*Immunology* 1979 **36** 199

# Macrophage fatty acid composition and phagocytosis:

## EFFECT OF UNSATURATION ON CELLULAR PHAGOCYTIC ACTIVITY

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*Received* 16 *March* 1978; *accepted for publication* 8 *May* 1978

**Summary.** In order to manipulate the physical properties of the macrophage membrane, methods were developed which potentiated the incorporation of exogenously supplied fatty acids into membrane lipids. Chromatograms of macrophages which were grown in the presence of a variety of fatty acids demonstrated that exogenously supplied unsaturated fatty acids (palmitoleic, oleic, elaidic, linoleic, linolenic and arachidonic acids) were readily incorporated into the cells and selectively altered the fatty acyl composition of macrophage phospholipids. Up to 38% of the total cellular phospholipids were found to be derived from the exogenously added fatty acid supplements.

The incorporation of the different fatty acids into cellular phospholipids had striking effects on cellular phagocytic activity. These effects were found to correlate with the degree of unsaturation, and the cis- or trans-double bond configuration. Thus, macrophage phagocytic ingestion rates of $^{125}$I-labelled *Shigella flexneri* were found to alter by more than 2-fold after the cells were cultivated in the presence of cis unsaturated fatty acids.

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0019-2805/79/0200-0199$02.00


## INTRODUCTION

The macrophage plasma membrane, as other biological membranes, is composed of a fluid lipid matrix. This has been demonstrated by direct physical measurements of the membrane lipid core using electron paramagnetic resonance (EPR) spectroscopic analysis (Schroit, Gallily & Rottem, 1976). Furthermore, biological studies have demonstrated that the engulfment of opsonized particles requires free Fc receptors for multiple and sequential interactions between the Fc portion of the particle-bound IgG (Griffin & Silverstein, 1974). Accordingly, the rate of receptor-dependent ingestion of particulate material by macrophages should depend upon the physico-chemical properties of the membrane lipid phase. This would allow for either random diffusion or controlled migration of these receptors in the membrane plane. According to the fluid mosaic model of cell membrane structure (Singer, 1971; Singer & Nicholson, 1972) macrophage phagocytic activity should be particularly susceptible to changes in membrane fluidity which itself is

dependent upon the lipid composition of the lipid bilayer matrix.

The effect of lipids on the activity of the reticuloendothelial (RES) system has been studied in a variety of experimental systems. It has been shown that triglycerides of a variety of fatty acids (Cooper & West, 1962; Cooper, 1964) and polyunsaturated fatty acids (Spratt & Kratzing, 1975; Meade & Mertin, 1976) stimulate RES activity, whereas alcohol esters (Diluzio & Wooles, 1964; Blickens & DiLuzio, 1965) and cholesterol (Dianzani, Torrielli, Canuto, Garcea & Feo, 1976) depress RES function and macrophage phagocytic activity, respectively. These alterations of macrophage function have not been explained, though suggestions have been made which range from activation of inactive cells to toxicity following intravenously administered lipid emulsions. Interestingly, cellular plasma membranes have rarely been analysed to detect compositional alterations resulting from these treatments. Recently, however, it has been shown that an increased plasma membrane cholesterol level (Dianzani *et al.*, 1976) and changes in membrane fatty acid composition (Schroit *et al.*, 1976; Mahoney, Hamill, Scott & Cohn, 1977) play a role in phagocytosis as in other eukaryotic plasma membrane oriented processes.

The present communication extends these findings and relates them to the role of the membrane lipid phase on the phagocytic process by determining phagocytic ingestion rates of compositionally modified mouse peritoneal macrophages in monolayer culture.

## MATERIALS AND METHODS

*Macrophages*

Macrophages obtained from the peritoneal exudates of female C57Bl/6 mice 4 days after an intraperitoneal injection of 2 ml thioglycollate were washed and suspended in Dulbeccos' modified Eagles medium (DMEM) containing $2 \times 10^{-2}$ M dl-desthiobiotin (DMEM + db) (Wisnieski, Williams & Fox, 1973). The cells were cultured overnight in tissue culture microtest plates (6 mm well diameter; $10^5$ cells/0·1 ml growth media/well; Nunc, Denmark) or in plastic petri dishes (90 mm diameter; $25 \times 10^6$ cells/30 ml growth media/plate; Nunc, Denmark) at 37° in a 5% $CO_2$ humidified incubator. Non-adhering cells were then removed by intensive rinsing and fresh medium was added. The remaining plastic-adhering cell population consisted of more than 95% macrophages as determined by morphological and phagocytic criteria.

*Macrophage fatty acid replacements*

Macrophage cultures prepared as described above were grown overnight. The medium was then replaced with DMEM + db containing 24 μg/ml fatty acids (Sigma Chemical Co., Mo.) prepared by adding 0·1 ml fatty acid stock solution (12 mg/ml in ethanol/dimethylsulphoxide [DMSO], 1 : 9) to 50 ml growth medium. The fatty acid supplemented growth medium was replaced with freshly prepared medium every 48 h for 4 days. Following an additional 48 h incubation, the various assays were carried out.

*Fatty acid analysis*

Cells grown as described above for 7 days were removed from the petri dishes with a rubber policeman and washed twice in saline. Lipids were extracted from the cells by treatment with chloroform/methanol (2 : 1) (Folch, Lees & Sloane-Stanley, 1957) for 30 min at room temperature. Neutral lipids were separated from polar lipids by chromatography through a column (9 × 60 mm) of activated silicic acid (100 mesh, Mallinckrodt Chemical Works, Mo.) prewashed with chloroform. The neutral and polar lipids were eluted from the column with 30 ml chloroform and 30 ml chloroform/methanol (1 : 1), respectively (Hanahan, 1960).

Aliquots of the total phospholipid fraction (1 : 1 chloroform/methanol silicic acid eluate) were evaporated under $N_2$ and transmethylated with 3 ml methanolic boron trifluoride in sealed tubes under $N_2$ for 10 min at 100° (Morrison & Smith, 1964). Water (2 ml) was then added to the cooled tubes and the methyl esters were extracted twice using 5 ml petroleum ether. The petroleum ether was then evaporated under $N_2$. The fatty acid methyl esters were dissolved in hexane and analysed by gas chromatography using a Packard model 840 instrument equipped with a polar column (200 × 0·3 cm, 15% diethyleneglycol adipate on chromosorb W). Fatty acids were identified by their retention times relative to standard methyl ester mixtures (Supelco, Inc., Bellefonte, Pa.) and quantitated by a computerized area computation system.

*Determination of lipid phosphorus*

Aliquots from total cell lipid extracts were evaporated under $N_2$ until dry, ashed with 0·03 ml 10% $Mg(NO_3)_2$ in 95% ethanol, and hydrolysed with 0·3 ml 0·5 N HCl for 15 min at 100°. The

amount of inorganic phosphate was then determined according to the phosphomolybdate complex-ascorbic acid reduction method of Ames (1966). Ashed $KH_2PO_4$ served as a phosphorus standard. Samples were read at 820 $\mu$m.

*Determination of cholesterol*

Macrophage cholesterol was determined by a modification of the procedure developed by Moore, Patzer, Barenholtz & Wagner (1977), using the enzyme cholesterol oxidase to determine serum cholesterol (Allain, Poon, Chan, Richmond & Fu, 1974). Aliquots of the total cell lipid extracts and standard cholesterol solution were evaporated under $N_2$ until dry and solubilized in 0·03 ml 10% taurodeoxycholate (Sigma Chemical Co., Mo.). The solution was diluted to 1 ml with phosphate buffered saline (pH 7·2) and 0·01 ml cholesterol oxidase (2·5 u/ml; Supelco, Inc., Bellefonte, Pa.) was added. The reaction mixtures were incubated at 37° for 3 h to ensure complete cholesterol oxidation. The obtained cholest-4-en-3-one was separated from the reaction mixture as described by Moore *et al.* (1977).

*Phagocytic assay*

Alcohol-killed *Shigella flexneri* were fixed with 0·25% glutaraldehyde and iodinated with Na[$^{125}$I] (carrier free; Amersham, England) according to the chloramine T procedure of Carpenter (1966). The specific activity of the labelled bacteria was $10^{-2}$ c.p.m./bacterium.

The phagocytic ingestion rate of macrophages which had been grown in tissue culture microtest plate wells was assayed by incubating the cells with 0·05 ml of graded amounts of $^{125}$I-labelled *Shigella* for 60 min at 37°. Thereafter, the macrophage monolayers were intensively washed to remove non-phagocytosed particles. Phagocytosis of $^{125}$I-labelled *Shigella* was assessed by counting each individual well of a cut tissue culture micro-test plate in a gamma scintillation spectrometer (Packard model 5110).

*Quantitation of macrophages by incorporation of $^{86}$rubidium ($^{86}$Rb)*

The number of cells in different fatty acid-treated macrophage populations was determined by the $^{86}$Rb uptake assay as described by Walker & Lucas (1972).

*Quantitation of phagocytic data*

Maximal initial rates of macrophage phagocytosis (Vmax values) have been quantitated by regression analysis of double reciprocal plots of $^{125}$I-labelled *Shigella* added versus $^{125}$I-labelled *Shigella* ingested. These calculations are based on the findings that the phagocytic process follows enzyme kinetics in a manner analogous to carrier-mediated membrane transport mechanisms (Schroit & Gallily, 1977). Measured values of $^{125}$I-labelled *Shigella* uptake have been corrected for the number of viable macrophages present in each fatty acid-treated macrophage population by introducing a viability factor obtained from the $^{86}$Rb assay described above.

*Statistics*

All of the above experiments were done in sextuplicate, 3–4 times. The statistical significance of the data was determined according to Student's *t* test. All data demonstrated a *P* value of 0·05 or less.

## RESULTS

**Incorporation of exogenously supplied fatty acids into macrophages**

In order to manipulate the physical properties of the macrophage membrane, methods were developed which enabled the cells to incorporate exogenously supplied fatty acids. The data presented in Table 1 demonstrates that exogenously supplied fatty acids present in the growth medium are incorporated into macrophages and alter the fatty acyl composition of the cellular phospholipids. The optimal procedure for these experiments included the use of serum-free Dulbecco's modified Eagle's medium supplemented with dimethylsulphoxide-fatty acid solutions. Macrophages grown under these conditions for seven days were more than 98% viable and morphologically identical to freshly explanted plastic adhering cell populations. Analysis by gas chromatography of macrophages grown in medium containing the various fatty acids showed that all of the unsaturated fatty acids employed were selectively incorporated into macrophage phospholipids. The results presented in Table 1 show that the specific enrichment of the phospholipid fatty acid moities ranged from 7·7% to 27·7% in macrophages cultivated in medium containing archidonic (20 : 4) and linoleic (18 : 2) acids, respectively. Moreover, of the total phospholipids of cells grown with palmitoleic (16 : 1), oleic (18 : 1), linoleic (18 : 2), linolenic (18 : 3) and arachidonic (20 : 4) acids, 16·7%, 36·9%, 38·1%, 16·3% and 22·3% of the fatty acid moities were

**Table 1.** Phospholipid fatty acyl composition macrophages cultivated in fatty acid supplemented growth media

| | Phospholipid fatty acyl composition (%) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Growth medium | 12:0 | 14:0 | 15:0 | 16:0 | 16:1 | 18:0 | 18:1* | 18:2 | 18:3 | 20:4 | Net increase (%) |
| DMEM+db+DMSO | 0·4 | 0·4 | 0·1 | 20·7 | 3·3 | 24·7 | 25·2 | 10·4 | | 14·6 | |
| DMEM+db+16:1 | 1·3 | 1·2 | 0·4 | 22·1 | 16·7 | 18·4 | 20·0 | 9·1 | | 10·8 | 13·4 |
| DMEM+db+18:1 | 2·0 | 0·6 | 0·4 | 20·0 | 5·3 | 18·7 | 36·9 | 7·1 | | 9·0 | 11·7 |
| DMEM+db+18:2 | 0·9 | 0·5 | 0·3 | 20·4 | 4·0 | 22·6 | 6·6 | 38·1 | | 6·6 | 27·7 |
| DMEM+db+18:3 | 1·4 | 1·1 | 0·4 | 23·5 | 3·2 | 22·7 | 7·2 | 7·7 | 16·3 | 16·5 | 16·3 |
| DMEM+db+20:4 | 3·0 | 0·6 | 0·3 | 31·6 | 2·1 | 25·1 | 8·6 | 6·4 | | 22·3 | 7·7 |
| DMEM+db+18:1t | 0·6 | 0·2 | 0·4 | 17·7 | 10·0 | 19·7 | 30·4 | 9·4 | | 11·6 | ND† |

* Fatty acid 18:1 includes the total of oleate (18:1, cis) and elaidate (18:1, trans).
† Not determined.

derived from the fatty acids supplemented in the growth media, respectively (Table 1). Macrophages cultivated in growth medium, in growth medium containing dl-desthiobiotin (DMEM+db) or in growth medium containing dl-desthiobiotin and ethanol/DMSO (DMEM+db+solvent) were essentially identical with respect to their fatty acid composition, phagocytic activity and morphology.

**The effect of macrophage fatty acid composition on cellular phagocytic activity**

Preliminary experiments demonstrated that the addition of fatty acids to the growth medium of macrophage cultures had striking effects on cellular phagocytic activity. It can be seen that the addition of various fatty acids to the growth medium resulted in alterations in macrophage phagocytic activity in a manner which appeared to correlate the degree of unsaturation of the fatty acid supplement (Table 2). Thus, macrophage phagocytic activity was enhanced when the cells were cultivated with fatty acids of increasing unsaturation. Furthermore, significant differences in phagocytic activity were observed in macrophages grown in the presence of fatty acids which contain the cis (oleic acid) or trans (elaidic acid) double bond configurations being reduced in the latter (Table 2).

In order to determine whether the observed fatty acid-induced alterations in macrophage phagocytic activity could be due to gross composition/structural differences in cellular composition, several biochemical analysis on the fatty acid enriched macrophages were carried out. The data presented in Table 2 shows that the enrichment of macrophage phospholipids with the various fatty acid moieties directly affected the cholesterol/phospholipid, saturated/unsaturated fatty acid ratios, and the index of unsaturation. Depending upon the fatty

**Table 2.** Phagocytic activity and gross lipid composition of macrophages cultivated in fatty acid supplemented growth media

| Growth medium | Vmax Bacteria/cell/h | Cholesterol* / Phospholipid | % Saturated / % Unsaturated | Index of unsaturation† |
|---|---|---|---|---|
| DMEM+db+DMSO | 184± 6 | 0·572±0·002 | 46·1/53·9 | 1·088 |
| DMEM+db+16:1 | 157± 3 | 0·364±0·011 | 43·4/56·6 | 0·981 |
| DMEM+db+18:1 | 149± 8 | 0·383±0·001 | 41·7/58·3 | 0·924 |
| DMEM+db+18:2 | 173 ±6 | 0·481±0·009 | 44·7/55·3 | 1·132 |
| DMEM+db+18:3 | 211± 6 | 0·370±0·006 | 49·1/50·9 | 1·407 |
| DMEM+db+20:4 | 254±12 | 0·364±0·015 | 60·6/39·4 | 1·127 |
| DMEM+db+18:1t | 87± 2 | 0·409±0·023 | 38·6/61·4 | 1·056 |

* Molar Ratio

† $\frac{(\%\text{ unsaturated fatty acid X no. double bonds})}{100}$

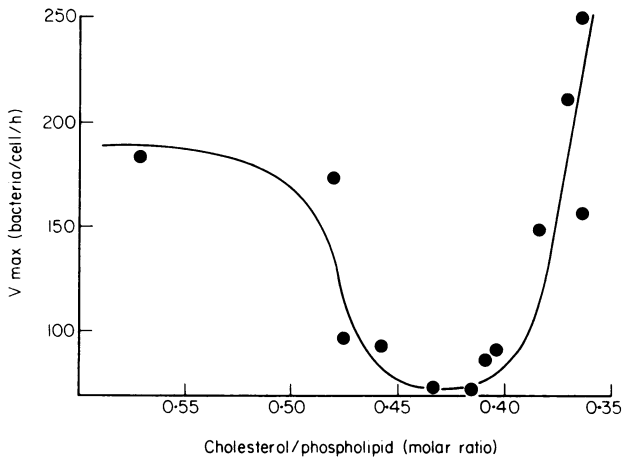


**Figure 1.** The relation of macrophage phagocytic activity to the cholesterol/phospholipid ratio of macrophage lipids.

acid moiety supplemented to the growth medium, the saturated/unsaturated fatty acid ratio and the index of unsaturation varied 2·4 and 1·5 fold, respectively.

Interestingly, the index of unsaturation and saturated/unsaturated fatty acid ratios presented in Table 2 were found to linearly correlate with macrophage phagocytic activity. This is particularly evident from the results obtained using 18 carbon chain length fatty acids of increasing unsaturation (18 : 1, 18 : 2, 18 : 3). Control experiments employing identical culture conditions carried out at 4° or at 37° in the presence of 0·1 M Na azide did not reveal any changes in the capacity of the cells to bind $^{125}$I-labelled *Shigella*.

The dependence of macrophage phagocytic activity on cellular cholesterol/phospholipid ratio is shown in Fig. 1. In contrast to the linear relation of the obtained Vmax values to both the index of unsaturation and the saturated/unsaturated fatty acid ratios, its association to the cellular cholesterol/phospholipid ratios was found to be of the optimal curve type. The results shown in Fig. 1 demonstrate that minimal Vmax values are associated with cholesterol/phospholipid ratios of approximately 0·425 and gradually increase at both reduced and elevated ratios.

## DISCUSSION

Macrophage phagocytic activity is a major membrane-associated event and should, therefore, be dependent upon membrane structure, in particular on the membrane fluidity imposed upon it by its lipid composition. In order to delineate the role of the lipid phase on macrophage phagocytic activity, methods were developed to enable the cells to incorporate exogenously supplied fatty acids of defined physiochemical properties into membrane phosphatides. This approach is based upon the observation that cells grown in medium containing serum or medium containing physiologically important fatty acids utilize these fatty acids. Thus, it has been shown in several instances that the fatty acid composition of the cell reflects that of exogenously supplied fatty acids (Wisnieski *et al.*, 1973; Horwitz, Hatten & Burger, 1974; Mahoney *et al.*, 1977).

The gas-liquid chromatograms of macrophages which were grown in the presence of a variety of fatty acids demonstrated that exogenously supplied unsaturated fatty acids were readily incorporated into the cells and that they selectively altered the fatty acyl composition of macrophage phospholipids. The results obtained by measuring the phagocytic activity of these compositionally altered macrophages demonstrated that the composition of the cellular phospholipid fatty acyl residues greatly affected cellular phagocytic activity. Thus, macrophage phospholipid fatty acyl enrichments with fatty acids of specific molecular properties selectively altered the index of unsaturation and the ratio of saturated to unsaturated fatty acids. Increases in both these parameters were followed by a parallel increase in macrophage phagocytic activity as

determined by measuring and quantitating macrophage Vmax ingestion rates. Moreover, the molar ratio of cellular cholesterol to phospholipids was also found to correlate with macrophage phagocytic activity. As a result of cultivating macrophages in the presence of various fatty acids, different cholesterol to phospholipid ratios were obtained. Although these ratios were found to be dependent on the degree of incorporation of the different fatty acids into the cellular phosphatides, the optimum type of curve obtained does suggest that cholesterol has a regulatory role in the maintenance of cell membrane integrity and phagocytic capability.

The mechanisms and molecular dynamics responsible for the observed increases in macrophage phagocytic activity which closely paralleled the unsaturated fatty acid content of the cell may be due to a parallel decrease in membrane viscosity. Indeed, 'fluidity' of the plasma membrane would be dependent essentially on the fatty acid and cholesterol composition of the lipid bilayer matrix, and possibly on secondary interactions of the lipid with protein elements.

Comparative analysis of the phospholipid composition of polymorphonuclear leucocyte phagolysosomal membranes with total cellular membrane phospholipid has shown that phagocytic vesicles have more saturated fatty acid phospholipids (Mason, Stossel & Vaughan, 1972; Smolen & Shohet, 1974) and have higher cholesterol-to-phospholipid ratios (Mason *et al.*, 1972) than the membrane (Smolen & Shohet, 1974) or the whole cells (Mason *et al.*, 1972), respectively. It has been suggested that this is due to oxidation of fatty acids within the phagocytic vesicle. An alternate interpretation suggests that local aggregation of relatively saturated fatty acids occurs at the site of internalization due to phospholipid partitioning (Berlin, 1976). Recently, Berlin & Fera (1977) have elaborated on the latter interpretation showing that phagocytosis of oil emulsions or polystyrene latex beads by polymorphonuclear cells induces a marked decrease in plasma membrane viscosity. These workers have suggested that the binding of a particle to the membrane of a phagocyte induces a local accumulation of less fluid (more saturated) lipid, which is subsequently internalized, thus resulting in a more fluid cytoplasmic membrane. However, a relatively high membrane unsaturated fatty acid phospholipid composition such as we have obtained here might minimize the frequency of occurrence and availability of saturated fatty acid membrane zones. Accordingly, such a situation would be expected to cause a decrease in phagocytic ingestion rates if phagocytosis was solely dependent upon saturated fatty acid partitioning.

Our results show that the enrichment of cellular membrane phosphatides with unsaturated fatty acids enhanced macrophage phagocytic activity. This enhancement can be explained on the basis of a predisposed 'fluid' membrane which affords rapid membrane component movement and diffusibility. Accordingly, if optimal clustering between the particle and membrane bound particle receptors into an aggregate of critical size and shape is a prerequisite of ingestion by macrophages (Van Oss, Gillman & Neuman, 1974), then lateral diffusion of specific membrane receptors would be of crucial importance to the rate of the ingestion process. Indeed, it has previously been shown that binding and ingestion of antibody-coated erythrocytes by Fc receptors requires movement of these receptors from a random distribution into aggregates or clusters on the macrophage surface to form multiple bridges at points of contact between the cell and the particle (Singer, 1974).

In conclusion, our results on the correlation of macrophage phagocytic activity with cellular fatty acid phospholipid composition suggests that the macrophage membrane lipid phase plays a significant role in cellular endocytic mechanisms.

## ACKNOWLEDGMENTS

This study was supported by research grants from Concern Foundation, Mr and Mrs Frank Lautenberg and Mr and Mrs Laurence Tisch.

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