SYNTHESIS OF LEUKOTRIENE C AND OTHER ARACHIDONIC ACID METABOLITES BY MOUSE PULMONARY MACROPHAGES*

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Recent work from this laboratory (1, 2) has demonstrated that mouse resident peritoneal macrophages release the slow-reacting substance (SRS), leukotriene C [5(S)-hydroxy-6(R)-glutathionyl-7,9,11,14-eicosatetraenoic acid; LTC] in response to a phagocytic stimulus. No data are as yet available concerning leukotriene synthesis by other mouse macrophage populations. It is of particular interest to know whether pulmonary macrophages are also able to produce large quantities of leukotrienes because of the sensitivity of the peripheral airways of the lung to SRS (3, 4). In this report, we present the results of studies of zymosan-induced leukotriene synthesis by mouse pulmonary alveolar macrophages, obtained by bronchoalveolar lavage and by pulmonary interstitial macrophages, isolated by enzymatic digestion of lung tissue.

Materials and Methods

Pulmonary Macrophage Cultures. Pulmonary alveolar macrophages (PAM) and pulmonary tissue macrophages (PTM) were obtained from male and female ICR mice weighing 25–30 g (Trudeau Institute Inc., Saranac Lake, NY) by a modification of the method of Blussé Van Oud Alblas and Van Furth (5). Each mouse was anesthetized with an intraperitoneal injection of 5 mg of sodium pentobarbital (Abbott Laboratories, North Chicago, IL). The chest cavity was opened, and the aorta and inferior vena cava were cut. Blood was removed from the lungs by perfusion with calcium- and magnesium-free phosphate-buffered saline containing 1 mM EDTA, which was injected into the right ventricle of the heart until the lung tissue was uniformly white. The trachea was then cannulated, and the lungs were washed five times with 1 ml of buffered saline containing EDTA at 37°C. These washes, which contained PAM, were combined and placed on ice. For culture, the cells were washed once with buffered saline and resuspended at a cell density of 1 × 10⁶/ml in minimum essential alpha medium (α-MEM; Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) containing 10% fetal calf serum. The cell suspensions were then added to 60-mm Diam plastic tissue culture dishes (3 ml), 15-mm culture wells (0.3 ml), or 12-mm glass cover slips (0.1 ml). After 2 h of incubation at 37°C in 95% air/5% CO2, the cultures were washed vigorously to remove nonadherent cells and overlaid with fresh α-MEM containing 10% serum. Cultures contained 2.5 ± 0.1 × 10⁵ adherent cells/60-mm dish.

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Abbreviations used in this paper: BHT, butylated hydroxytoluene; HETE, hydroxyeicosatetraenoic acid; HHT, 12-L-hydroxy-5,8,10-heptadecatrienoic acid; HPLC, high-pressure liquid chromatography; KRPG, Krebs-Ringer phosphate buffer containing 5.4 mM glucose; LTC, LTD, LTE, leukotrienes C, D, and E; α-MEM, minimum essential alpha medium; PAM, pulmonary alveolar macrophages; PGE2, prostaglandin E2; PTM, pulmonary tissue macrophages; SRS, slow-reacting substances; 20:4, arachidonic acid; TXB2, thromboxane B2.

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PTM were isolated from the lung tissue after completion of lavage. The heart and lungs were removed en bloc; the heart, trachea, bronchi, and hilar connective tissue were carefully dissected away. PTM were obtained by mincing the lungs with scissors and incubating the resulting fragments for 60 min at 37°C in Krebs-Ringer phosphate buffer supplemented with 5.4 mM glucose (KRPG) containing trypsin (200 U/ml), collagenase (18 U/ml), and DNAse (140 U/ml). All enzymes were from Worthington Biochemical Corp., Freehold, NJ. The tissue was filtered through sterile gauze, and the cells were washed twice with KRPG. Culture conditions were the same as for PAM except that initial cell suspensions contained 1 × 10^7 cells/ml of medium. Approximately 2.2 ± 0.2 × 10^6 adherent cells/60-mm dish were recovered.

In the case of PTM or PAM cultures to be maintained for longer than 24 h, the washed monolayers were treated with trypsin (50 U/ml in α-MEM) at 37°C for 20 min to remove contaminating fibroblasts. The cells were then washed again with buffered saline and overlaid with fresh α-MEM containing 10% serum.

Characterization of Macrophage Populations. Macrophages cultured on glass coverslips were stained for peroxidase by the method of Kaplow (6) and for nonspecific esterase by the method of Li et al. (7). The phagocytosis of zymosan was quantitated by direct phase contrast microscopy of cells fixed in 2.5% glutaraldehyde following a 60 min exposure to zymosan. Cell viability was assessed by trypan blue exclusion, and cells remained >95% viable throughout all of the experimental procedures.

Release of Arachidonic Acid (20:4) Metabolites by Macrophage Cultures. For the study of 20:4 release and metabolism, macrophages were incubated for 12 h in α-MEM containing 10% serum and 0.5 μCi/ml of [5,6,8,9,11,12,14,15-3H]20:4 (sp act 78 Ci/mmol; New England Nuclear, Boston, MA). Unless otherwise stated, cells were labeled immediately after the adherence purification step. After the labeling period, the cells were washed three times with buffered saline and placed in serum-free α-MEM containing 160 μg/ml of unopsonized zymosan (ICN K & K Laboratories, Inc., Plainview, NY) prepared by the method of Bonney et al. (8). The cells were then cultured for the designated time periods at 37°C in 95% air/5% CO2 to permit the release of 20:4 metabolites into the culture medium.

Studies of the time course of total 20:4 release by PTM used cells cultured in 15-mm wells. At the end of the culture period with zymosan, the medium was removed and placed in glass scintillation vials for radioactivity measurements. Cell monolayers were washed twice and scraped from the wells into 0.3 ml of a solution of 0.05% Triton X-100 (Rohm and Haas Co., Philadelphia, PA). The wells were scraped again with 0.3 ml of the Triton solution, and the radiolabel content of the cell lysates was determined.

For the characterization of 20:4 metabolites released by macrophages, [3H]20:4-labeled cultures of PAM or PTM in 60-mm dishes were used. Aliquots (50 μl) of the medium were taken initially for radioactivity measurements, and the cells were then incubated for 4 h in the presence or absence of zymosan. After aliquots were again removed to determine radiolabel content, the medium was placed on ice. The cells were washed and scraped into 0.05% Triton X-100 (2 × 0.75 ml). Portions (100 μl) of the lysates were assayed for radiolabel content, and protein was determined by the method of Lowry et al. (9).

The 20:4 metabolites in the culture medium were extracted by the method of Unger et al. (10). Briefly, to 1 ml of medium was added 1 ml ethanol and 10 μl 88% (wt/wt) formic acid, and the resultant solutions were extracted twice with 1 ml of chloroform containing 0.005% (wt/vol) butylated hydroxytoluene (BHT; Sigma Chemical Co., St. Louis, MO). The lower (chloroform) phases were combined, evaporated to dryness under nitrogen, and dissolved in the appropriate starting solvent for further purification by reversed-phase high-pressure liquid chromatography (HPLC). A column of 5 μ Ultrasphere ODS, 4.6 mm × 25 cm (Altex, Rainin Instruments, Woburn, MA) eluted at a flow rate of 1 ml/min was used for all HPLC purifications. Two separate solvent systems were used. System 1: the first solvent was 60 ml of methanol/water/acetic acid (65:34.9:0.1, vol/vol/vol) adjusted to pH 5.4 with ammonium hydroxide, followed by 40 ml of methanol/acetic acid (100:0.01, vol/vol). System 2: elution was with 120 ml of water:acetonitrile/benzene/acetic acid (76.7:23:0.2:0.1, vol/vol/vol/vol) followed by 40 ml of methanol/acetic acid (100:0.01, vol/vol). Fractions of 1 ml were collected, and the radiolabel content of whole fractions or aliquots was determined. The elution characteristics of prostaglandins (PG), leukotrienes, monohydroxyeicosatetraenoic acids (mono-
HETEs), and unreacted 20:4 were determined by the use of pure radiolabeled standards (see below). Those of di-HETEs and 12-1-hydroxy-5,8,10-heptadecatrienoic acid (HHT) were elucidated by comparison with the corresponding 20:4 metabolites produced by polymorphonuclear leukocytes (11) and platelets (12), respectively. In addition, the elution of these di-HETEs from the reversed phase HPLC column using methanol/water/acetic acid (75:25:0.01, vol/vol/vol) as the solvent was identical to that reported by others (9–19 min [11]). Except in the case of LTC, all 20:4 metabolites were identified on the basis of their HPLC elution time.

**Purification of Leukotrienes.** Chemically pure samples of leukotrienes for ultraviolet or amino acid analysis were obtained from the medium of cells that had been labeled with [3H]20:4 and exposed to zymosan for 4 h. The 20:4 metabolites were extracted from the medium as described above, and the extracts were concentrated under nitrogen. The concentrated extract from ~4 ml of medium was applied in a total volume of 100 µl of 80% ethanol to a column (0.6 × 10 cm) of activated silicic acid (Silicar CC-7; Mallinckrodt Inc., St. Louis, MO) packed in chloroform (2).

The columns were eluted sequentially with 5 ml of chloroform, 35 ml of 20% (vol/vol) methanol in chloroform and 20 ml of methanol, all containing 0.005% BHT. The methanol eluates containing the leukotrienes were combined, concentrated under reduced pressure, and rechromatographed as above on a single silicic acid column, with BHT omitted from the eluting solvents. The methanol eluate from this column was concentrated under reduced pressure and subjected to HPLC using system 1.

**Amino Acid Analysis of LTC.** Fractions from HPLC containing purified LTC were dried under reduced pressure and hydrolyzed in evacuated ampoules in 6 M HCl for 20 h at 110°C. Amino acid analysis was performed on a Durrum D-500 automatic amino acid analyzer (Dionex Corp. Sunnyvale, CA) by previously described methods (13, 14) using norleucine as an internal standard.

**Bioassay of LTC.** The bioassay for SRS activity was performed using an isolated guinea pig ileum in Tyrode's solution in the presence of atropine (1 µM) and pyrilamine maleate (1 µM), by the method of Chakravarty (15). One unit of SRS activity was defined as that amount producing a contraction equal to that elicited by 5 ng of histamine.

**Macrophage Lipid Extraction, Separation, and Analysis.** Macrophages prelabeled for 12 h with [3H]20:4 were washed, placed in α-MEM with or without zymosan (160 µg/ml), and incubated for 4 h. Aliquots of the medium were taken for radioactivity measurements before and after the 4 h incubation. The cells were washed and scraped into 0.9% saline, and cell lipids were extracted by the procedure of Bligh and Dyer (16). Concentrated lipid extracts were applied to 0.4-g columns of activated silicic acid (Silicar CC-4; Mallinckrodt Inc.). Neutral lipids and phospholipids were sequentially eluted with 10 ml each of chloroform and methanol, respectively (17). Fatty acid methyl esters of phospholipids were prepared by transesterification in methanolic HCl and analyzed by gas-liquid chromatography on 1/8-in × 10-ft columns of 10% SP-2330 on 100/200 Chromosorb WA/W (Supelco, Inc., Bellefonte, PA) at 180°C with a carrier gas flow rate of 30 ml/min (17).

To determine the localization of [3H]20:4 in individual cell phospholipids, extracts of radiolabeled PMT that had not been exposed to zymosan were subjected to two-dimensional thin-layer chromatography on plates of silica gel (Redi-Coat plates, Supelco, Inc.). To facilitate the visualization of lipid on the plates, phospholipid (0.1 µmol) extracted from unlabeled J774 cells (a macrophage-like cell line) was added to the samples. Plates were developed in the first dimension with chloroform/methanol/ammonium hydroxide (65:35:5, vol/vol/vol) and in the second dimension with chloroform/acetone/acetic acid/methanol/water (30:40:10:10:5, vol/vol/vol/vol/vol) (18). The lipid-containing regions were visualized by exposing the plates to iodine vapors, and the silica gel was scraped from the plates into scintillation vials for radioactivity determinations.

Macrophage phospholipid phosphorus content was determined on cell lipid extracts by the method of Ames and Dublin (19).

**Assay of γ-Glutamyl Transpeptidase Activity.** Assay solutions contained 0.5 M Tris HCl (pH 8.5), 25 mM glycylglycine, and 2.5 mM L-γ-glutamyl-p-nitroanilide (Sigma Chemical Co.). Macrophages cultured in a 60-mm dish were washed twice with buffered saline and overlaid with 1 ml of the reaction solution. After incubation for 6 h at 37°C with gentle agitation, 0.5
ml of the solution was removed and combined with 0.25 ml ice-cold 15% (wt/vol) trichloroacetic acid. The solutions were placed on ice for 15 min, and protein was removed by centrifugation. The optical density was measured at 410 nm after neutralization with solid sodium bicarbonate (20). One unit of γ-glutamyl transpeptidase activity is defined as 1 pmol of p-nitroaniline produced per hour at 37°C.

Radioactivity Measurements. The radiolabel content of samples was measured by liquid scintillation counting in Hydrofluor (National Diagnostics, Inc., Somerville, NJ) using an LKB Ultradelta 1210 scintillation counter (LKB Instruments, Inc., Rockville, MD). Corrections were made for counting efficiency (≈40% for 3H).

Radiolabeled Standards of 20:4 Metabolites. The recovery of 20:4 metabolites through extraction and purification procedures and their elution times on HPLC were monitored with the aid of radiolabeled standards. [5,8,9,11,12,14,15-3H]6-ketoPGF1α (80–120 Ci/mmol), [5,6,8,9,11,12,14,15-3H]thromboxane B2 (TXB2, 100–150 Ci/mmol), [9-3H]PGF2α (5–15 Ci/mmol), and [5,6,8,9,11,12,14,15-3H]PGE2 (100–200 Ci/mmol) were purchased from New England Nuclear. [3H]12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) and [3H]-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) were obtained from calcium ionophore A23187-stimulated human blood platelets (12) and polymorphonuclear leukocytes (21), respectively, and were the generous gift of Dr. Nicholas Pawlowski of The Rockefeller University. [3H]LTC was obtained from [3H]-labeled mouse peritoneal macrophages (1). [3H]Leukotriene D [5(S)-hydroxy-6(R)-cysteynlyglycyl-7,9,11,14-eicosatetraenoic acid; LTD] was derived from the incubation of [3H]LTC with γ-glutamyltranspeptidase (22). [3H]Leukotriene E [5(S)-hydroxy-6(R)-cysteinly-7,9,11,14-eicosatetraenoic acid; LTE] was similarly obtained as a reaction product of [3H]LTD with leucine aminopeptidase (22). All leukotrienes were purified by silicic acid column chromatography and HPLC as described above. Standards were dissolved in α-MEM from cultures of unlabeled macrophages challenged with zymosan for extraction and HPLC purification.

Results

Mouse Pulmonary Macrophage Populations. Two macrophage populations were obtained from lungs on the basis of their accessibility to lavage. PAM were readily obtained by washing the tracheo-bronchial tree five times with saline, whereas PTM included those cells remaining in the alveoli after the lavage in addition to interstitial macrophages. The cytochemical and phagocytic properties of the two cell populations as well as cell recoveries are summarized in Table I. These data indicate that typical macrophages comprised at least 80 and 95% of PTM and PAM populations, respectively.

Uptake of [3H]20:4 by Pulmonary Macrophages and Its Distribution in Cell Lipid. During 12 h of exposure to [3H]20:4, the uptake of the radiolabel by PAM and PTM cultures was similar (82 ± 16% for PAM and 74 ± 21% for PTM). Two-dimensional thin-

Table I

Characteristics of Mouse Pulmonary Macrophage Populations*

| Cell type | Cells/mouse (× 10⁶) | µg cell protein/10⁶ cells | Percent phagocytic | Zymosan positive | Percent peroxidase positive |
|-----------|---------------------|--------------------------|-------------------|-----------------|---------------------------|
| PAM       | 1.6 ± 0.4           | 114                      | 96                | 99              | 1                         |
| PTM       | 3.8 ± 0.4           | 92                       | 81                | 80              | 9                         |

* Assays were carried out on purified cell populations after 12 h of incubation.
‡ The number of adherent cells recovered per mouse.
§ The percent of the total number of cells ingesting zymosan during 1 h of exposure to a maximal dose of 160 µg/ml.
∥ The average number of zymosan particles ingested per cell.
layer chromatography of PTM lipid extracts indicated that ~40% of the $^3$H was localized in phosphatidylcholine, whereas phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine contained 23, 16, and 7%, respectively. The remaining 14% was recovered from neutral lipid. Less than 1% of the cell-associated radiolabel was present as the free fatty acid.

Release of $[^3]H$20:4 by Pulmonary Macrophages in Response to Zymosan. When $[^3]H$20:4-labeled cultures of PTM or PAM were challenged with a phagocytic stimulus of unopsonized zymosan, they released $^3$H selectively from their phospholipid pool, and this radiolabel was recovered in the culture medium. After 4 h of exposure to 160 μg/ml of zymosan, a dose that elicited a maximal response, PTM and PAM released 13.7 ± 5.1% and 5.5 ± 1.9% of their phospholipid $[^3]H$20:4, respectively. In contrast, cells not challenged with zymosan released 2.9 ± 1.1% of the radiolabel in their phospholipid pool over a 4-h period.

Fig. 1 shows the time course of the release of $[^3]H$20:4 from PTM phospholipid after the cells were challenged with zymosan (160 μg/ml). An initial burst of $^3$H release that lasted for 30–60 min after the addition of zymosan was followed by a slower rate of release which continued for up to 4 h. Because culture of macrophages in serum-free medium for longer than 4 h after the addition of zymosan was associated with a loss in cell viability, later time points were not evaluated.

The effect of culture time on $[^3]H$20:4 release by pulmonary macrophages was examined in parallel cultures of PTM and PAM incubated for 12 or 60 h before the zymosan challenge. The results indicated that increased culture time resulted in a decrease in $[^3]H$20:4 release in response to zymosan. PTM cultured for 60 h released 6.0 ± 0.5% of their phospholipid $[^3]H$20:4 during a 4-h exposure to zymosan, in
contrast to 13.2 ± 1.1% [³H]20:4 release by 12-h PTM cultures. Corresponding values for PAM phospholipid [³H]20:4 release were 2.5 ± 0.4% by 60-h cultures and 4.8 ± 0.5% by 12-h cultures. The decrease in 20:4 release was not the result of cell loss or death, and 60-h PTM and PAM cultures phagocytized zymosan to an extent equal to that of 12-h cultures. In contrast, no effect of culture time was seen in 20:4 released in response to 10 μg/ml calcium ionophore A 23187. Thus, 12-h PTM cultures released 11.4% of their phospholipid fatty acid 20:4 after ionophore challenge, whereas 60-h cultures released 10.0%. PAM cultured for 12 and 60 h released 9.2% and 10.3% of their 20:4, respectively, in response to the ionophore.

Synthesis of LTC by PTM and PAM. Initial studies of the radiolabeled products released by [³H]20:4-labeled PTM indicated that LTC was a major 20:4 metabolite of these cells. This metabolite was subsequently purified from PTM culture medium by extraction, silicic acid column chromatography, and HPLC (Fig. 2). A peak of radioactivity was obtained from HPLC, which co-migrated with LTC isolated from mouse peritoneal macrophages (elution time = 19 min). The ultraviolet spectrum of this material (Fig. 2) indicated an absorption maximum at 280 nm with shoulders at 270 nm and 292 nm, confirming the presence of a conjugated triene structure. Amino acid analysis yielded 0.82 nmol of glutamic acid and 1.0 nmol of glycine per nanomole of leukotriene as determined by absorbance at 280 nm (ε = 40,000 [23]). Other amino acids, including cysteine, were recovered at <0.1 nmol/nmol leukotriene. This result was consistent with the structure of LTC, but not of LTD or LTE. The failure to quantitatively recover cysteine in acid hydrolysates of leukotrienes has been reported by others (23). Bioassay of the 20:4 metabolite in a standard guinea pig ileum preparation (Fig. 2) indicated a bioactivity of ~2 U of SRS activity per picomole of LTC.

Approximately 3.2 nmol of LTC (based on the absorbance at 280 nm) was isolated from PTM cultures totaling 3.7 mg protein (4.1 × 10⁷ cells). The recovery of both LTC and LTD through the extraction and purification procedure was ~25%. Thus, PTM produced 3.4 pmol of LTC/μg cell protein. No LTD was obtained.

Leukotriene synthesis was also studied in parallel PAM cultures. As in the case of PTM, LTC was the major leukotriene synthesized by PAM; however, small quantities of two other ³H-labeled metabolites were isolated from PAM culture medium. These have been tentatively identified as LTD and LTE on the basis of co-migration with radiolabeled standards on HPLC (Fig. 2). Because of the small numbers of cells available and the relatively low release of [³H]20:4 in response to zymosan, insufficient quantities of the leukotrienes were obtained from PAM cultures to allow spectral or amino acid analyses.

γ-Glutamyl Transpeptidase Activity in PTM and PAM Cultures. The enzyme γ-glutamyl transpeptidase catalyzes the conversion of LTC to LTD (22). Assay of this enzyme in 12-h cultures of PTM and PAM indicated low levels of activity (37 U/μg cell protein in PAM and 13 U/μg cell protein in PTM), in comparison with other cell types such as lymphoid cell lines (50-1,000 U/μg cell protein [24]) or human blood platelets (2,100 U/μg cell protein [20]). Culture of PTM for 60 h did not result in a significant change in enzymatic activity (18 U/μg cell protein). The low levels of enzymatic activity in pulmonary macrophage cultures correlates well with the synthesis of LTC as the major leukotriene metabolite.

Total 20:4 Metabolites of PTM and PAM. We next examined total 20:4 metabolites
Fig. 2. $^3$H elution profiles from final HPLC purification of leukotrienes produced by $[^3H]20:4$-labeled PTM (---) and PAM (-----) challenged with zymosan. Leukotrienes released into the culture medium were extracted and purified by silicic acid column chromatography followed by HPLC system 1. Relevant elution times are: LTC, 19-20 min; LTD, 34-35 min; LTE, 46-47 min.

Top: ultraviolet spectrum of LTC isolated from PTM culture medium. Bottom: response of a standard guinea pig ileum preparation to LTC purified from PTM culture medium. The first arrow indicates sample addition, and the second arrow indicates washout.

synthesized by zymosan-challenged PTM and PAM using HPLC system 1, which allows the mutual separation of cyclo-oxygenase and lipoxygenase products. A representative chromatogram for PTM cultures is shown in Fig. 3. In profiles from both PTM and PAM cultures, LTC was evident as a prominent peak of radiolabel (elution time = 19 min) together with significant quantities of cyclo-oxygenase products that constitute the major radiolabeled species in the peak eluting at 4-15 min. Minor amounts of HETEs and unreacted 20:4 were also evident, as were, in the case of PAM, small peaks of LTD (35 min) and LTE (46 min). Separation of the individual cyclo-oxygenase products formed by pulmonary macrophages using HPLC system 2 indicated that both PAM and PTM formed TXA$_2$ (isolated as TXB$_2$) and PGE$_2$. In addition, PTM, but not PAM, synthesized low levels of prostacyclin (isolated as 6-
Fig. 3. HPLC chromatogram of 20:4 metabolites released by \(^{3}H\)20:4-labeled PTM. Cells were incubated for 4 h with (— — —) or without (---) 160 µg/ml zymosan. 20:4 metabolites in the medium were extracted and subjected to HPLC using system 1. The elution times for relevant standards are: PG and TXB\(_{2}\), 4–15 min; LTC, 19–20 min; di-HETEs, 20–50 min; HHT, 57–58 min; mono-HETEs and unreacted 20:4, 70–90 min. The recovery of 20:4 metabolites through the extraction and HPLC was 70–75%.

keto-PGF\(_{1\alpha}\) (Fig. 4). The specific release of all 20:4 metabolites was calculated from radiolabel data and is expressed in Table II as percent of total 20:4 release obtained after zymosan treatment. It is evident that lipoxygenase products (LTC and HETEs) represent the major portion of the identified 20:4 metabolites of these cells.

**Alterations in PTM Phospholipids with Phagocytosis.** Direct fatty acid analysis indicated that the phagocytosis of zymosan was associated with a 15% reduction in the 20:4 content of PTM phospholipid (Table III). This value was in good agreement with the specific release (zymosan-treated cultures − control cultures) of \(^{3}H\) from the cells, which indicated a loss of 14% of the phospholipid radiolabel. In a second experiment, values for specific release of phospholipid 20:4 were 10% from fatty acid analysis and 12% from radiolabel data.

PTM cultures contained 302 ± 18 pmol of phospholipid/µg cell protein, a value that did not change with phagocytosis (295 ± 5 pmol/µg cell protein). Therefore, the 20:4 content of PTM was 140 pmol/µg cell protein, and a specific release of 11% of phospholipid \(^{3}H\)20:4 represented a loss of 15 pmol of 20:4/µg cell protein.

**Discussion**

In these studies, we have examined 20:4 metabolism in two mouse pulmonary macrophage populations. These include PAM, which were obtained by lavage of the airways, and PTM, which were isolated by enzymatic digestion. Of the two cell populations, PTM released greater quantities (two- to threefold) of 20:4 metabolites than did PAM under identical culture conditions. Because the PTM cultures were undoubtedly a mixture of interstitial and alveolar macrophages of unknown proportions, it is possible that the capacity of the pulmonary interstitial macrophage to
release 20:4 metabolites is actually greater than indicated by the data for PTM. The differences in 20:4 release observed between PTM and PAM populations did not appear to result from the use of proteolytic enzymes to isolate PTM, because exposure of PAM to these enzymes did not alter the zymosan-induced 20:4 release by those cells. Recent studies by Blussé Van Oud Alblas and Van Furth (5) have suggested that alveolar macrophages originate as pulmonary interstitial macrophages which, in turn, are derived from blood monocytes. Once in the alveolar space, the macrophages are continually exposed to foreign particulate matter and are very actively phagocytic. It is possible that this constant stimulation of macrophages in the alveoli results in changes in the cells that are accompanied by a down regulation of 20:4 metabolism as observed in PAM cultures.

The release of 20:4 in response to zymosan decreased with culture time in both PTM and PAM populations. Treatment of 60-h PTM cultures with the proteolytic enzymes used for their isolation failed to restore 20:4 release to levels observed in 12-h cultures, which indicates that these changes were not an artifact of the isolation procedure. Furthermore, time in culture did not affect 20:4 release in response to calcium ionophore A23187. It has been suggested that unopsonized zymosan interacts...
TASLE II

20:4 Metabolites Synthesized by PAM and PTM in Response to Zymosan*

| 20:4 Metabolite | PTM  | PAM  |
|-----------------|------|------|
| Prostacyclin    | 5-7  | —    |
| TXA2            | 6-10 | 7-12 |
| PGE2            | 10-15| 10-15|
| HETES + 20:4    | 9-12 | 14-18|
| di-HETEs        | 20-25| 20-25|
| LTC             | 20-25| 10-15|
| LTD             | —    | 1-2  |
| LTE             | —    | 1-2  |
| Unidentified‡   | 10-30| 20-30|

* Values are expressed as the percent of total 20:4 specifically released in response to zymosan and were calculated as follows: 20:4 metabolite as percent of total 20:4 = \( \frac{M_t - M_c}{M_t - M_c} \times 100\% \), where Met = dpm of the metabolite recovered from zymosan treated cultures; Mete = dpm of the metabolite recovered from parallel control cultures; Mt = dpm in the medium from zymosan treated cultures; and Mc = dpm in the medium from parallel control cultures.

† Unidentified products consisted primarily of radiolabel co-migrating with cyclo-oxygenase products in HPLC system 1, but not in system 2.

TASLE III

Effect of Phagocytosis on PTM Phospholipid Fatty Acid Composition*

| Fatty acid | Mole percent of fatty acid |
|------------|---------------------------|
|            | Control | Zymosan | Δ    |
| 14:0       | 8.1 ± 0.3‡  | 8.0 ± 0.3 | −0.1 |
| 16:0       | 29.0 ± 1.3  | 31.6 ± 1.0 | +2.0 |
| 18:0       | 18.7 ± 0.6  | 20.3 ± 0.2 | +1.6 |
| 18:1       | 14.1 ± 0.4  | 15.4 ± 0.3 | +1.3 |
| 18:2       | 6.7 ± 1.5   | 5.0 ± 0.9 | −1.7 |
| 20:4       | 23.4 ± 0.3  | 19.8 ± 1.1 | −3.6 |

* Cultures of PTM were incubated for 4 h in the presence or absence of unopsonized zymosan (160 μg/ml). The cells were then washed and scraped into isotonic saline. The fatty acid content of isolated phospholipids was determined by gas-liquid chromatography after transesterification.

‡ Each value is the mean ± SD of four chromatographic analyses of a single sample.

with the macrophage membrane by binding to the receptor for mannose-terminal glycoproteins (25). Preliminary studies also indicated that 20:4 metabolism stimulated by opsonized zymosan and immune complex-coated Sephadex beads decreases with time. These observations suggest that an alteration in receptor-mediated 20:4 metabolism occurs in cultured pulmonary macrophages.

Spectroscopic quantitation of purified [3H]LTC obtained from radiolabeled PTM cultures indicated that its specific activity (69 dpm/pmol LTC) was in good agreement with that of the total cell phospholipid 20:4 (70 dpm/pmol 20:4). Furthermore, fatty acid analysis indicated that the release of 3H from cell phospholipid provided an accurate measurement of the 20:4 released by the cells in response to zymosan. Similar results were obtained in [3H]20:4-labeled mouse resident peritoneal macrophages,
which were also shown to release \[^{3}H\]PGE\(_2\) having a specific activity equivalent to that of the total phospholipid 20:4 pool (1, 26). These considerations indicate that the specific activity of the \[^{3}H\]20:4 in macrophage phospholipids may be used to calculate molar quantities of isolated 20:4 metabolites from their radiolabel content. In Table IV are the results of such calculations for the 20:4 metabolites of PTM. Also included are corresponding values for resident peritoneal macrophages cultured under similar conditions, providing a direct comparison of two macrophage populations from distinct body sites in a single species. Both PTM and peritoneal macrophages are rich sources of 20:4 metabolites; however, there are differences in the proportions of the released 20:4 that is metabolized by the cyclo-oxygenase and lipoxygenase pathways. Lipoxygenase products predominate in PTM 20:4 metabolites and LTC is the single major compound. In contrast, peritoneal macrophages secrete relatively more cyclo-oxygenase products, with PGE\(_2\) predominating.

The finding that pulmonary macrophages are rich sources of LTC suggests that these cells may play a role in inflammatory or immunologic reactions that result in SRS formation in the lung. Considerable work in the past has shown that specific antigen challenge of IgE-sensitized lung tissue results in significant SRS production; however, studies of purified pulmonary mast cells have indicated that these cells are not capable of releasing large quantities of leukotrienes (27, 28). The possibility must therefore be considered that the macrophage is the source of SRS in these IgE-mediated reactions. The present studies demonstrate LTC production in response to phagocytic stimuli, and, at present, macrophage 20:4 metabolism in response to IgE

| 20:4 Metabolite | Peritoneal macrophages | PTM |
|----------------|------------------------|-----|
| Prostacyclin    | 5.5-7.2                | 0.7-1.1 |
| TXA\(_2\)       | 0.9-1.5                | 0.9-1.5 |
| PGE\(_2\)       | 15-20                  | 1.5-2.3 |
| HETEs + 20:4    | 2.1-4.6                | 4.3-5.6 |
| LTC             | 3.5-7.0                | 3.0-3.8 |
| Unidentified    | 1.5-4.5                |       |

*Values given are picomoles of 20:4 metabolite per microgram of cell protein specifically released in response to a maximal zymosan stimulus. PTM were cultured for 4 h and peritoneal macrophages for 2 h after zymosan addition. Total release of 20:4 metabolites was calculated by the formula: pmol metabolite/µg protein = \(\frac{\text{Met}}{(M + C - M_0)P \times A}{R}\), where Met = dpm of isolated metabolite recovered from HPLC; M = dpm of medium after the incubation with or without zymosan; C = dpm of cells after the incubation with or without zymosan; M\(_0\) = dpm in the medium before the incubation with or without zymosan; R = the recovery of the metabolite through extraction and HPLC; P = the fraction of total 20:4 incorporated into cell phospholipid (0.70 for peritoneal macrophages and 0.86 for PTM); and A = the phospholipid 20:4 content (86 pmol/µg cell protein for peritoneal macrophages, 140 pmol/µg cell protein for PTM).

The specific release of 20:4 metabolites is defined as the release by zymosan treated cultures minus the release by control cultures. The specific release of total 20:4 metabolites by peritoneal macrophages and PTM was approximately 35 pmol/µg cell protein and 15 pmol/µg cell protein, respectively.
and antigen has not been reported. However, the binding of IgE to the macrophage membrane as well as cell-cell interactions between mast cells and mononuclear phagocytes have been suggested as possible mechanisms for the stimulation of LTC release by macrophages (28, 29).

The synthesis of LTC by pulmonary macrophages in response to phagocytic stimuli raises other questions concerning the possible involvement of SRS generation during acute and chronic inflammatory states. The recently reported vascular effects of LTC and LTD suggest that these substances are potent proinflammatory agents (30). Thus, it is likely that the role of SRS formation in the lung is not limited to immediate hypersensitivity reactions.

Summary

Mouse resident pulmonary macrophages were subdivided into alveolar (PAM) and interstitial (PTM) populations on the basis of accessibility to pulmonary lavage, and zymosan-induced arachidonic acid (20:4) metabolism was examined in both populations labeled with [3H]20:4. Maximal phagocytic doses of unopsonized zymosan induced the specific release of 11% of phospholipid 20:4 by PTM and 4.6% by PAM. Direct fatty acid analysis of [3H]20:4-labeled PTM cultured in the presence or absence of zymosan indicated that the specific activity of the [3H]20:4 in cell phospholipid provided an accurate measure of 20:4 released by the cells, and could therefore be used to quantitate the synthesis of 20:4 metabolites by PTM in vitro. The single major 20:4 metabolite of PTM was the slow-reacting substance leukotriene C, which was synthesized in quantities of 3-4 pmol/μg cell protein (280-370 pmol/10⁶ cells), and comprised 20-25% of the released 20:4. PTM also synthesized prostaglandin E₂, prostacyclin, thromboxane A₂, and hydroxyeicosatetraenoic acids. In contrast, PAM produced leukotrienes D and E in addition to leukotriene C, prostaglandin E₂, thromboxane A₂, and hydroxyeicosatetraenoic acids. Prostacyclin formation by PAM was not observed. These studies define a set of experimental conditions for the study of 20:4 metabolism by pulmonary macrophages, and demonstrate that these cells are rich sources of LTC as well as other 20:4 oxygenated products.

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