 to 2.1, and lowered the index of unsaturation from 1.0 to 0.5 (Table 3). Addition of a polyunsaturated fatty acid to the culture medium increased markedly the proportion of polyunsaturated fatty acids present in the phospholipid fraction. For example, addition of eicosapentaenoate increased the proportion of polyunsaturated fatty acids in the phospholipid fraction to 41%, decreased the saturated/unsaturated fatty acid ratio to 0.8 and increased the index of unsaturation to 1.9 (Table 3). Addition of the fish-oil-derived fatty acids eicosapenaenoate and docasahexaenoate had the largest effect on these parameters.

In most cases, the phospholipid fraction became enriched solely in the fatty acid added to the medium. For example, addition of linolenate increased the proportion of this fatty acid in the phospholipid fraction from non-detectable to 16% of the total (Table 3). Addition of fatty acids other than arachidonate resulted in a decrease in the proportion of arachidonate in the phospholipid (Table 3). Addition of arachidonate to the medium increased the proportion of arachidonate in the phospholipid fraction from 15% to 28% (Table 3). Addition of fish-oil-derived fatty acids increased the proportion of $n-3$ fatty acids in the phospholipid fraction from 2% to 22% (for docosahexaenoate-grown cells) or 31% (for eicosapentaenoate-grown cells); the proportion of arachidonate was decreased to less than 10% (Table 3). Addition of eicosapentaenoate or docosahexaenoate increased the proportion of C_{22} fatty acids, some containing fewer double bonds than the fatty acid added (Table 3). This suggest that elongation and saturation of these fatty acids can occur in macrophages.

Addition of linoleate caused a marked decrease in the proportion of arachidonate in the phospholipid fraction of the macrophages, despite the incorporation of linoleate (Table 3). This finding supports the view that these cells lack the $\delta-6$ desaturase enzyme responsible for the initial step in the conversion of linoleate to arachidonate; Chapkin *et al.* [29] have reported that macrophages did not incorporate ^{14}C from linoleate into arachidonate.

In contrast to the situation with the neutral lipid fraction, there was no change in the phospholipid content of macrophages cultured in the presence of different fatty acids (Table 1). Since there was no change in the absolute amount of phospholipid, an increase in the proportion of a fatty acid in the phospholipid fraction will be accompanied by an increase in the absolute amount of that fatty acid, and by a decrease in proportions and amounts of other fatty acids. The changes in fatty acid composition of the phospholipid fraction which occur without an increase in the amount of phospholipid present in the cells are consistent with a high rate of turnover of fatty acids in this fraction.

Macrophage adhesion

The effect of the addition of fatty acids to the culture medium for 48 h on the ability of macrophages to remain adherent to two surfaces, tissue culture plastic and bacterial plastic, was tested. Adhesion to either surface was unaffected by replacement of FCS by albumin (Table 4). In agreement with the results of Rosen &

Table 4. Adhesion of fatty-acid-enriched murine thioglycollate-elicited peritoneal macrophages to different surfaces

Macrophages were cultured on tissue-culture-treated plastic or bacterial plastic as described in the Materials and methods section. The medium was supplemented with FCS, a serum-free medium supplement, albumin or fatty acids (33 μM) complexed to albumin. At the end of the culture period adhesion to the two surfaces was tested as described in the Materials and methods section. Data are means ± S.E.M. ($n = 6$) of absorbance of Giemsa at 460 nm; this absorbance is directly proportional to the number of adherent cells [28]. Statistical significance (Student's t test): versus Nutridoma+BSA (on the same surface): $*P < 0.001$; versus palmitate (on the same surface): $\dagger P < 0.05$, $\ddagger P < 0.01$, $\S P < 0.001$.

	A_{460}	
Supplement	Tissue culture plastic	Bacterial plastic
FCS	0.198 ± 0.018†	0.117 ± 0.008‡
Nutridoma-SP	0.194 ± 0.012‡	0.106 ± 0.009‡
Nutridoma-SP + BSA	0.215 ± 0.020	0.105 ± 0.007§
+ Myristate	0.230 ± 0.016	0.127 ± 0.013
+ Palmitate	0.249 ± 0.011	0.159 ± 0.009*
+ Stearate	0.242 ± 0.013	0.118 ± 0.008‡
+ Oleate	0.242 ± 0.013	0.120 ± 0.004‡
+ Linoleate	0.218 ± 0.008†	0.123 ± 0.012†
+ Linolenate	0.209 ± 0.011†	0.115 ± 0.015†
+ Arachidonate	0.207 ± 0.014†	0.102 ± 0.008§
+ Eicosapentaenoate	0.205 ± 0.012†	0.095 ± 0.006§
+ Docosahexaenoate	0.198 ± 0.015†	0.089 ± 0.005§

Gordon [28], macrophages adhered less well to bacterial plastic than to tissue culture plastic. In the presence of serum, approx. 60% of the cells which were adherent to tissue culture plastic were also adherent to bacterial plastic; this proportion was 55% in the absence of serum, 50% in the presence of albumin, and 45–64% in the presence of various fatty acids.

Compared with the controls (Nutridoma plus BSA), there was a tendency for addition of polyunsaturated fatty acids to decrease adhesion and for addition of saturated fatty acids to increase adhesion on both surfaces (Table 4). However, except for that obtained for cells cultured on bacterial plastic in the presence of palmitate, these results are not statistically significant (Table 4). The greatest degree of adhesion to both surfaces was observed with palmitate addition, whereas the poorest adhesion was observed with the addition of the fish-oil-derived $n-3$ fatty acids (Table 4). Macrophages cultured in the presence of palmitate showed significantly greater adhesion to bacterial plastic than those cultured in the presence of each of the other fatty acids, except for myristate (Table 4). Macrophages cultured in the presence of palmitate showed significantly greater adhesion to tissue culture plastic than those cultured in the presence of linoleate, linolenate, arachidonate, eicosapentaenoate or docosahexaenoate (Table 4). Addition of arachidonate, eicosapentaenoate or docosahexaenoate resulted in 80–85% and 55–65% of the adhesion observed with palmitate to tissue culture or bacterial plastic respectively.

Cultures were performed with medium containing fatty-acid–BSA complexes but in the absence of cells. After 48 h there was no detectable protein adherent to the dishes (results not shown). Inclusion of [^{14}C]methylated BSA in the culture medium did not result in any detectable radioactivity adherent to the dishes whether they contained cells or not; in all cases > 98%, and in most cases 100%, of the label was recovered in the medium after 48 h (results not shown). Consequently, it is considered that the effects described here are due to alteration of the macrophages by the fatty acids rather than to an interaction between the culture dishes and the fatty-acid–BSA complexes.

Phagocytosis

Phagocytosis of unopsonized zymosan particles was measured at 37 °C after 48 h of culture in the presence of various fatty acids. The uptake of zymosan by cells cultured in medium containing FCS was $(3.6 \pm 0.28) \times 10^6$ zymosan particles/2 h, equivalent to approx. 12 particles/cell. Zymosan uptake by cells cultured in FCS was linear with time for 60 min, after which it plateaued (results not shown). In contrast, zymosan uptake by cells cultured in the absence of serum (including those cultured in medium supplemented with fatty acids) was linear for up to 120 min (results not shown). The rate of phagocytosis of unopsonized zymosan by cells cultured in the absence of serum is shown in Table 5. Cells cultured in the absence of serum showed a reduced rate of phagocytosis. The rate for cells cultured in the presence of albumin was $(5.7 \pm 0.44) \times 10^5$ particles/h; approx. 16% of the rate for serum-grown cells. Addition of the saturated fatty acids myristate or palmitate caused a significant decrease in the rate of phagocytosis, which was lowered by 28% (myristate) or 21% (palmitate). The effects of stearate or oleate, which both lowered the rate of phagocytosis, were not statistically significant. Addition of myristate caused the lowest rate; i.e. $(4.1 \pm 0.34) \times 10^5$ particles/h. Addition of polyunsaturated fatty acids increased phagocytosis by 25–55% in comparison with controls (Table 5). Addition of arachidonate resulted in the greatest rate of phagocytosis; $(8.8 \pm 0.44) \times 10^5$ particles/h. In general, phagocytic rate increased with increasing degree of unsaturation of the added fatty acid, except for the two fish-oil-derived fatty acids ($C_{20:5,n-3}$, $C_{22:6,n-3}$), which showed lower phagocytic activity than expected based upon their degree of unsaturation (Table 5).

Table 5. Phagocytosis of unopsonized zymosan particles by murine macrophages enriched in various fatty acids

Macrophages were cultured as described in the Materials and methods section in serum-free medium supplemented with 33 μM fatty acid (added as a BSA–fatty acid complex; the BSA/fatty acid ratio was 1:1). After 48 h the medium was replaced with fresh medium also containing 50 μg of zymosan/ml. Phagocytosis was measured after 2 h at 37 °C, as described in the Materials and methods section. Results are shown as means ± S.E.M. of particle uptake/h from three to six separate measurements. Statistical significance versus BSA control (Student's t test): *$P < 0.05$; †$P < 0.02$; ‡$P < 0.01$; §$P < 0.001$.

Medium supplement	Phagocytosis	
	Rate (particles/h)	(% of control)
BSA	572500 ± 44900	100
+ Myristate	414700 ± 34400†	72
+ Palmitate	451600 ± 29500*	79
+ Stearate	488900 ± 31500	85
+ Oleate	511400 ± 45100	89
+ Linoleate	830100 ± 38200‡	145
+ Linolenate	847400 ± 55700‡	148
+ Arachidonate	884100 ± 44300§	154
+ Eicosapentaenoate	723000 ± 45600†	126
+ Docosahexaenoate	773000 ± 37900‡	135

In the experiments described here, fatty-acid–BSA complexes were included in the medium in which phagocytosis was measured. To rule out the possibility that coating of the zymosan particles with lipid–BSA may have had an effect upon binding of the particles, experiments were performed in which the fatty acids were omitted from the medium during phagocytosis. The rates of phagocytosis were almost identical with those measured in the presence of fatty acids (results not shown). Furthermore, the rate of phagocytosis by cells cultured in the presence of serum was identical whether the uptake was measured in the presence or absence of serum, or in the presence of fatty acids (results not shown).

The rate of phagocytosis of zymosan particles opsonized with fresh PO mouse serum has also been investigated; the rate of phagocytosis of these particles was greater than that of unopsonized zymosan (results not shown). Fatty acids caused similar effects to those observed with unopsonized zymosan: saturated fatty acids decreased the rate of phagocytosis, whereas polyunsaturated fatty acids increased the rate (results not shown).

DISCUSSION

Interactions of macrophages with the extracellular matrix and with other cells are important in tissue repair, inflammation, immunity and atherosclerosis [28]. Many of these interactions, including binding to other cell types such as endothelial cells during tissue repair, migration into tissues, interaction with T-lymphocytes during an immune response, uptake of lipoproteins and stimulation by growth factors secreted by other cell types, depend upon specific plasma membrane receptors [10,30]. The fatty acid composition of the membrane influences membrane fluidity [31,32]; changes in membrane fluidity would be expected to modify receptor function and/or the activity of signal transduction mechanisms. For example, the number of insulin receptors and their affinity for insulin are strongly influenced by the lipid composition of the plasma membrane in the liver [33], erythrocyte [34] and adipocyte [35], and there are specific effects

of unsaturation of fatty acids in the membrane upon these properties in some cell lines [36,37]. Furthermore, adenylate cyclase activity is influenced by the fatty acid composition of membranes in fibroblast [38,39] and ovary [40] cell lines.

Mahoney *et al.* [14] have calculated that, under steady-state conditions, fluid-phase endocytosis results in internalization of an amount of the macrophage plasma membrane equivalent to the entire surface area within 33 min. Membrane internalization during phagocytosis depends upon the size and number of particles ingested. However, uptake of a single sheep erythrocyte results in internalization of approx. 7% of the macrophage surface area [14]. These calculations suggest that turnover of phospholipids in the macrophage membrane is rapid so that incorporation of fatty acids provided extracellularly into the macrophage plasma membrane should occur readily. Furthermore, if fatty acid is present in the medium, then synthesis of fatty acids *de novo* by mammalian cells in culture is inhibited [12]. Under these conditions, extracellular fatty acids are taken up into macrophages, incorporated into cellular membranes [14–16], stored intracellularly [15,41] or oxidized, depending upon the conditions and availability of other oxidizable substrates [42–44].

In this study we have investigated the effects of the addition of nine fatty acids to the culture medium on lipid content and the fatty acid composition of the neutral lipid and phospholipid fractions of macrophages after 48 h in culture. The effects of the resultant changes in composition of the lipid upon two macrophage functions that involve the plasma membrane, i.e. adhesion and phagocytosis, were also studied. The concentration of each fatty acid in the culture medium (33 μM) is a physiological concentration; the total plasma free fatty acid concentration in the fed state is approx. 0.3 mM [45], so the individual concentrations of the major fatty acids would be in the range 30–130 μM. The fatty acids were added as complexes with albumin to mimic the physiological situation and to avoid possible toxic effects of the free acids or solvent; the fatty acid/BSA ratio was maintained at 1:1; the physiological ratio rarely exceeds 2:1 [46].

The rates of fatty acid uptake measured here (Table 1) are similar to those calculated from rates of incorporation of [^{14}C]-palmitate, -oleate and -elaidate into cellular material of the macrophage [14,16,42]. Once taken up, fatty acids are incorporated into both neutral and phospholipid fractions, and the fatty acid composition of these lipid fractions is changed. These experiments provide, therefore, a range of cells enriched in saturated, monounsaturated or polyunsaturated fatty acids (Tables 2 and 3), and these cells differ from one another in saturated/unsaturated fatty acid ratio, percentage polyunsaturated fatty acid and index of saturation.

The fatty acid composition of the phospholipid fraction of murine peritoneal macrophages cultured in FCS (Table 3) was similar to the composition reported previously [14]. However, the phospholipid fraction in the present work contained lower proportions of palmitate and stearate, but similar proportions of oleate and arachidonate. We confirmed the observation of Mahoney *et al.* [14] that culture of macrophages in the absence of serum does not significantly alter the phospholipid fatty acid composition (Table 3).

Although modification of the total lipid fatty acid profile of macrophages by addition of fatty acids to culture medium has been previously reported [16–19], the range of fatty acids used in a single investigation has been limited, and reports of the effects of fatty acids on the composition of the phospholipid fraction are few [14,20]. There have been no previous reports of the effects of fatty acids on the composition of the neutral lipid fraction of macrophages.

Little is known about the process of macrophage adhesion, which is important during interactions *in vivo* such as binding to endothelial cells and migration into tissues, although Rosen & Gordon [28] have identified the involvement of various receptors and defined several requirements for this process using '*in vitro*' adhesion assays. Macrophage phagocytic activity is a major membrane-associated event in which particles are bound to specific or non-specific membrane receptors and then surrounded by the cell membrane, forming phagocytic vesicles. The processes of internalization, fusion with lysosomes and digestion which follow require attachment of macrophage receptors to ligands on the particle. If the ligands are not present at sufficient density for recognition to occur or if the receptors are not sufficiently mobile in the membrane, this process stops at the binding stage [10,30]. It is likely that the processes of macrophage adhesion and phagocytosis will be influenced by membrane structure, in particular by the fluidity of the membrane; the latter can be influenced markedly by the lipid composition [31,32].

There have been no previous reports of the effect of alteration of the fatty acid composition of phospholipids upon macrophage adhesion. However chick neural retinal cells which were enriched with saturated fatty acids (myristate, palmitate, stearate, arachidate) showed increased cell adhesion, whereas those enriched with unsaturated fatty acids (oleate, linoleate, linolenate, arachidonate) showed decreased adhesion [47]; it was suggested that these effects were due to changes in cell surface fluidity [47]. In the present work there was a tendency for the enrichment of macrophage phospholipids with saturated fatty acids to increase adhesion and for enrichment with polyunsaturated fatty acids to decrease adhesion (Table 4). Adhesion of macrophages that were enriched with saturated fatty acids was greater than that of those cells enriched with unsaturated fatty acids. This suggests involvement of membrane fluidity in the adhesion process, perhaps in the maintenance of the correct environment for receptor action. This effect may be important in the development of atherosclerosis, which is characterized by an initial adhesion of monocytes to the site of endothelial injury, followed by release of growth factors and chemoattractants which recruit more monocytes and macrophages to the damaged area. Later there is accumulation of lipid by these cells which may then develop into foam cells [8,9]. There is a positive correlation between the intake of diets rich in saturated fatty acids and the development of atherosclerosis, and it is considered that this is due to the presence of an increased plasma concentration of LDL-cholesterol in subjects ingesting such a diet [48–50]. However, the present results suggest another causal link; the saturated fatty acid diet may decrease the level of unsaturation of the monocyte and macrophage membranes, so promoting adhesion to damaged endothelial cells, a process important in the development of an artherosclerotic plaque. Diets rich in $n-6$ polyunsaturated fatty acids are associated with a lower rate of development of atherosclerosis; such diets have a cholesterol-lowering effect [51]. Fish-oil ($n-3$) fatty-acid-rich diets also have a hypolipidaemic effect [11], and this has been linked with the observed lowered incidence of coronary heart disease in individuals who consume large amounts of fish [1–3]. The present results suggest one further factor that is influenced by the polyunsaturated fatty acids, i.e. a decrease in monocyte/macrophage adhesion.

There have been three previous studies on the effects of modification of the fatty acid composition upon phagocytosis by murine macrophages [14,16,20]. Mahoney *et al.* [14] showed decreased phagocytosis of antibody-coated sheep red blood cells by macrophages which had been enriched with nonodecanoate or elaidate, both of which increased the saturated/unsaturated fatty acid ratio in the phospholipids. Schroit & Gallily [20] showed a 2-fold difference in phagocytosis between macrophages enriched with palmitoleic, oleic, linoleic, linolenic, arachidonic or elaidic acids, and the phagocytic activity was correlated

linearly with index of unsaturation and saturated/unsaturated fatty acid ratios of the phospholipids; highest phagocytic activity was observed for arachidonate-enriched cells [20]. Phagocytic uptake of sheep red blood cells was reduced by 75% for palmitate-enriched macrophages and by 20% for those enriched with oleate [16].

In the present study, macrophages enriched in the saturated fatty acids myristate or palmitate showed decreased rates of uptake of unopsonized zymosan particles by phagocytosis, whereas those enriched in polyunsaturated fatty acids displayed increased phagocytic activity (Table 5). This suggests that membrane fluidity is an important determinant of phagocytic activity; macrophage phagocytic activity was correlated with parameters of membrane fluidity, determined from phospholipid fatty acid composition. There was a strong positive correlation between phagocytosis and the percentage polyunsaturated fatty acid content ($r = 0.928$). The relationships between phagocytosis and the saturated/unsaturated fatty acid ratio or the index of unsaturation were also good ($r = -0.747$ and $r = 0.783$ respectively). The index of unsaturation is probably most closely related to membrane fluidity, since it takes into account not only the proportion of unsaturated fatty acids but also the number of double bonds. Macrophages enriched in fish-oil-derived fatty acids show a lower phagocytic activity than expected on the basis of the expected change in fluidity of the plasma membrane of these cells. These results indicate that membrane fluidity is important in determining macrophage phagocytic activity, but that this may be partially overcome if the cells are enriched with fish-oil-derived fatty acids. Such an effect, combined with the possible decreased adhesivity of macrophages enriched with $n-3$ polyunsaturated fatty acids (see above), may be important in the treatment of inflammatory and autoimmune disorders.

One further factor that may be of importance when considering treatment of such disorders is the different profile of eicosanoids that would be produced by macrophages enriched with $n-3$ fatty acids [4,52,53]. Determination of the effects of fatty acids upon receptor activity and secretion of eicosanoids and cytokines by macrophages will be necessary to provide more information on basic mechanisms for the effect of diets containing such fatty acids.

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