Arachidonic acid turnover in response to lipopolysaccharide and opsonized zymosan in human monocyte-derived macrophages

Christina C. LESLIE*†‡ and Diane M. DETTY*

*Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, and †Department of Pathology, University of Colorado Health Science Center, Denver, CO, U.S.A.

Macrophages are an important source of the lipid mediators, arachidonic acid metabolites and platelet-activating factor (PAF), produced during inflammation. Studies were undertaken to identify the phospholipid substrates that can serve as a source of arachidonic acid in human monocyte-derived macrophages exposed to the inflammatory stimuli bacterial lipopolysaccharide (LPS) and opsonized zymosan (OpZ). Since PAF is derived from 1-alkyl-2-acyl-glycerophosphocholine, it was of interest to determine if this phospholipid precursor could also serve as a source of arachidonic acid. The day-5 macrophages incorporated 38% of the available [^3H]arachidonic acid into lipid by 4 h, 54% of which was in phospholipid [phosphatidylcholine (PC) > phosphatidylethanolamine (PE) > phosphatidylinositol (PI)]. The proportion of label incorporated into ether-linked PC and PE increased with time. After prelabelling with [^3H]arachidonic acid, the effect of stimuli on the redistribution of label within phospholipids was followed. Without stimulus there was a loss of label from PC, PI and phosphatidic acid by 3 h, but an increase of label in PE. The [^3H]arachidonic acid that was lost from PC in the absence of stimulus was derived solely from the 1-acyl-linked species of PC, whereas an increase in label occurred in the 1-alkyl-linked species of PC. By contrast, LPS stimulation resulted in a preferential, dose-dependent loss of label from PC and PI, which was maximal between 1 and 3 h after adding the LPS. In addition, LPS induced a 35% decrease in the molar quantity of PI in the macrophages but had no effect on the quantity of PC, PE or phosphatidylserine. Stimulation with OpZ also resulted in a loss of label, mainly from PC and PI. Of the total label lost from PC in response to LPS or OpZ, approx. 50% was derived from the 1-alkyl-linked species. The results suggest that phospholipase C- and phospholipase A_2 -mediated mechanisms for arachidonic acid release are activated in human macrophages exposed to the inflammatory stimuli LPS and OpZ. In addition, 1-alkyl-linked PC can serve as a source of arachidonic acid and as a precursor for PAF production in the stimulated macrophages.

INTRODUCTION

Macrophages play a central role in inflammation, tumour cell killing, and regulation of the immune response. The ability of macrophages to synthesize and secrete a multitude of bioactive mediators is critical to their involvement in these processes. Lipid metabolites represent an important class of mediators produced by macrophages (Chensue & Kunkel, 1983; Gemsa *et al.*, 1982). In response to a variety of external stimuli, macrophages synthesize and secrete oxygenated metabolites of arachidonic acid from both the cyclo-oxygenase and lipoxygenase pathways (Kurland & Bockman, 1978; Hseuh, 1979; Rouzer *et al.*, 1980; Hseuh & Sun, 1982). Under similar conditions, these cells also produce the bioactive ether-linked phospholipid, PAF (Roubin *et al.*, 1983). Since the elucidation of the structure of PAF as 1-alkyl-2-acetyl-GPC (Demopoulos *et al.*, 1979), the biosynthesis and biological effects of this lipid mediator have been extensively studied (Benveniste & Vergaftig, 1983; Albert & Snyder, 1983; Mueller *et al.*, 1983).

When macrophages are exposed to a variety of inflammatory stimuli they exhibit increased phospholipid

turnover and the production of these lipid mediators. The enhanced turnover of PI has been observed in many stimulated secretory cells (Michell, 1975), including macrophages (Ogmundsdottir & Weir, 1979; Emilsson & Sundler, 1984; Homma *et al.*, 1982). PI turnover is mediated by the activation of phospholipase C leading to diacylglycerol production (Berridge, 1984). The diacylglycerols are subsequently hydrolysed by a diacylglycerol lipase, resulting in the release of arachidonic acid. Arachidonic acid can also be directly released from phospholipid via the activation of phospholipase A_2 enzymes. The activation of a phospholipase A_2 is also postulated to be the first step in the production of PAF (Benveniste & Vergaftig, 1983). Recent evidence suggests that arachidonic acid metabolism and PAF production may be co-ordinately regulated (Chilton *et al.*, 1983, 1984). The hydrolysis of 1-alkyl-2-arachidonyl-GPC by a phospholipase A_2 would result in the release of arachidonic acid and the formation of lyso-PAF which could then be acetylated to PAF. Release of arachidonic acid from 1-alkyl-linked PC has been demonstrated in rabbit neutrophils (Swendson *et al.*, 1983) and rat alveolar macrophages (Albert & Snyder, 1984).

Abbreviations used: PAF, platelet-activating factor; LPS, lipopolysaccharide; OpZ, opsonized zymosan; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; -GPC, -glycerophosphocholine; PPP, platelet-poor plasma; HBSS, Hanks' balanced salt solution.

‡ To whom correspondence and reprint requests should be sent at: Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson Street, Denver, CO 80206, U.S.A.

The present study was conducted to investigate the pathways involved in arachidonic acid turnover in stimulated human monocyte-derived macrophages. There is little information available on the regulation of arachidonic acid release in human macrophages. Identification of the phospholipid substrates that act as a source of arachidonic acid would provide information as to the nature of the phospholipases activated in these cells in response to physiological inflammatory stimuli. Our results demonstrate that both LPS and OpZ induce PI turnover as well as arachidonic acid release from both 1-acyl- and 1-alkyl-linked species of PC in human monocyte-derived macrophages prelabelled with arachidonic acid.

MATERIALS AND METHODS

Materials

[5,6,8,9,11,12,14,15-³H]Arachidonic acid (87 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). LPS from *E. coli* 0111:B4 was a chromatographically purified Westphal preparation obtained from List Biological Laboratories, Inc. (Campbell, CA, U.S.A.). OpZ was prepared by boiling zymosan (Koch-Light, Haverhill, Essex, U.K.) in saline for 1 h and then washing twice with saline. The zymosan was incubated for 30 min in a freshly prepared human serum pool at 37 °C and then washed twice with saline. LPS-free human serum albumin (Cutters Laboratory, Emeryville, CA, U.S.A.) was dissolved (100 mg/ml) in saline containing 0.1 mg of EDTA/ml, and extracted with di-isopropyl ether/butanol (3:2, v/v) for 30 min (Cham & Knowles, 1976). The extracted albumin solution was evaporated to half the starting volume under N₂ and then dialysed against saline to remove the remaining butanol. A *Limulus* amoebocyte lysate kit from Associates of Cape Cod, Inc. (Woods Hole, MA, U.S.A.) was used to test reagents for LPS. The lower limit of detection for LPS using this assay was 0.1 ng/ml. The phospholipid and neutral lipid standards for t.l.c. were purchased from Serdary Research Laboratories, Inc. (London, Ontario, Canada). The fatty acid methyl ester standards used for gas liquid chromatography were purchased from Supelco, Inc. (Bellefonte, PA, U.S.A.).

Cell isolation and culture

Human mononuclear cells were isolated from the blood of healthy donors on plasma/Percoll gradients (Haslett *et al.*, 1985). This technique minimizes exposure of the cells to LPS. Venous blood was collected in 3.8% citrate and then centrifuged at 300 *g* for 20 min. The platelet-rich plasma was collected and a portion was centrifuged at 2500 *g* for 15 min to produce platelet-poor plasma (PPP). The remaining platelet-rich plasma was clotted at 37 °C after adding CaCl₂ (final concentration 20 mM). This autologous serum was used for culturing the monocytes. The blood cells were resuspended in saline and the erythrocytes sedimented at 1 *g* in 0.6% dextran. The leukocyte-rich fraction was centrifuged at 275 *g* for 6 min and then the pellet resuspended in PPP. The cells were underlaid with 42% Percoll in PPP followed by 51% Percoll in PPP and the gradient was centrifuged for 10 min at 275 *g*. The mononuclear cell fraction was removed from the interface of the plasma and 42% Percoll layer and then centrifuged through 26% Percoll

in PPP at 275 *g* for 10 min. Contaminating platelets remained above the Percoll layer and the mononuclear cells pelleted. The pellet was resuspended in endotoxin-free saline and centrifuged for 7 min at 275 *g*. This wash step was repeated and the pellet was resuspended in HBSS. The mononuclear cells were plated at 1.5×10^7 cells in 35 mm tissue culture wells in HBSS containing 0.1% autologous serum. After a 1 h incubation the adherent monolayer was vigorously rinsed with HBSS to remove the non-adherent lymphocytes. The adherent monocyte monolayer was greater than 97% esterase-positive. Medium M199 containing 5% autologous serum, 20 mM-Hepes buffer and penicillin/streptomycin (100 units/ml and 100 µg/ml) was added to the monocytes and they were visually examined by phase contrast microscopy for platelet contamination. Routinely, the platelet contamination was approx. 10 platelets per 100 monocytes including both free platelets and those adhering to monocytes. For unknown reasons, occasionally a monocyte preparation was more heavily contaminated with platelets and these preparations were not used. The monocytes were cultured for 5 days at 37 °C in a humidified incubator containing air/CO₂ (19:1) with a change of medium on day 3. During this time they acquired both morphological and biochemical characteristics of macrophages (Johnson *et al.*, 1977; Zuckerman *et al.*, 1979; Musson *et al.*, 1980; Newman *et al.*, 1980). As an estimate of cell number, nuclei counts were determined by treating the macrophages with Zapoglobin (Coulter Electronics, Hialeah, FL, U.S.A.) and counting the released nuclei on a Coulter counter (model ZH). Routinely there were 8×10^5 nuclei/well.

Turnover of [³H]arachidonic acid

Day-5 macrophages were incubated for 2 h in serum-free medium M199 (1 ml/well) containing 0.5 µCi of [³H]arachidonic acid/ml complexed to delipidated albumin (0.025% final concentration). Some of the wells were harvested immediately after the labelling period (zero-time). The rest of the wells were rinsed twice with HBSS to remove the label and then reincubated in fresh, serum-free medium M199 containing 0.25% delipidated albumin and either LPS (0.5 µg/ml), OpZ (30–50 particles/cell), or no stimulus. Except for the time-course experiments the macrophages were incubated in the absence of label for 3 h. To harvest the macrophages, the wells were placed on ice, the medium was removed and cold saline was added. The monolayers were rinsed once and then scraped into cold saline with a rubber policeman.

Lipid analysis

Total lipids were extracted from the macrophages (Bligh & Dyer, 1959), evaporated under N₂, and then dissolved in chloroform/methanol (9:1, v/v). Neutral lipids were separated by t.l.c. on silica gel G plates using a solvent system of hexane/diethyl ether/acetic acid (80:20:1, by vol.). For the [³H]arachidonic acid uptake and turnover experiments, the phospholipids were separated by two-dimensional t.l.c. on Redi-coat plates (Supelco, Bellefonte, PA, U.S.A.). The plates were developed first with chloroform/methanol/NH₄OH (13:5:1, by vol.), and in the second dimension with chloroform/acetone/methanol/acetic acid/water (3:4:1:1:0.5, by vol.). After separation, lipids were visualized with I₂ vapour and then scraped into vials

Table 1. Fatty acid profile of human monocytes and monocyte-derived macrophages

Human mononuclear cells were incubated in medium M199 containing 5% autologous serum. After 2 h, half of the wells were rinsed and then harvested (day 0), and the remaining cells were incubated for 5 days. The lipids were extracted, separated into neutral lipid and phospholipid and then fatty acid methyl esters were prepared for g.l.c. The values are the means $\pm$ S.E.M. from three different donors.

Fatty acid	Composition (mol %)		
	Day 0 Phospholipid	Day 5	
		Phospholipid	Neutral lipid
14:0	0.6 $\pm$ 0.1	1.0 $\pm$ 0.6	1.2 $\pm$ 0.1
14:1	0.1 $\pm$ 0.01		0.1 $\pm$ 0.02
15:0	2.4 $\pm$ 0.2	3.4 $\pm$ 0.2	0.2 $\pm$ 0.1
16:0	13.1 $\pm$ 1.9	11.4 $\pm$ 1.4	19.8 $\pm$ 2.0
16:1	1.2 $\pm$ 0.2	1.1 $\pm$ 0.4	3.6 $\pm$ 0.5
17:0	0.4 $\pm$ 0.1	0.3 $\pm$ 0.1	0.3 $\pm$ 0.1
17:1	1.8 $\pm$ 0.4	1.9 $\pm$ 0.3	1.3 $\pm$ 1.1
18:0	25.2 $\pm$ 1.6	20.8 $\pm$ 1.8	9.3 $\pm$ 2.5
18:1	19.4 $\pm$ 0.6	24.2 $\pm$ 1.0	36.0 $\pm$ 1.8
18:2	7.1 $\pm$ 0.5	9.4 $\pm$ 0.5	12.9 $\pm$ 1.5
18:3	0.3 $\pm$ 0.1	0.3 $\pm$ 0.2	0.3 $\pm$ 0.1
20:4	22.5 $\pm$ 0.9	19.9 $\pm$ 0.3	2.2 $\pm$ 0.5
Unidentified	5.8 $\pm$ 0.5	6.3 $\pm$ 1.6	12.9 $\pm$ 2.5

containing 0.5 ml of water. The amount of radioactivity was measured using a Beckman (model LS 8000) liquid-scintillation counter.

To determine the amount of [^3H]arachidonic acid incorporated into ether- and ester-linked species of PC and PE the phospholipids were separated by t.l.c. on silica gel H plates (Analtech, Newark, DE, U.S.A.) using a solvent system of chloroform/methanol/acetic acid/water (50:25:8:4, by vol.). The PC and PE areas were scraped and extracted from the silica gel (Bligh & Dyer, 1959). The PC was hydrolysed with phospholipase C (*Bacillus cereus*, type XIII; Sigma Chemical Co., St. Louis, MO, U.S.A.) and the diacylglycerols were converted to the acetate derivatives with acetic anhydride (Waku *et al.*, 1974). The 1-alkyl- and 1-acyl-linked diacylglycerol acetates were separated by t.l.c. on silica gel G plates using a solvent system of hexane/diethyl ether (3:2, v/v). Samples were visualized with I_2 vapour and then 0.25 cm strips were scraped to determine the distribution of radioactivity. The PE was spotted on silica gel G plates and then exposed to HCl vapour for 30 min to hydrolyse the plasmalogen species (Sun & Horrocks, 1969). PE was separated from lyso-PE on silica gel H plates using a solvent system of chloroform/methanol/acetic acid/water (60:35:8:4, by vol.). To determine the efficiency of hydrolysis with HCl vapour, authentic PE plasmalogen (Avanti Polar Lipids, Inc., Birmingham, AL, U.S.A.) was subjected to the same hydrolysis procedure and then the amount of lyso-PE formed was quantified by lipid phosphorus determination (Rouser *et al.* 1966). Exposure to HCl vapour for 30 min routinely resulted in 71% hydrolysis of the plasmalogen.

G.l.c.

Total lipid extracts of day-0 and day-5 macrophages were separated into total neutral lipid and total phospholipid fractions on silicic acid columns. The neutral lipids were eluted with chloroform and the phospholipids with methanol. Fatty acid methyl esters

were prepared by dissolving the dried lipids in methanol containing 2% H_2SO_4 , flushing with N_2 , and then heating at 70 °C for 1 h. The methyl esters were extracted into hexane and then analysed with a Varian 6000 gas chromatograph with a CDS 401 data processor. A 4 m glass column containing Supelcoport 100/200 coated with 5% DEGS/PS (Supelco, Bellefonte, PA, U.S.A.) was used. With N_2 as the carrier gas, the methyl esters were separated isothermally at a column temperature of 190 °C. The injector and detector temperatures were 200 °C and 205 °C, respectively.

Quantification of lipid content

The total lipid and neutral lipid content of day-5 macrophages were quantified by weight determinations of dried lipid extracts using a Cahn 25 Electrobalance. The total neutral lipid fraction was isolated on silicic acid columns. Total phospholipids were quantified by lipid phosphorus determination (Rouser *et al.*, 1966). The triacylglycerols were separated by t.l.c. with hexane/diethyl ether/acetic acid (80:20:1, by vol.) and extracted from the silica gel (Bligh & Dyer, 1959). Fatty acid methyl esters of the triacylglycerols were prepared and quantified by g.l.c.

Assay for platelet-activating factor

Day-5 human macrophages were incubated for 1 h in serum-free M199 containing 0.25% delipidated albumin in the presence and absence of LPS (*E. coli* 0111:B4, 0.5 $\mu\text{g}/\text{ml}$) or OpZ (30–50 particles/cells). The medium was removed and the monolayers rinsed with saline which was pooled with the medium. The macrophages were scraped in saline and then the cells and medium were extracted separately (Bligh & Dyer, 1959). The phospholipids were separated on silica gel G plates with chloroform/methanol/water (65:35:4, by vol.). The area co-migrating with authentic [^3H]PAF standard was scraped from the plate and extracted from the silica gel (Bligh & Dyer, 1959). The PAF activity was quantified

Fig. 1. Time course of [^3H]arachidonic acid incorporation into phospholipids

Day-5 macrophages were incubated in serum-free medium containing $0.5 \mu\text{Ci}$ of [^3H]arachidonic acid/ml. At the indicated times the cells were extracted and the radioactive lipids separated by two-dimensional t.l.c. The values are the means $\pm$ S.E.M. for four separate experiments.

by measuring [^3H]serotonin release from rabbit platelets (Henson, 1976).

RESULTS

Fatty acid composition

Human monocytes (Stossel *et al.*, 1974; Pawlowski *et al.*, 1983) and tissue macrophages from a variety of animal species (Mason *et al.*, 1972; Scott *et al.*, 1980) contain a high proportion of arachidonic acid esterified to phospholipid compared with other cell types. However, culturing human monocytes for 5 days in serum-containing medium could potentially have had an effect on their fatty acid composition. Consequently, the fatty acid composition of phospholipids from freshly plated human monocytes was compared with that of monocytes cultured for an additional 5 days in 5% serum. The relative amount of arachidonic acid in phospholipids of day-0 compared with day-5 monocytes decreased only slightly from 22.5% to 19.9%, respectively (Table 1). Larger changes occurred in the C_{18} fatty acids as a result of the 5-day incubation with a decrease in stearic acid and increases in oleic acid and linoleic acid. Monocytes cultured for 5 days in the presence of 5% serum accumulated large amounts of neutral lipid. The day-5 macrophages contained 0.3 ± 0.07 mg of total lipid/mg of protein, of which $41.4 \pm 6.3\%$ was neutral lipid and most of the neutral lipid ($83.3 \pm 3.5\%$) (means $\pm$ S.E.M., $n = 3$) was triacylglycerols. The fatty acid composition of the neutral lipids of day-5 macrophages was determined since the triacylglycerols could potentially serve as a source of

arachidonic acid in stimulated cells. In contrast with the phospholipid fraction, arachidonic acid was not a major fatty acid in the neutral lipid fraction and constituted on 2.2% of the total fatty acids (Table 1). The major fatty acids in the neutral lipid fraction were oleic acid > palmitic acid > linoleic acid. The fatty acid profile of the neutral lipid fraction was essentially identical with the fatty acid profile of isolated triacylglycerols (results not shown). The freshly plated monocytes (day 0) contained a negligible amount of neutral lipid; consequently, the fatty acid composition was not determined.

Uptake of [^3H]arachidonic acid

The time course of [^3H]arachidonic acid incorporation into total lipid of day-5 macrophages was determined. Macrophages incubated for 30 min, 2 h and 4 h incorporated $17.0 \pm 1.8\%$, $32.5 \pm 3.8\%$ and $37.8 \pm 4.8\%$ (means $\pm$ S.E.M., $n = 5$) of the label in the medium into total lipid. By 2 h, $53.5 \pm 2.9\%$ (mean $\pm$ S.E.M., $n = 4$) of the label incorporated was in phospholipid and this proportion of label in the phospholipid fraction was constant irrespective of the labelling time (from 10 min up to 24 h). Most of the remaining label was in triacylglycerols ($40.9 \pm 3.1\%$; mean $\pm$ S.E.M., $n = 4$) and only $1.9 \pm 0.1\%$ (mean $\pm$ S.E.M., $n = 4$) of the total label was free fatty acid.

The time course of [^3H]arachidonic acid incorporation into the major phospholipid classes is shown in Fig. 1. Labelling of PC, PI and PS had reached a constant level between 2 and 4 h, whereas the incorporation of label into PE continued to increase. The amount of [^3H]arachidonic acid incorporated into PA at 30 min, 1 h and 4 h was 1250 ± 110 d.p.m., 1740 ± 180 d.p.m. and 1640 ± 160 d.p.m., respectively. By 4 h most of the labelled arachidonic acid was in PC (39.5%), followed by PE (31.9%), PI (23.0%), PS (4.9%) and then PA (0.7%).

Since it was of interest to determine if ether-linked phospholipids could serve as a source of arachidonic acid in the human macrophages, the extent of [^3H]arachidonic acid labelling of these phospholipid species was determined. The time course of [^3H]arachidonic acid incorporation into the ether- and ester-linked species of PC and PE is shown in Fig. 2. The 1-acyl-linked species of PC and PE were preferentially labelled with [^3H]arachidonic acid at early time points, reaching a maximum by 2 h. Labelling of the ether-linked species of PC and PE increased more slowly with time. By 4 h 28% of the label in PC was in the 1-alkyl-linked species and 64% of the label in PE was in PE plasmalogen.

Arachidonic acid turnover

Experiments were conducted to identify the lipid substrates that could serve as a source of arachidonic acid in response to LPS or OpZ in macrophages prelabelled with [^3H]arachidonic acid. Day-5 macrophages were labelled for 2 h with [^3H]arachidonic acid, some of which were harvested immediately (zero-time). The remaining cultures were incubated for 3 h in fresh, unlabelled medium with and without either LPS or OpZ. The changes that occurred in the amount of label in the phospholipids of unstimulated cells and in response to either LPS or OpZ over the 3 h incubation period are shown in Fig. 3. In the absence of stimulus there was a decrease of label in PA, PC, and PI and an increase of label in PE. The major effects of incubating the macrophages with LPS were to diminish the apparent loss

Fig. 2. Time course of [^3H]arachidonic acid incorporation into ether- (○) and ester- (●) linked species of PC (a) and PE (b)

Day-5 macrophages were incubated in serum-free medium containing $0.5 \mu\text{Ci}$ of [^3H]arachidonic acid/ml. At the indicated times the cells were extracted and the phospholipids separated by one-dimensional t.l.c. The PC and PE were isolated and processed as described in the Materials and methods section. The values are the means $\pm$ S.E.M. for three separate experiments.

Fig. 3. Effect of LPS and OpZ on [^3H]arachidonic acid turnover

Day-5 macrophages were incubated in serum-free medium containing $0.5 \mu\text{Ci}$ of [^3H]arachidonic acid/ml. After 2 h, a portion of the cells was harvested immediately (zero-time; ■) and the rest was further incubated for 3 h in unlabelled medium containing either LPS ($0.5 \mu\text{g}/\text{ml}$; □), OpZ (50 particles/cell; ▨), or no stimulus (▤). The cells were extracted and the lipids separated by two-dimensional t.l.c. The values represent the means $\pm$ S.E.M. for 20 separate experiments for no stimulus and LPS, and 10 separate experiments for OpZ.

of label from PA and to stimulate a further 27% and 24% release of [^3H]arachidonic acid from PI and PC, respectively. Similar results were obtained using LPS from other strains of *E. coli* including 026:B6, 055:B5, and K235. In addition, a similar pattern of arachidonic acid mobilization was observed in response to LPS in macrophages prelabelled for 30 min or 4 h (results not shown). The phagocytic stimulus, OpZ, induced a loss of label from PC, PI and PS and also prevented, in part, the apparent decrease of label from PA, which occurred in

the absence of stimulus. In addition, the increase of labelling in the PE fraction that occurred in the absence of stimulus was no longer evident in OpZ-treated macrophages.

The time course of [^3H]arachidonic acid release from PC and PI in response to LPS is shown in Fig. 4. Due to the large amount of variability in the response of cells from different donors, a consistent effect of LPS on arachidonic acid release could not be detected until 1 h after exposure to the stimulus. As shown in Fig. 5 there

Fig. 4. Time course of [^3H]arachidonic acid release from phospholipids of LPS-stimulated macrophages

Details of the experiment are as described in the legend to Fig. 3. The values represent the means $\pm$ S.E.M. for four separate experiments.

was a dose-dependent loss of arachidonic acid from PC and PI in response to LPS.

Since a large proportion of the [^3H]arachidonic acid incorporated into macrophage lipids was in triacylglycerols, the ability of this neutral lipid class to serve as a source of arachidonic acid was determined. In the absence of stimulus for 3 h there was an $7.6 \pm 5.3\%$ loss of labelled arachidonic acid from triacylglycerols. In contrast, LPS and OpZ induced a $12.8 \pm 5.9\%$ and $34.3 \pm 4.9\%$ increase of [^3H]arachidonic acid in triacylglycerols, respectively. This increase of [^3H]arachidonic acid in triacylglycerols in response to LPS or OpZ was only evident by 3 h. At earlier time points (10 min–1 h) there was no consistent effect of the inflammatory stimuli on triacylglycerol labelling. These data suggest that triacylglycerols do not serve as a source of arachidonic acid in response to the inflammatory stimuli. Overall, LPS and OpZ induced a $7.7 \pm 2.5\%$ and $16.5 \pm 3.5\%$ loss of labelled arachidonic acid from total lipid over the 3 h incubation period, respectively, compared with a $5.3 \pm 3.0\%$ loss of label from total lipid in unstimulated macrophages.

Effect of LPS on the quantity of phospholipids

A deacylation–reacylation mechanism for arachidonic acid release mediated by phospholipase A_2 enzymes would not be expected to result in a decrease in the quantity of the phospholipid in the cell. To investigate this possibility, the effect of LPS on the phospholipid content of day-5 macrophages was determined. Incubation of the macrophages with LPS for 3 h had no consistent effect on the content of PC, PE, PS, or sphingomyelin but did induce a $31.2 \pm 30\%$ decrease (mean $\pm$ S.E.M., $n = 4$) in the quantity of PI in the cells. PC ($38.9 \pm 1.8\%$)

was the major phospholipid in day-5 macrophages followed by PE ($29.5 \pm 1.1\%$), PS ($9.8 \pm 0.9\%$) and then PI ($4.3 \pm 0.8\%$). Sphingomyelin constituted $17.5 \pm 0.7\%$ of the phospholipid fraction (means $\pm$ S.E.M., $n = 4$).

Arachidonic acid release from 1-alkyl- and 1-acyl-linked species of PC

Experiments were conducted to determine if the labelled arachidonic acid released from PC, in response to LPS or OpZ, was derived from the 1-acyl- or 1-alkyl-linked species. As shown in Fig. 6, the [^3H]arachidonic acid that was released from PC in the absence of stimulus was derived solely from the 1-acyl-linked species (36% loss after 3 h). In contrast, there was an 11% increase of [^3H]arachidonic in the 1-alkyl-linked species after the 3 h incubation in the absence of stimulus. The loss of [^3H]arachidonic acid from 1-acyl-linked PC, accompanied by an increase of label in 1-alkyl-linked PC, suggests that arachidonic acid was mobilized from the diacyl into the ether-linked species of PC. In response to either LPS or OpZ, there was a further 12% and 14% loss of label, respectively, from the 1-acyl-linked species compared with unstimulated control cells. LPS and OpZ also induced a release of labelled arachidonic acid from the 1-alkyl-linked species resulting in decreases of 35% and 38% , respectively, compared with unstimulated control cells. As shown in Fig. 2, there was a greater amount of label in the 1-acyl-linked species of PC. Consequently, of the total label lost from PC compared with the unstimulated control, LPS and OpZ induced a $52.3 \pm 12.9\%$ and $49.7 \pm 2.3\%$ loss from the 1-alkyl-linked species and a $47.7 \pm 12.9\%$ and $50.3 \pm 2.3\%$ loss from the 1-acyl-linked species, respectively.

PAF production

The LPS- or OpZ-induced release of arachidonic acid from alkyl-linked PC would result in the formation of

Fig. 5. Effect of LPS dose on [^3H]arachidonic acid release

Details of the experiment are as described in the legend to Fig. 3. The values represent the means $\pm$ S.E.M. for four separate experiments.

Fig. 6. Effect of LPS and OpZ on $[^3\text{H}]$ arachidonic acid release from 1-acyl- and 1-alkyl-linked species of PC

Details of the experiment are as described in the legend to Fig. 3. The PC was separated by one-dimensional t.l.c., extracted from the silica gel and subjected to phospholipase C hydrolysis. The diacylglycerols were converted to the acetate derivatives and these were separated by t.l.c. as described in the Materials and methods section. The values represent the means $\pm$ S.E.M. for four separate experiments. ■, zero-time; ▨, 3 h incubation with no stimulus (▨), LPS (□) or OpZ (▤).

lyso-PAF. The ability of LPS or OpZ to promote PAF production was determined. After a 1 h incubation with the stimuli, the total amount of PAF activity produced by the cultures was 4.9 ± 0.7 and 10.9 ± 4.0 ng/ 10^7 cells for LPS and OpZ, respectively, compared with 2.0 ± 1.0 ng/ 10^7 cells (means $\pm$ S.E.M., $n = 4$) produced by unstimulated cells. Of the PAF produced by LPS- or OpZ-treated macrophages, only 23.4% and 16.8%, respectively, was secreted by the cells into the medium, the rest being cell associated. These results demonstrate that in the human macrophages, 1-alkyl-2-arachidonyl-GPC can serve as a precursor for both arachidonic acid and PAF in response to inflammatory stimuli.

DISCUSSION

There is little information available on the regulation of arachidonic acid metabolism and lipid mediator production in human macrophages due to the difficulty in obtaining human material for study. Peripheral blood monocytes are the most readily available source of mononuclear cells from man in which to study these important processes. Human monocytes cultured in the presence of serum have been demonstrated to undergo maturation *in vitro*, acquiring both morphological and biochemical characteristics of macrophages (Johnson *et al.*, 1977; Zuckerman *et al.*, 1979; Musson *et al.*, 1980, 1982; Newman *et al.*, 1980). However, a potential problem in using human monocytes is the possibility of platelet contamination (Pawlowski *et al.*, 1983). The

isolation technique we employed to isolate mononuclear cells using plasma/Percoll gradients resulted in approx. 10 platelets/100 monocytes. There are several characteristics of the responses we observed which indicate that the results are not due to the presence of platelets. The time-course of the LPS-induced turnover of arachidonic acid is more typical of macrophages compared with the rapid responses of platelets. Also, human platelets do not respond to the stimuli used in this study (LPS and OpZ). Finally, it is unlikely that the platelets in the monocyte cultures remain functional after 5 days of incubation. However, it cannot be ruled out that the initial interaction of platelets with the monocytes did not have an effect on the response of the day-5 macrophages to the stimuli.

In human monocyte-derived macrophages treated with either LPS or OpZ, the greatest loss of labelled arachidonic acid occurred in PC and PI. Certain aspects of our results suggest that PI turnover induced by LPS is mediated by a phospholipase C. The decrease in labelled arachidonic acid from phospholipids induced by LPS or OpZ was accompanied by an increase of label in PA compared with unstimulated control cells. It is well documented that the turnover of PI via a phospholipase C is associated with the production of PA (Michell, 1975). However, in unstimulated macrophages there was a relatively large amount of endogenous arachidonic acid release from PA. Therefore, the stimuli could be acting either by suppressing this endogenous turnover or by having no effect on the pool of PA that is turning over endogenously but inducing formation of another pool of PA. LPS also resulted in a decrease in PI content of the macrophages. This decrease would not be expected if PI degradation occurred by a direct deacylation mechanism, since lyso-phospholipids are rapidly reacylated. Also, we were unable to detect any phospholipase A_2 activity toward PI in cell-free homogenates of the human macrophages, whereas activity against PC was demonstrable (C. C. Leslie & D. M. Detty, unpublished work). Although these results suggest that PI turnover induced by LPS may be mediated by a phospholipase C, we were unable to detect the production of diacylglycerols when tested between 10 min and 3 h after adding stimulus (results not shown). It is possible that diacylglycerol production in these cells is very transient, making its detection difficult. Further studies are needed to clarify the mechanism of PI turnover in LPS-stimulated human macrophages.

The pattern of $[^3\text{H}]$ arachidonic acid incorporation and turnover that occurred in unstimulated human macrophages suggests that arachidonic acid is mobilized from diacyl species of phospholipid into the ether-linked species of PC and PE. Although $[^3\text{H}]$ arachidonic acid was preferentially incorporated in the 1-acyl-linked species of PC and PE at early time points, the proportion of label in ether-linked PC and PE continued to increase after the diacyl species had reached a constant level of labelling. When human macrophages were prelabelled with $[^3\text{H}]$ arachidonic acid and then incubated in the absence of any stimulus, the amount of label in PE increased. At the same time the amount of label in PC, PI and PA decreased, suggesting that these latter species turn over rapidly and that the arachidonic acid lost from these species may be incorporated into PE. A similar phenomenon has been observed in rabbit alveolar macrophages except that there was no decrease of label

in PI; consequently, PC was the source of label for incorporation into PE (Sugiura *et al.*, 1984). In platelets, arachidonic acid was transferred from both PI and PC and PE plasmalogen in response to thrombin (Rittenhouse-Simmons *et al.*, 1977). In our study, LPS had no apparent effect on this transfer phenomenon into PE, whereas the increase of label in PE that occurred in the absence of stimulus was no longer evident in OpZ-treated cells. Compared with LPS, OpZ may induce the production of greater amounts of arachidonic acid metabolites, which would make less arachidonic acid available for transfer to PE.

The mobilization of arachidonic acid from 1-acyl- to 1-alkyl-linked PC is supported by the data showing that when cells prelabelled with [3 H]arachidonic acid were reincubated in the absence of stimulus there was a loss of label from the 1-acyl-linked species of PC with a concomitant increase of label in the 1-alkyl-linked species. These results indicate that diacyl-PC turns over rapidly and the arachidonic acid lost from this species is incorporated into 1-alkyl-linked PC. Similarly, arachidonic acid mobilization from 1-acyl- to 1-alkyl-linked PC has been observed in rabbit alveolar macrophages (Sugiura *et al.*, 1984). Recently, microsomes from rabbit alveolar macrophages have been shown to catalyse the direct transfer of arachidonic acid from 1-acyl- to 1-alkyl-linked PC via a CoA-independent mechanism (Sugiura & Waku, 1985). In response to LPS or OpZ, however, both the 1-acyl- and 1-alkyl-linked species of PC exhibited an apparent loss of arachidonic acid. Approx. 50% of the label lost from PC was derived from the 1-alkyl-linked species, similar to results reported for rat alveolar macrophages (Albert & Snyder, 1984) and rabbit neutrophils (Swendsen *et al.*, 1983) with calcium ionophore as a stimulus. An important consideration in the interpretation of this data is the possibility that the observed decrease of label from 1-acyl-linked PC in LPS- or OpZ-stimulated cells does not represent the release of free arachidonic acid but rather represents label that has been mobilized into the 1-alkyl-linked species. If the latter possibility is correct then the 1-alkyl-linked species of PC could actually represent the major source of free arachidonic acid from PC in the stimulated macrophages. Arachidonic acid release from PC occurs in a variety of cell types and is postulated to be mediated by the activation of phospholipase A_2 enzymes. Cell-free homogenates of human monocyte-derived macrophages exhibit phospholipase A_2 activity towards arachidonyl-containing 1-acyl- and 1-alkyl-linked PC (C. C. Leslie & D. M. Detty, unpublished work).

Our results demonstrate a complex pattern of arachidonic acid turnover in phospholipids of human macrophages. In unstimulated cells, arachidonic acid is preferentially incorporated into PI and the diacyl species of PE and PC and is then mobilized to the ether-linked species of PE and PC. In response to inflammatory stimuli several phospholipid species, primarily PI and the ether- and ester-linked species of PC, serve as potential sources of arachidonic acid for oxygenation into the various bioactive mediators. Thus, in stimulated human macrophages, both phospholipase C and A_2 enzymes appear to be activated. In addition, the phospholipase A_2 enzyme(s) that is activated can hydrolyse alkyl-linked PC, thus providing substrate for PAF production. Elucidation of the mechanisms involved in lipid mediator production induced by inflammatory stimuli are crucial to the

understanding of how macrophages function during inflammation.

This work was conducted in the F. L. Bryant, Jr., Research Laboratory for the Mechanisms of Lung Disease and was supported by NIH grant HL 32015. C.C.L. is a Parker B. Francis fellow of the Puritan Bennett Foundation.

REFERENCES

- Albert, D. H. & Snyder, F. (1983) *J. Biol. Chem.* **258**, 97–102
 Albert, D. H. & Snyder, F. (1984) *Biochim. Biophys. Acta* **796**, 92–101
 Benveniste, J. & Vergafig, B. B. (1983) in *Ether Lipids: Biochemical and Biomedical Aspects* (Mangold, H. & Paltauf, F., eds.), pp. 355–376, Academic Press, New York
 Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
 Bligh, E. G. & Dyer, W. I. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
 Bonney, R. J., Wightman, P. D., Davies, P., Sadowski, S. J., Kuehl, F. A. & Humes, J. L. (1978) *Biochem. J.* **176**, 433–442
 Cham, B. E. & Knowles, B. R. (1976) *J. Lipid Res.* **17**, 176–181
 Chensue, S. W. & Kunkel, S. L. (1983) *Clin. Lab. Med.* **4**, 677–694
 Chilton, F. H., O'Flaherty, J. T., Ellis, J. M., Swendsen, C. L. & Wykle, R. L. (1983) *J. Biol. Chem.* **258**, 7268–7271
 Chilton, F. H., Ellis, J. M., Olson, S. J. & Wykle, R. L. (1984) *J. Biol. Chem.* **259**, 12014–12019
 Demopoulos, C. A., Pinckard, R. N. & Hanahan, D. J. (1979) *J. Biol. Chem.* **254**, 9355–9358
 Emilsson, A. & Sundler, R. (1984) *J. Biol. Chem.* **259**, 3111–3116
 Gerns, D., Leser, H., Seitz, M., Deimann, W. & Barlin, E. (1982) *Mol. Immunol.* **19**, 1287–1296
 Haslett, C., Guthrie, L. A., Kopaniak, M. M., Johnston, R. B. & Henson, P. M. (1985) *Am. J. Pathol.* **119**, 101–110
 Henson, P. M. (1976) *J. Exp. Med.* **143**, 937–952
 Homma, Y., Onozaki, K., Hashimoto, T., Nagai, Y. & Takenawa, T. (1982) *J. Immunol.* **129**, 1619–1626
 Hsueh, W. (1979) *Am. J. Pathol.* **97**, 137–147
 Hsueh, W. & Sun, F. F. (1982) *Biochem. Biophys. Res. Commun.* **106**, 1085–1091
 Johnson, W. D., Mei, B. & Cohn, Z. A. (1977) *J. Exp. Med.* **146**, 1613–1626
 Kurland, J. I. & Bockman, R. (1978) *J. Exp. Med.* **147**, 952–957
 Mason, R. J., Stossel, T. P. & Vaughn, M. (1972) *J. Clin. Invest.* **51**, 2399–2407
 Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
 Mueller, H. W., O'Flaherty, J. T. & Wykle, R. L. (1983) *J. Biol. Chem.* **258**, 6213–6281
 Musson, R. A., Shafran, H. & Henson, P. M. (1980) *J. Reticuloendothel. Soc.* **28**, 249–264
 Musson, R. A., McPhail, L. C., Shafran, H. & Johnston, R. B. (1982) *J. Reticuloendothel. Soc.* **31**, 261
 Newman, S. L., Musson, R. A. & Henson, P. M. (1980) *J. Immunol.* **125**, 2236–2244
 Ogmundsdottir, H. M. & Weir, D. M. (1979) *Immunology* **37**, 689–696
 Pawlowski, N. A., Kaplan, G., Hamill, A. L., Cohn, Z. A. & Scott, W. A. (1983) *J. Exp. Med.* **158**, 393–412
 Rittenhouse-Simmons, S., Russel, F. A. & Deykin, D. (1977) *Biochim. Biophys. Acta* **488**, 370–380
 Roubin, R., Tence, M., Mencia-Huerta, J. M., Arnoux, B., Ninio, E. & Benveniste, J. (1983) *Lymphokines* **8**, 249–276
 Rouzer, G., Siakotos, A. N. & Fleischer, S. (1966) *Lipids* **1**, 85–86
 Rouzer, C. A., Scott, W. A., Cohn, Z. A., Blackburn, P. & Manning, J. M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4928–4932
 Scott, W. A., Zrike, J. M., Hamill, A. L., Kempe, J. & Cohn, Z. A. (1980) *J. Exp. Med.* **152**, 324–335

- Stossel, T. P., Mason, R. J. & Smith, A. L. (1974) *J. Clin. Invest.* **54**, 638–645
- Sugiura, T., Katayama, O., Fukui, J., Nakagawa, Y. & Waku, K. (1984) *FEBS Lett.* **165**, 273–276
- Sugiura, T. & Waku, K. (1985) *Biochem. Biophys. Res. Commun.* **127**, 384–390
- Sun, G. Y. & Horrocks, L. A. (1969) *J. Lipid. Res.* **10**, 153–157
- Swendsen, C. L., Ellis, J. M., Chilton, F. H., O'Flaherty, J. T. & Wykle, R. L. (1983) *Biochem. Biophys. Res. Commun.* **113**, 72–79
- Waku, K., Ito, H., Bito, T. & Nakagawa, Y. (1974) *J. Biochem. (Tokyo)* **75**, 1307–1319
- Zuckerman, S. H., Ackerman, S. K. & Douglas, S. D. (1979) *Immunology* **38**, 401–411

Received 9 October 1985/30 December 1985; accepted 16 January 1986