REGULATION OF ARACHIDONIC ACID
METABOLITES IN MACROPHAGES*

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During the course of our studies on the lipid composition of macrophage membranes, it was noted that 25% of the total fatty acid content of this cell was composed of arachidonic acid (20:4).[1] Similar values have been reported for both rabbit (1) and human mononuclear phagocytes (2), whereas the fatty acid complement of most other cell types rarely exceeds a few percent. This unusual enrichment with esterified 20:4 has focused attention on the ability of mononuclear phagocytes to produce prostaglandins (PG) from endogenous stores. PG, depending upon their structure, are thought to play a variety of important roles in the initiation and control of the inflammatory process (3).

It is now well established that mononuclear phagocytes produce PG in response to a variety of stimuli that perturb their surface membranes. These include phagocytizable particulates such as zymosan (4) and immune complexes (5) or soluble agents such as phorbol myristate acetate and lipopolysaccharide (6, 7). Bonney et al. (4) have reported that the major synthetic product of mouse peritoneal cells is PGE with smaller amounts of 6-oxo-PGF$_{1\alpha}$

Unfortunately, there is little information concerning the regulation and control of the oxygenation products of 20:4. For this reason we have established a group of defined conditions in which we have examined the relationship between phagocytosis and PG synthesis, the localization and fate of 20:4 in the phospholipid pool of resident peritoneal macrophages, and the proportions of other 20:4 products released upon a zymosan challenge.

Materials and Methods

Cell Cultures. Primary cultures of peritoneal macrophages were established from resident cells of female NCS mice that weighed 25–30 g, as previously described (8). For measurements of PG synthesis, 6×10^6 peritoneal cells were added in minimum essential medium alpha medium (α-MEM) that contained 10% fetal calf serum (FCS) to 35-mm-diameter plastic culture dishes For determinations of phagocytosis and cell viabilities, 7×10^5 peritoneal cells were added to 12-mm-diameter glass coverslips placed in similar dishes After 2 h at 37°C in 5% CO_2, the cultures were washed three times in α-MEM to remove nonadherent cells and incubated overnight (16 h) in fresh α-MEM plus 10% FCS

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[1] *Abbreviations used in this paper* α-MEM, minimum essential medium alpha medium, FCS fetal calf serum, HETE, hydroxy-eicosatetraenoic acid(s); α-MEM, minimum essential medium alpha medium, PG, prostaglandin(s), RIA, radioimmunoassay, 16 0, palmitic acid, 18.0, stearic acid, 18·1, oleic acid, 18 2, linoleic acid; 18·3, linolenic acid, 20.4, arachidonic acid, [^{3}H]20 4, [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]20 4

Preparation of Unopsonized Zymosan.   Zymosan was purchased from ICN K & K Laboratories Inc., Plainview, N. Y., and stock solutions in α-MEM were prepared as described (4).

Synthesis of Oxygenated Products of 20:4.   1 μCi of [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]20:4 ([^{3}H]20:4) (62.2 Ci/mmol sp act; New England Nuclear, Boston, Mass.) was added to appropriate cultures before the 16-h incubation period. At the end of the labeling period, macrophage cultures were washed three times with α-MEM, overlaid with α-MEM (no serum), and unopsonized zymosan was added. After incubation for the appropriate periods under 5% CO_2 at 37°C, media were removed. Cell monolayers were overlaid with 0.05% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.), and the dishes were scraped. Protein was determined by the method of Lowry et al. (9). The protein content of cultures in 35-mm-diameter dishes was 83 ± 16 μg ($n = 50$).

Where appropriate, macrophage cultures were labeled with other fatty acids, including [9,10-^{3}H]palmitic acid (16:0) (10 Ci/mmol sp act; New England Nuclear), [1-^{14}C]oleic acid (18:1) (50 mCi/nmol sp act; Amersham Corp., Arlington Heights, Ill.), or [1-^{14}C]linolenic acid (18:3) (50 mCi/mmol sp act; Amersham Corp.), as described above.

PG and other 20:4-derived products were extracted from culture media following the procedure of Unger et al. (10). In brief, 1 vol of ethanol was added, and media were extracted twice with 2 vol each of petroleum ether (30–60°C). The petroleum ether phases were discarded. After acidification with 10 μl of formic acid (final pH ~3), the aqueous phases were extracted twice with 1 vol each of chloroform. The chloroform phases were pooled and taken to dryness under a stream of nitrogen. The residue was resuspended in 1 ml of chloroform, again dried under nitrogen to remove traces of formic acid, and redissolved in a final vol of 0.2 ml of chloroform.

Recoveries of PGE and 20:4 were determined by extraction of media that contained 1 μCi of [^{3}H]20:4 or 0.25 μCi of [^{14}C]PGE$_2$ (55 μCi/mmol sp act; New England Nuclear). Yields ranged from 75 to 90%. Similar recoveries of the radioactivity released by [^{3}H]20:4-labeled macrophages treated with zymosan were obtained.

Oxygenated products of 20:4 were separated by chromatography of concentrated chloroform extracts on 0.3-g columns of silicic acid (Unisil, 100–200 mesh; Clarkson Chemical Co., Inc., Williamsport, Pa.) using a modification of a previously described procedure (11). The silicic acid was activated for 1 h at 110°C, suspended in chloroform, and transferred to a Pasteur pipet plugged with glass wool. Columns were washed with chloroform and eluted step-wise with the following solvents after addition of samples: Solvent 1: 15 ml of chloroform, solvent 2: 10 ml of 2.5% methanol in chloroform, solvent 3: 5 ml of 5.0% methanol in chloroform, solvent 4: 5 ml of 20.0% methanol in chloroform.

Routinely, the four solvent fractions were collected separately in glass scintillation vials and evaporated under a stream of air. Radioactivity was measured by liquid scintillation counting in Aquasol (New England Nuclear). 1-ml fractions were collected when elution profiles of standards and chloroform extracts of radiolabeled media were monitored

Elution of PGE, PGF, and 20:4 from silicic acid columns was determined with the aid of standards 20:4 was recovered in solvent 1, PGE in solvent 2, and PGF in solvent 3. All three compounds were well resolved Separations were checked with each new batch of silicic acid. As noted by others (12), resolution of PG can vary, and it was necessary to adjust the volumes of the solvent fractions. Similar separations were obtained with ethyl acetate, benzene, and methanol as eluting solvents (13). However, with other separation techniques, including thin-layer chromatography (14) and paper chromatography (4), high levels of background radio-activity in sample lanes of chloroform-extracted media often contaminated PGE and presumably resulted from streaking of compounds that migrated near the solvent front This problem was not evident with silicic acid chromatography.

For purposes of identification, the radiolabeled material eluted from silicic acid columns by solvent 1 (chloroform) was subjected to thin-layer chromatography. Samples were concentrated to small volumes (50 μl) and spotted on silica gel H plates (Analtech, Inc , Newark, Del.). Chromatograms were developed in hexane/ethyl acetate/acetic acid (1/1/0.005, vol/vol/vol) (15). Sample lanes were divided into 2-cm sections and the silica gel of each was scraped into scintillation vials. The radioactivity was determined in 4 ml of Aquasol after the addition of 0.4 ml of water.

Radioimmunoassay (RIA) Measurements of PGE.   Macrophage cultures were treated as described above except that [^{3}H]20:4 was omitted from media during the overnight incubation. After treatment with unopsonized zymosan, media were extracted by the procedure of Unger et al (10) omitting the petroleum ether step. The chloroform extract was dried under a stream of nitrogen, then resuspended in chloroform, and the PGE was isolated by silicic acid chromatography The 2 5% methanol effluent was taken to dryness and resuspended in Tris-gelatin buffer Alternately, evaporated chloroform extracts of media were immediately suspended in Tris-gelatin buffer Comparable levels of PG were detected in samples prepared by both methods

Approximately 4,000 dpm of [^{14}C]PGE$_2$ were added to the media before extraction to monitor recoveries. Values of PGE release were corrected for extraction efficiencies (69.8 ± 7 0%, n = 24) Competitive binding between [^{3}H]PGB$_1$, authentic PGB$_2$, or test solutions after conversion of PGE to PGB by treatment with alkali (16), and specific PGB antibody was determined with a double-antibody RIA (Clinical Assays Inc., Div of Travenol Laboratories, Inc., Cambridge, Mass).

Lipid Extraction, Separation, and Analysis.   Macrophage monolayers were rinsed, scraped into isotonic saline, and the lipids extracted by the procedure of Bligh and Dyer (17). Neutral lipids and phospholipids were separated on columns that contained 0.4 g of acid-washed silicic acid (Unisil). Individual phospholipids were separated on plates of silica gel (Redi-Coat plates; Supelco, Inc., Bellefonte, Pa.) by two-dimensional thin-layer chromatography. Plates were developed in the first direction with chloroform/methanol/ammonium hydroxide (65/35/5, vol/vol/vol) and in the second direction with chloroform/acetone/acetic acid/methanol/H$_2$O (30/40/10/10/5, vol/vol/vol/vol/vol). Lipid-containing regions were visualized by exposing the chromatograms to iodine vapors, and the silica gel was scraped into scintillation vials that contained 1 ml of water before the determination of radioactivity in 10 ml of Aquasol

Fatty acid methyl esters of total cell phospholipids and neutral lipids were prepared by transesterification in methanolic HCl and purified by thin-layer chromatography (18). The methyl esters were then analyzed by gas-liquid chromatography on 1/8-in. × 6-ft columns of 10% SP-2330 on 100/200 Chromosorb WA/W (Supelco, Inc.) at 180°C with a carrier gas-flow rate of 30 ml/min.

Phagocytosis.   Macrophage cultures on coverslips were exposed to zymosan for various periods of time, washed three times in phosphate-buffered saline to remove nonadherent particles, fixed in 2.5% glutaraldehyde, and mounted. The number of ingested particles was determined by visual counts, and the phagocytic index was calculated (19). Viability of cultures following ingestion was monitored by trypan blue exclusion Viability remained >95% for up to 4 h following ingestion of zymosan by serum-free cultures of macrophages.

Results

Uptake of [^{3}H]20:4 by Macrophages and Distribution of [^{3}H]20:4 in Macrophage Lipids.   Optimal conditions for the uptake and incorporation of exogenously supplied [^{3}H]20:4 by explanted macrophages were determined. As shown in Fig. 1 A, initial rates of fatty acid uptake were influenced by the serum concentration of the medium. High levels of serum promoted uptake; however, comparable amounts (~90%) of 20:4 were removed from the medium by 24 h, regardless of the serum concentration.

Approximately 70% of the [^{3}H]20:4 incorporated by macrophages was recovered in cell phospholipids, with the remainder in the neutral lipid fraction. Although this distribution remained invariant throughout the 24-h labeling period, the relative levels of [^{3}H]20:4 incorporated in individual phospholipids changed. In the initial portion of the labeling period, the highest percentage of [^{3}H]20:4 was recovered in phosphatidylinositol and the major phospholipid, phosphatidylcholine (Fig. 1 B). At subsequent times, the relative levels of radiolabel in both phospholipids decreased with a concomitant 3.5-fold increase in the [^{3}H]20:4 content of phosphatidylethanolamine
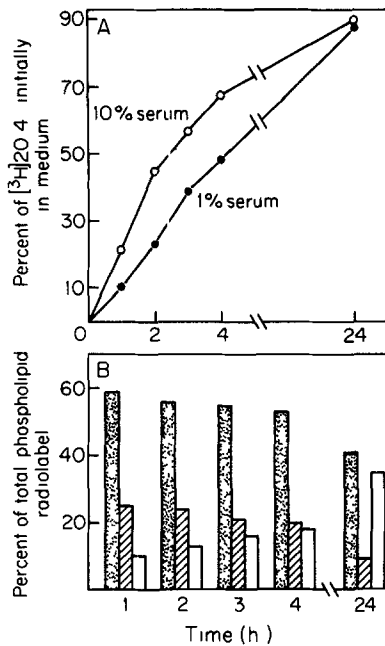
Fig 1   Uptake of [³H]20.4 by macrophage cultures, and distribution of [³H]20:4 among macrophage phospholipids. (A) Time-course of [³H]20.4 incorporation into total macrophage lipids. Peritoneal exudates were incubated for 2 h in 35-mm-diameter plastic dishes, washed three times with α-MEM to remove nonadherent cells, and overlaid with fresh α-MEM that contained 10% FCS At time = 0, 1 μCi of [³H]20:4 was added to each culture. The medium was removed from duplicate cultures at the indicated times. Cell monolayers were washed, scraped into isotonic saline, and then the lipids were extracted Radioactivity was determined in aliquots of lipid extracts and medium at time = 0 and after incubation Data are expressed as the percentage of [³H]20.4 initially added to cultures that was recovered in macrophage lipid extracts Thin-layer chromatography of the lipid extracts indicated that >98% of the radiolabel taken up by macrophages was incorporated into cell lipids. (B) Percentage of phospholipid radiolabel in individual macrophage phospholipids as a function of exposure time to [³H]20·4 Extracted lipids were separated by two-dimensional thin-layer chromatography. Areas of chromatograms that contained individual phospholipids were scraped, and the radioactivity of each was determined. ▨, phosphatidylcholine, ▨, phosphatidylinositol; □, phosphatidylethanolamine The percentage of phospholipid radiolabel in other macrophage phospholipids, including phosphatidylserine, sphingomyelin, and cardiolipin, remained constant throughout the labeling period The relative levels of radiolabel in these phospholipids were 5-7, 1-2, and 3 5-5%, respectively

The synthesis of PGE and other oxygenated products of 20:4 was estimated from the radiolabel released by macrophages prelabeled with [³H]20:4 and corrected for recovery assuming that the specific activity of each product was that of the phospholipid 20:4. The specific activity of the macrophage phospholipid 20:4 was calculated from known quantities, which included the specific activity of the exogenously added [³H]20:4, the phospholipid content of macrophage cultures (172 nmol/mg cell protein [18]), and the 20:4 content of the cultures (Table I). As a measure of the validity of the radiolabel assay, levels of PGE synthesis were compared with the release of PGE determined by RIA measurements. PGE release (4.30 μg/mg cell protein) calculated from the radiolabel assay (Fig. 4; 160 μg zymosan) was within 18% of the value (5.27 μg/mg cell protein) found by RIA (Fig. 3).

TABLE I

Effect of Phagocytosis on Macrophage Phospholipid Fatty Acid Composition *

Fatty acid	Mole percent of fatty acid		
	Control	Zymosan	Δ
	%	%	%
16 0	35.9	39 5	3 6
18·0	24 1	30 8	6 7
18:1	9.8	12.0	2 2
18.2	5 2	5 7	0 5
20 4	25 3	12 1	−13 2
Percent saturated/ unsaturated	1.49	2 36	

* After the initial 2-h incubation period, cultures were washed to remove nonadherent cells and incubated for an additional 16 h in α-MEM plus 10% FCS One-half of the cultures were treated with 160 μg of zymosan for 3 h in the absence of serum Controls were similarly treated omitting zymosan The media were removed, and lipids were extracted from cells scraped into isotonic saline. Fatty acid analyses of isolated phospholipids were determined by gas-liquid chromatography after transesterification

Phagocytosis and the Fate of Macrophage 20:4

FATTY ACID COMPOSITION. The effects of zymosan ingestion on the fatty acid composition of total macrophage phospholipids are summarized in Table I. After a 3-h exposure to unopsonized zymosan (160 μg) in the absence of serum, the 20:4 content of macrophage phospholipids was reduced by 48%. Increases in the relative content of other phospholipid fatty acids following phagocytosis were evident with the greatest changes in saturated fatty acid (16:0 and 18:0) levels. As a result of these changes in fatty acid composition, the degree of saturation in macrophage phospholipids increased 1.6-fold after zymosan treatment. This result is of interest considering our recent findings (20) that reductions in endocytic activities in the macrophage are associated with saturated fatty acid or *trans*-unsaturated fatty acid enrichment.

20:4-DERIVED PRODUCTS RELEASED BY MACROPHAGES. Approximately 67% of the radiolabel released by [^{3}H]20:4-labeled macrophages following exposure of cultures to zymosan was recovered as PG. The remainder of the released radiolabel eluted in the chloroform fraction on silicic acid chromatography of extracted media, which indicated that it is less polar than PG. The time-course for the release of this material was similar to that of PGE (Fig. 2). Although 20:4 eluted in the chloroform fraction of silicic acid columns, only small amounts (<10%) of the radiolabel released by macrophages comigrated with 20:4 standards on thin-layer chromatography. The bulk of the apolar radiolabeled products had R_f values ranging from ~0.35 to 0.60, intermediate between those of 20:4 and PGE standards and similar to those reported (15) for hydroxy-eicosatetraenoic acid (HETE), which suggested that this material was a product of lipoxygenase reaction (21). Preincubation of macrophages for 1 h at 37°C with 1–10 μg/ml indomethacin before zymosan treatment inhibited PG synthesis but had no effect on the release of the less polar products, which confirmed that they are not derived from the cyclooxygenase pathway of PG synthesis (22).

EFFECT OF PHAGOCYTOSIS ON THE RELEASE OF FATTY ACIDS OTHER THAN 20:4. Macrophage cultures were labeled overnight with radiolabeled 16:0, 18:1, and 18:3
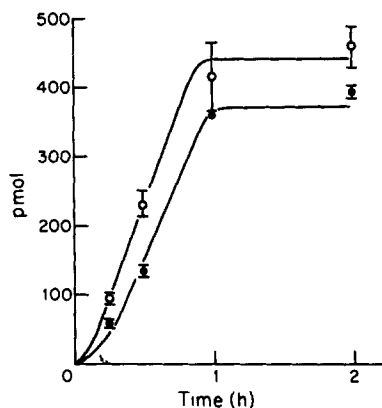
Fig 2. Time-course for the release of radiolabeled PGE and apolar (HETE and 20:4) compounds by macrophages treated with unopsonized zymosan. Cultures in 35-mm-diameter plastic dishes were labeled for 16 h with 1 μCi of [^{3}H]20.4. After washing, the cultures were exposed to 50 μg of zymosan at time = 0 in α-MEM (no serum). Media were removed from duplicate cultures at the indicated times, extracted, and the radiolabeled compounds were isolated by silicic-acid chromatography. HETE plus 20·4 and PGE were recovered in the chloroform and 2.5% methanol effluents, respectively, of silicic acid columns. Values are the means ± range. O, PGE; ●, HETE plus 20 4.

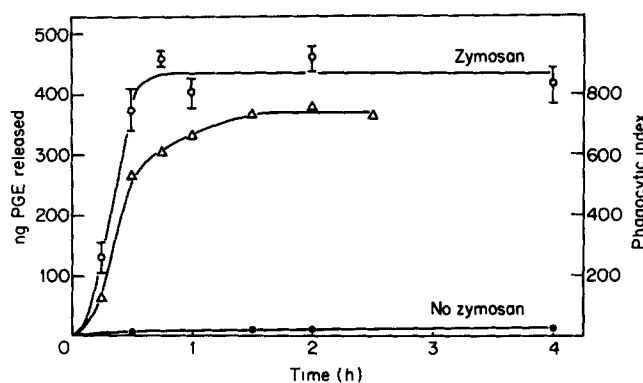Fig 3 Time-course of PGE release and zymosan ingestion by macrophages Cultures were incubated overnight (no [^{3}H]20 4), washed, and exposed to 160 μg of zymosan at time = 0 in α-MEM (no serum) Media from duplicate cultures were extracted at each time point, and the PGE levels were determined in duplicate by RIA. Zymosan was omitted from control cultures Values are the means ± SE. Phagocytosis was measured in parallel cultures on glass coverslips similarly treated Phagocytic index (△), PGE release by zymosan-treated (O) and control (●) cultures

as described for 20:4. Following a 1-h exposure to zymosan (160 μg) in the absence of serum, levels of radiolabel in media were not significantly different from the low amounts in media of control cultures (no zymosan). Likewise, no release of fatty acids could be detected by gas-liquid chromotography analysis of extracted media from zymosan-treated cultures. Together, these data indicated that the release of fatty acids other than 20:4 by macrophages challenged with zymosan was insignificant.

KINETICS OF PGE RELEASE AND ZYMOSAN INGESTION. The time-course of zymosan ingestion by macrophage cultures maintained in the absence of serum is shown in Fig. 3. PGE release as measured by RIA followed similar kinetics. The addition of

zymosan (160 µg) to cultures promoted PGE release after a lag period of 5–7 min The amounts of PGE in the medium increased for up to 60 min and then abruptly ceased. PGE levels in the medium remained constant at 5.24 ± 0.6 µg/mg cell protein (mean ± SE) for up to 4 h, which suggested that exogenous PG is not metabolized by macrophages. Beyond 4 h, loss of cells and decreased cell viability were evident in serum-free cultures exposed to high concentrations of particles, and measurements were discontinued. Compared with control cultures (no zymosan), which released 0.12 ± 0.03 µg PGE/mg cell protein (mean ± SE), 160 µg of zymosan elicited a 40.7-fold increase in PGE release.

Influence of Zymosan Concentration on the Time-Course of PGE Synthesis

DOSE RESPONSE. As noted above, PGE release closely paralleled the ingestion of zymosan by macrophages. To evaluate the relationship between phagocytosis and PGE synthesis, dose-response relationships were established between PGE release and zymosan concentration by using macrophage cultures prelabeled with [³H]20:4. As shown in Fig. 4 A, the initial rate of PGE synthesis was determined by the particle concentration in the medium. PGE synthesis was directly proportional to the zymosan dose and, hence, to phagocytosis. Interestingly, the duration of PGE synthesis continued for a finite period (~60 min) regardless of the particle concentration. As a result, initial rates of synthesis determined the total amount of PGE formed

The phagocytic indices of cultures and the extent of ingestion by individual cells within cultures were assessed at all zymosan concentrations. As shown in Fig. 4 B, the majority of the macrophages were engaged in the phagocytic event even at low doses of zymosan. Thus, the data (Fig. 4 A) represent a valid dose-response relationship. Zymosan concentrations of 160 µg/ml resulted in maximal levels of phagocytosis (phagocytic indices ranged from 800 to 1,100), and a twofold increase (320 µg/ml) did not lead to either significantly increased ingestion (Fig. 4 B) or PGE synthesis (data not shown).

RESTIMULATION OF PGE SYNTHESIS BY MACROPHAGES. We explored the question of whether macrophages treated with zymosan can be stimulated to synthesize additional amounts of PGE on exposure to a second dose of particles. In the experiment shown in Fig. 5, cultures were initially treated with a low concentration of zymosan (50 µg/ ml). After 60–80 min, PGE synthesis ceased, as noted in the previous experiment (Fig. 4 A), and the cultures were challenged with an additional 110 µg of zymosan. The second zymosan treatment promoted a new round of PGE synthesis (Fig. 5). The total amount of PGE formed was additive in that the amount was comparable to that produced by cultures given a single dose of 160 µg of zymosan (compare with Fig 4 A).

EFFECT OF SERUM ON PGE SYNTHESIS BY MACROPHAGES. The preceding experiments examined PGE synthesis by macrophage cultures treated with zymosan in the absence of serum. Levels of PGE synthesis were also examined in cultures prelabeled with [³H]20:4 and exposed to zymosan in α-MEM that contained from 0.1 to 10% FCS. All concentrations of serum reduced the rate of PGE release and the total amount produced (Fig. 6) compared with serum-free cultures. It was likely that serum lipids were incorporated by macrophages during phagocytosis (23) leading to a reduction in the specific activity of the phospholipid [³H]20:4 and, as a consequence, an underestimate of PGE synthesis. However, RIA measurements of PGE release in
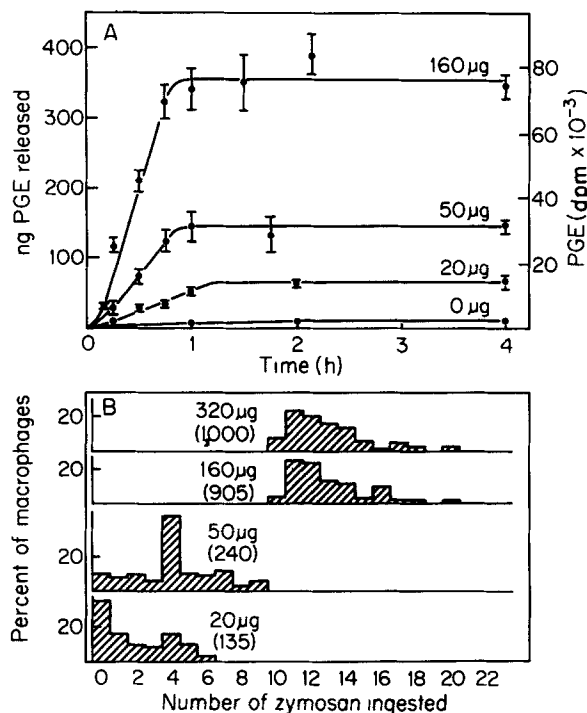
Fig 4   Effect of zymosan concentration on PGE synthesis and phagocytosis by macrophages (A) PGE synthesis Experimental details are those given under Fig 2 except that the cultures were treated with the indicated amounts of zymosan. The results represent three experiments for 160 μg zymosan, three experiments for 50 μg zymosan, one experiment for 20 μg zymosan, and two experiments for controls (no zymosan). (B) Phagocytosis of unopsonized zymosan Macrophage cultures on glass coverslips in 35-mm-diameter culture dishes were incubated overnight, washed, overlaid with fresh α-MEM (no serum), and treated for 1 h at 37°C with the same amounts of zymosan per culture dish as in Fig 4 A. After washing to remove nonadherent particles, the cultures were fixed with glutaraldehyde, mounted, and the number of ingested particles per cell and phagocytizing cells in random fields were counted. The phagocytic index calculated for duplicate cultures is given in parentheses Viability of macrophages remained >95% as determined by trypan blue exclusion

parallel cultures (Fig. 6) exposed to zymosan in 10% serum confirmed the results of the radiolabel assay, thereby ruling out this possibility.

The total amount of radiolabel released by macrophages treated with zymosan in low serum (0.1–1.0%) was comparable to that of control cultures (no serum) and was increased 1.75-fold in cultures that contained 10% serum. Thus, high levels of serum appeared to promote 20:4 release, but serum suppressed PGE synthesis at all concentrations tested.

Discussion

The results of this paper provide a set of specified conditions for assessing the release of 20:4-derived products by explanted peritoneal macrophages in response to a zymosan challenge. In a previous report (18), we had shown that macrophage cultures readily incorporate long-chain saturated fatty acids in the absence of appreciable fatty acid synthesis. Exogenously supplied 20:4 is similarly taken up by macrophages.
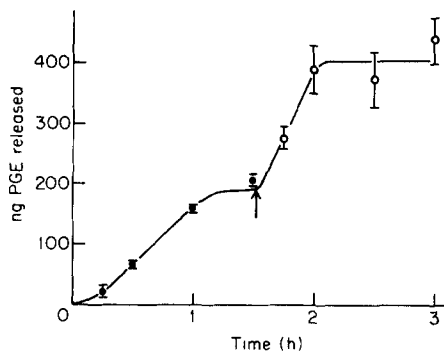
Fig 5. Restimulation of PGE synthesis by macrophages. After the 16-h labeling period with 1 μCi [³H]20:4, the cell cultures were washed and treated with 50 μg of zymosan at time = 0 in α-MEM (no serum) Media were removed from duplicate cultures at the indicated times, extracted, and the PGE was isolated by silicic-acid chromatography After 90 min (indicated by the arrow), an additional 110 μg of zymosan was added to the remaining cultures, the media harvested at subsequent times, and similarly processed. Values are the means ± range
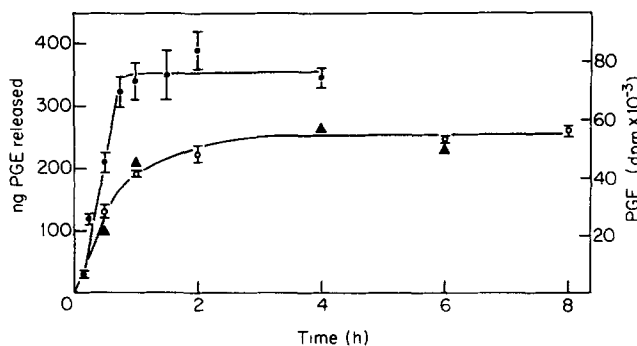

Fig 6 Effect of serum on PGE synthesis by macrophages The procedures are those described under Figs. 2 and 3 except that the cultures were treated with 160 μg of zymosan in α-MEM that contained 10% FCS at time = 0 Values are the means ± SE. O, radiolabeled PGE released by cultures prelabeled with [³H]20·4; ▲, PGE release determined by RIA from nonlabeled cultures; ●, radiolabeled PGE released by [³H]20:4-labeled cultures exposed to 160 μg of zymosan in the absence of serum (redrawn from Fig 4 A for comparison)

During extended labeling periods, the distribution of [³H]20:4 among macrophage phospholipids shifted with progressively larger percentages incorporated into phosphatidylethanolamine. The distribution of 20:4 in the phospholipids of the macrophage-like cell line J774 is highly asymmetric, with phosphatidylethanolamine accounting for ~70% of the total (W. A. Scott. Unpublished results.). A similar localization of 20:4 may exist in peritoneal macrophage phospholipids. Because of this possibility, we employed a 16-h labeling period, which is considerably longer than that used by other investigators.

By using cultures prelabeled with [³H]20:4, it was possible to quantitate the amount and fate of 20:4 released by macrophages as a result of a zymosan challenge. The lack of appreciable fatty acid biosynthesis in phagocytizing macrophage cultures (W. A. Scott. Unpublished results.) maintained in the absence of serum insured that dilution of [³H]20:4 incorporated into cell lipids did not occur and may be a contributing factor to the accuracy of radiolabel assay as a measure of 20:4 release. Although it is

difficult to prove that small amounts of fatty acids other than 20:4 were not released in response to a phagocytic stimulus, the weight of the evidence suggests that the release of 20:4 is specific, which supports earlier observations (4).

Maximal levels of phagocytosis (160 μg zymosan) promoted the release of 28–30% of the [^{3}H]20:4 incorporated by macrophages. This amount is less than the depletion of 20:4 (48%) estimated by fatty acid analysis of whole cell phospholipids. The apparent discrepancy is thought to result from the distribution of [^{3}H]20:4 in macrophage lipids. Assuming that the released 20:4 is derived solely from phospholipids (macrophage neutral lipids are essentially devoid of 20:4) that contain 70% of the incorporated [^{3}H]20:4, the extracellular radiolabel represents 40–42% of this pool and is in good agreement with the observed change in fatty acid composition. Thus, macrophages have the capacity to release between 40 and 50% of their 20:4 content in response to a phagocytic stimulus without loss of cell viability. It remains to be determined whether treatment of macrophages with other particles and agents that stimulate PG synthesis promotes similar levels of 20:4 release. As a result, the above value may be a minimal estimate. Unfortunately, little information is available in the literature concerning maximal levels of 20:4 release by other cell types. However, it is clear that macrophages are a potent source of oxygenated products of 20:4 considering their high content of esterified 20:4 and the large percentage released in response to a phagocytic stimulus.

Of the radiolabel released by cultures treated with zymosan, ~51% is recovered as PGE, 16% as 6-oxo-PGF$_{1\alpha}$, and the remainder as apolar products tentatively identified as HETE (25–30%) together with smaller amounts of 20:4 (3–8%). Our impression is that the percentage of these products is not influenced by the size of the phagocytic stimulus.

A detailed examination of the temporal relationship between zymosan ingestion and the release of PGE supported the idea that the synthesis and release of oxygenated products of 20:4 is a direct and immediate consequence of phagocytosis. Dose-response measurements further indicated that the initial rate of PGE synthesis is proportional to the amount of phagocytosis. However, the duration of PGE synthesis is constant (~60 min) and independent of the zymosan concentration. This suggests that either the phospholipase responsible for the release of 20:4 from cell phospholipids has a finite half-life ($\ll$60 min) or that select intracellular compartments are rapidly depleted of 20:4. It is obvious that the macrophage is capable of reactivating the phospholipase or activating new enzyme that acts on different membrane domains in that these cells are fully capable of responding to multiple exposures of zymosan provided that maximal levels of ingestion are not reached. In functional terms, the implication of these findings is that a phagocytic stimulus promotes a burst of PG synthesis and of other 20:4-oxygenated products, including HETE, the size of which is determined by the number of ingested particles.

Inclusion of 10% serum in phagocytizing cultures promotes the release of 20:4, but reduces the amount of PGE synthesis. The increased 20:4 release is concordant with the recent observations of Shier (24), which suggested that serum stimulates the hormone-activated phospholipase of 3T3 cells. It remains to be determined whether serum has a direct effect on the enzyme, or whether activity is increased because serum components, including albumin and lipoproteins, bind free 20:4. The latter effect may explain the decrease in PGE synthesis if the PG synthetase (cyclooxygenase)

uses only free 20:4 (25). Nevertheless, the effects of serum indicate that the amount of 20:4 release and the fate of 20:4 is influenced by factors other than the phagocytic stimulus.

Summary

The lipids of mouse peritoneal macrophages contain high levels (25 mole percent) of esterified arachidonic acid (20:4). Following in vitro exposure to unopsonized zymosan, these cells synthesize and release oxygenated products of 20:4. Maximal levels of zymosan ingestion promote the release of 40–50% of the 20:4 content of cultures without loss of viability. Release of radiolabel from macrophages prelabeled with [^{3}H]20:4 provides a quantitative measure for the synthesis of 20:4-derived products. Approximately 67% of the released 20:4 is recovered as prostaglandins (PG) (51% PGE and 16% 6-oxo-PGF$_{1\alpha}$) and the remainder as apolar products tentatively identified as hydroxy-eicosatetraenoic acids. The kinetics of synthesis are comparable for both sets of products.

A detailed examination of PGE synthesis indicated that PGE levels rise in parallel with phagocytosis during a continuous exposure of macrophages to zymosan. The concentration of particles determines the initial rate of PGE release, but the time-course of synthesis is finite (~60 min), regardless of the zymosan dose. These observations are compatible with the notion that phagocytosis results in a burst of PG synthesis, the size of which is determined by the phagocytic stimulus. This is supported by the finding that secondary challenges of zymosan promote new rounds of PG synthesis by macrophages.

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