Regulation of Prostaglandin Synthesis and of the Selective Release of Lysosomal Hydrolases by Mouse Peritoneal Macrophages

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Macrophages isolated from the peritoneal cavity of untreated mice and maintained in tissue culture synthesize and release prostaglandins when challenged with zymosan. These cells also selectively release lysosomal acid hydrolases under the same conditions. The major prostaglandins released into the media are found to be prostaglandins E₁, E₂ and 6-oxo-prostaglandin F_{1α}, whereas prostaglandin F_{2α} is not detected. Macrophages isolated from mice that have received an intraperitoneal injection of thioglycollate broth are far less responsive to zymosan challenge. These cells require 300 µg of zymosan to synthesize and release one-third the amount of prostaglandins released from non-stimulated macrophages exposed to 50 µg of zymosan. In addition, thioglycollate-stimulated macrophages release less than 10% of their lysosomal acid hydrolases when exposed to 300 µg of zymosan whereas non-stimulated cells release approximately 50% of these enzymes after treatment with 50 µg of zymosan. The zymosan-stimulated synthesis and release of prostaglandins are completely inhibited by indomethacin, whereas the increased selective release of lysosomal acid hydrolases is not affected. Macrophages, unlike fibroblasts, do not synthesize and release prostaglandins when exposed to serum or to bradykinin.

Mononuclear phagocytes are derived from a bone-marrow stem cell, which proliferates through several morphologically recognizable stages of maturation (Goud *et al.*, 1975) until its progeny are released into the circulation as monocytes. Monocytes in turn leave the circulation and migrate into a large number of organs and tissues of the body where they serve a wide variety of functions (Van Furth, 1975). It has become clear that in addition to their phagocytic function, first discovered by Metchnikoff, they also influence their pericellular environment through the release of a wide variety of synthesized products (Page *et al.*, 1978; Gordon, 1977). These include lysozyme (Gordon *et al.*, 1974), lysosomal acid hydrolases (Davies & Allison, 1976a), neutral proteinases (Werb & Gordon, 1975a,b; Vassalli & Reich, 1977), products affecting the proliferation of lymphocytes and other cells (Unanue *et al.*, 1976), components of the complement system (Colten & Einstein, 1976), and several others that are reviewed by Page *et al.* (1978). Many of these products probably contribute to the roles played by mononuclear phagocytes in maintaining homeostasis (Allison & Davies, 1975; Davies & Allison, 1976b).

Studies by other investigators have shown that the phospholipids of mononuclear phagocytes contain a particularly high proportion of their fatty acids as

arachidonate. Arachidonate constitutes 20% of the fatty acids of rabbit alveolar macrophages (Mason *et al.*, 1972) and of human peripheral monocytes (Stossel *et al.*, 1974). In view of the importance of the oxygenation products of arachidonate in many metabolic processes it was relevant to determine whether or not significant quantities can be synthesized and released by mononuclear phagocytes. Recent studies in our laboratory (Humes *et al.*, 1977) established that this was indeed so, confirming earlier observations by others that mononuclear phagocytes produced prostaglandins (Bray *et al.*, 1974; Gordon *et al.*, 1976).

In the present studies we describe conditions under which mononuclear phagocytes incorporate [³H]-arachidonate into cellular phospholipids and establish the capacity of certain phagocytic stimuli to cause the synthesis and release of arachidonate oxygenation products as well as to stimulate the selective release of lysosomal enzymes.

Experimental

Materials

Male Swiss Webster mice (HLA-SW/ICR SPF) were purchased from Hilltop Lab Animals, Scottdale,

PA, U.S.A. M199 medium, pig serum and foetal calf serum were from Grand Island Biological Co., Grand Island, NY, U.S.A. The pig serum and foetal calf serum were inactivated by heating at 56°C for 30 min. Nunclon tissue-culture dishes were from Vanguard International, Neptune, NJ, U.S.A. [5,6,8,9,11,12,14,15(n)-³H]arachidonic acid (sp. radioactivity 64 Ci/mmol), [8,9,11,12,14,15(n)-³H]-eicosatrienoic acid (sp. radioactivity 17.5 Ci/mmol) and [9,10(n)-³H]oleic acid (sp. radioactivity 6.4 Ci/mmol) were obtained from New England Nuclear, Boston, MA, U.S.A. Zymosan was from ICN, K and K Labs, Plainview, NY, U.S.A., and latex beads (0.8 µm diam.) were from Dow Chemicals, Indianapolis, IN, U.S.A. The zymosan particles were suspended in phosphate-buffered saline, boiled and centrifuged three times. The final pellet was suspended in phosphate-buffered saline at a concentration of 20 mg/ml and stored frozen. Latex beads were suspended in phosphate-buffered saline, centrifuged at 5000g and washed five times with phosphate-buffered saline. Suspensions of zymosan and latex were diluted in culture medium and sonicated immediately before addition to the cells. Thiolglycollate broth was supplied by BBL, Cockeysville, MD, U.S.A., and dissolved as described. *p*-Nitrophenyl *N*-acetyl-β-D-glucosaminide was from Sigma Chemical Co., St. Louis, MO, U.S.A. Phenolphthalein glucuronide and bradykinin were from Calbiochem, San Diego, CA, U.S.A. Prostaglandins F_{2α}, E₂ and D₂ were from Ono Pharmaceuticals, Osaka, Japan. SG-81 chromatographic paper was from Ace Scientific, Linden, NJ, U.S.A. Silica-gel GF t.l.c. plates were from Analtech, Newark, DE, U.S.A. Nanograde solvents from Mallinkrodt, St. Louis, MO, U.S.A., were used in the radioimmunoassay.

The antisera to prostaglandin F_{2α} (preparation 101-F) was a gift from Dr. H. Behrman, Yale University, and antiserum to prostaglandin E₂ (lot 61-335) was from Miles Laboratories, Elkhart, IN, U.S.A.

6-Oxoprostaglandin F_{1α} and thromboxane B₂ were prepared from labelled arachidonic acid by using rat stomach and guinea-pig lung homogenates respectively and were a gift from Dr. E. A. Ham of these laboratories.

Methods

Tissue culture. Macrophages were collected by peritoneal lavage from untreated mice or from mice that had received, 4 days previously, an intraperitoneal injection of 2 ml of thiolglycollate broth. The lavage medium was M199 containing 100 units of penicillin and streptomycin/ml, 20 units of heparin/ml, and 1% heat-inactivated pig serum. The cells were plated at 4 × 10⁶–5 × 10⁶ per 60 mm culture dish and incubated for 2 h at 37°C in 5% CO₂ in air. The non-adherent cells were removed by washing the

cell sheet four times with phosphate-buffered saline and the adherent cells were maintained overnight in 4 ml of medium M199 plus 10% heat-inactivated pig serum. After 24 h the media were removed and the cells were washed twice with 5 ml of phosphate-buffered saline. Fresh M199 medium (4 ml) containing 1% heat-inactivated pig serum was added and 1 µCi of ³H-labelled fatty acid was used.

Harvest of media and cells for prostaglandin and lysosomal hydrolase measurements. At the termination of the experiments, media were collected and divided for subsequent analysis. Then 2 ml of 0.9% NaCl containing 0.1% Triton X-100 was added to the plates and the lysed cells were removed with a rubber 'policeman'.

Enzyme assays. Lactate dehydrogenase was assayed by determining the rate of oxidation of NADH at A₃₄₀. *N*-Acetyl-β-D-glucosaminidase was assayed by the method of Woolen *et al.* (1961) with *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide as substrate dissolved in 0.1 M-citric acid/sodium phosphate dibasic buffer, pH 4.5, and β-glucuronidase by the method of Talalay *et al.* (1946). A unit of enzyme activity for *N*-acetyl-β-D-glucosaminidase and β-glucuronidase is defined as the amount of enzyme needed to cleave 1 nmol of substrate/h at 37°C.

Extraction and chromatography of ³H-labelled prostaglandins. The harvested culture media were acidified to 0.03 M with citric acid, and prostaglandins E₂ (50 µg) and F_{2α} (30 µg) and arachidonic acid (30 µg) were added as chromatographic standards. The acidified media were then extracted with three successive 3 ml portions of chloroform/methanol (2:1, v/v). The combined organic phases were washed with two successive 2 ml samples of methanol/water (2:1, v/v) and evaporated to dryness under a stream of N₂ at 45°C. The residues were dissolved in 25 µl of ethyl acetate/methanol (3:1, v/v), and quantitatively applied to 2 cm × 20 cm strips of SG-81 paper. The strips were developed by descending chromatography in the following systems: system 1, ethyl acetate/acetone/acetic acid (90:10:1, by vol.); system 2, ethyl acetate/acetic acid (99:1, v/v); system 3, ethyl acetate/acetic acid (99:1, v/v), followed by development in the same direction with *n*-hexane/diethyl ether/acetic acid (75:25:1, by vol.). The prostaglandin and fatty acid standards were detected by I₂ vapour.

In some experiments the radioactivity profile of the entire chromatogram was determined by scanning with a Packard 7201 radiochromatogram scanner. Quantification was achieved by cutting the chromatogram into small segments corresponding to the radioactivity peaks and the radioactivity was determined by counting of the samples in 10 ml of Aquasol in a Packard 3255 liquid-scintillation spectrometer.

In most experiments the area corresponding to authentic prostaglandin E₂ of chromatogram devel-

oped with system 2 was cut out and the radioactivity determined. With regard to 6-oxoprostaglandin $F_{1\alpha}$, a 2 cm area of the chromatogram developed with solvent system 2, which had an R_F value identical to that of authentic 6-oxoprostaglandin $F_{1\alpha}$, was cut out and counted. In some experiments with solvent system 1 radioactivity co-chromatographic with both 6-oxoprostaglandin $F_{1\alpha}$ and prostaglandin $F_{2\alpha}$ was cut out and counted.

The cellular distribution of incorporated arachidonate was determined by harvesting the cells in 2 ml of 0.03 M-citric acid followed by lipid extraction. The final residues were dissolved in ethyl acetate/methanol, applied to SG-81 paper and chromatographed with authentic phospholipid standards in chloroform/methanol/acetic acid/water (50:25:8:4, by vol.). The radioactivity was determined by scanning the chromatograms.

Radioimmunoassay for prostaglandins. The culture media containing released prostaglandins were collected, extracted and prostaglandins of the E and F series purified by t.l.c. on silica-gel G plates as described by Humes & Strausser (1974). Radioimmunoassays for prostaglandins E and F were carried out by the method of Orczyk & Behrman (1972).

Results

The procedure used to isolate and cultivate cells for these studies is highly reproducible. The cultures have been shown by morphological and functional criteria to be predominantly mononuclear phagocytes. Cells isolated from untreated mice or from thioglycollate-treated mice were strongly adherent, secreted lysozyme, possessed membrane receptors for the Fc portion of immunoglobulin molecules, and were highly phagocytic.

Labelling of cellular phospholipids with [3H]arachidonate

The depletion of [3H]arachidonate from the culture medium was proportional to the number of adherent cells. In 12 experiments with cultures containing 3×10^6 – 5×10^6 non-stimulated or thioglycollate-stimulated cells/dish, the incorporation of radioactive label ranged from 40 to 60% of the total added [3H]arachidonate in 4 h. Fig. 1 illustrates the time-dependent uptake of [3H]arachidonate by macrophages. In this experiment 43% of the label was taken up by non-stimulated macrophages and 62% by thioglycollate-stimulated macrophages in 4 h. This uptake increased by less than 20% by incubation for a further 20 h. The label could not be removed from the cells by four successive washes with phosphate-buffered saline.

The cellular distribution of the incorporated label

Fig. 1. Time-dependent uptake of [3H]arachidonate by cultured macrophages

Peritoneal macrophages were isolated from thioglycollate-stimulated (●) and from untreated mice (○). Approx. 4×10^6 cells were added to each dish and the cells cultured as described in the Experimental section. [3H]Arachidonate (1 μ Ci) was added to the culture medium (arrow) and the cells were harvested at various times. Radioactivity was determined in the culture media and in thrice-washed cells. The percentage of radioactivity associated with the cells was calculated. The results are the means $\pm$ s.d. for three determinations.

among cellular constituents shown in Table 1 demonstrates that the major phospholipid labelled with [3H]arachidonate in non-stimulated cells was phosphatidylcholine with lesser amounts of the label found in phosphatidylethanolamine and triacylglycerols. In contrast, in thioglycollate-stimulated cells, the radioactive label was distributed nearly equally between phosphatidylcholine and phosphatidylethanolamine. The label found in these three components represents 99% of the solvent-extractable radioactivity.

Zymosan-stimulated release of prostaglandins and lysosomal acid hydrolases from macrophages

Zymosan causes chronic inflammatory lesions, dominated by macrophages, in mice when injected intramuscularly (Schorlemmer *et al.*, 1977). This inflammatory stimulus has also been shown to cause the selective release of lysosomal enzymes from mouse peritoneal macrophages maintained in culture (Schorlemmer *et al.*, 1977). We now show that addition of zymosan to cultures of macrophages stimulates

Table 1. *The incorporation into cellular lipid of [^3H]arachidonate by non-stimulated and thioglycollate-stimulated macrophages and its mobilization from non-stimulated cells by zymosan*

To determine its extent of incorporation into various cellular lipids, [^3H]arachidonate (1 μCi) was incubated with macrophage cultures for 4h. Cells were harvested in 0.03M-citric acid and the lipids extracted as described in the Experimental section. The results are the mean $\pm$ s.d. for three determinations. To determine redistribution of label, [^3H]arachidonate-labelled cells were washed and fresh medium containing zymosan (50 $\mu\text{g}/\text{ml}$) was added. After incubation for 4h the cells were removed from the plates in 0.03M-citric acid and the lipids extracted as described in the Experimental section. Results are the means $\pm$ s.d. for three determinations.

Incorporation of [^3H]arachidonate		
Labelled component	Percentage of incorporated label	
	Non-stimulated	Thioglycollate-stimulated
Phosphatidylcholine	53 $\pm$ 0	36 $\pm$ 1
Phosphatidylethanolamine	31 $\pm$ 5	36 $\pm$ 2
Triacylglycerol	16 $\pm$ 3	35 $\pm$ 1
Mobilization of [^3H]arachidonate from non-stimulated cells by zymosan		
Component	Proportion of radioactivity	
	No addition	Zymosan
Phosphatidylcholine	57 $\pm$ 3	47 $\pm$ 3
Phosphatidylethanolamine	26 $\pm$ 1	26 $\pm$ 0
Triacylglycerol	18 $\pm$ 4	27 $\pm$ 4

the release of certain prostaglandins as well as lysosomal acid hydrolases in a dose- and time-dependent manner (Figs. 2 and 3). The release of both classes of products, prostaglandins and lysosomal acid hydrolases, increased in a similar manner as a function of zymosan concentration (Fig. 2). There was nearly a 30-fold increase in the release of prostaglandin E_2 and a 15-fold increase in 6-oxoprostaglandin $\text{F}_{1\alpha}$ over baseline values by non-stimulated macrophages treated with 200 μg of zymosan/ml. Similarly more than 50% of the total cellular activity of *N*-acetyl- β -D-glucosaminidase was released into the medium of cultures exposed to 200 μg of zymosan/ml for 4h. This release of lysosomal acid hydrolases was selective, since cellular concentrations of the cytoplasmic enzyme lactate dehydrogenase remained constant and no activity was detected in the media.

As shown in Fig. 3, significant release of prostaglandins and lysosomal acid hydrolases does not occur when macrophages are cultured in serum-free medium over a 24h period. However, the addition of 50 μg of zymosan/ml to cultures of non-stimulated macrophages caused a large increase in the release of at least two different prostaglandins and selective release of the lysosomal acid hydrolase *N*-acetyl- β -D-glucosaminidase. In contrast macrophages from thioglycollate-treated mice were less responsive to *in vitro* challenge with zymosan (Fig. 3). The release of prostaglandins from stimulated macrophages by 300 μg of zymosan was significantly less than that found from unstimulated cells treated with 50 μg of zymosan (Figs. 3a and 3b). In addition, there was

little or no zymosan-stimulated release of *N*-acetyl- β -D-glucosaminidase from stimulated cells (Fig. 3c).

Identification of released prostaglandins

The identity of the major prostaglandin components released into the medium after incubation with zymosan was determined by chromatography of the ^3H -labelled compounds in three different solvent systems, and the identity of prostaglandin E_2 was confirmed by radioimmunoassay.

Fig. 4 illustrates a typical radioscan of a chromatogram that had separated the various ^3H -labelled products found in the culture medium. It shows that the addition of 50 μg of zymosan/ml to unstimulated macrophages prelabelled with [^3H]arachidonate caused the release of a variety of ^3H -labelled components that separated into six major peaks of radioactivity (Fig. 4b). In this system, peak 0 co-chromatographs with phospholipids, peak 1 with 6-oxoprostaglandin $\text{F}_{1\alpha}$, peak 2 with prostaglandin E_2 , peak 3 does not co-chromatograph with known standards, peak 4 with 12-hydroxyeicosatetraenoate and peak 5 with arachidonate. It should be noted that the amount of material in peak 0 does not appreciably change after the addition of zymosan (Figs. 4a and 4b).

The cyclo-oxygenase inhibitor indomethacin completely abolished the synthesis of radioactive material corresponding to peaks 1 and 2, but had only a minimal effect on the radioactivity associated with peaks 3–5 (Fig. 4c). In the present paper we concern

Fig. 2. Effect of zymosan on the release of prostaglandins and the selective release of the lysosomal acid hydrolase *N*-acetyl- β -D-glucosaminidase

Cultures of non-stimulated macrophages were labelled with $1 \mu\text{Ci}$ of [^3H]arachidonate for 4 h and washed free of non-incorporated radioactive isotope. Various amounts of zymosan were added in 2 ml of culture media and the cells incubated for an additional 4 h. Media and cells were collected for subsequent prostaglandin and enzyme activity measurements. The amounts of radioactivity found in the media that co-chromatographed with prostaglandin E_2 ($\circ$) and 6-oxoprostaglandin $\text{F}_{1\alpha}$ (Δ) and the percentages of enzyme activity for *N*-acetyl- β -D-glucosaminidase ($\bullet$) and lactate dehydrogenase ($\square$) found in the media were calculated. Results are the averages $\pm$ s.d. for three determinations.

ourselves with the identity of the components associated with peaks 1 and 2.

Peak 1. This product, which is synthesized by cultures to which zymosan was added and whose formation is inhibited by indomethacin, has been identified as 6-oxoprostaglandin $\text{F}_{1\alpha}$ by the following criteria. (1) It co-chromatographs with authentic 6-oxoprostaglandin $\text{F}_{1\alpha}$ in the three solvent systems described in the Experimental section. (2) Further

Fig. 3. Time-dependent release of prostaglandin E_2 (a), 6-oxoprostaglandin $\text{F}_{1\alpha}$ (b) and acid hydrolase (c) from stimulated and non-stimulated macrophage

Macrophages were collected from thioglycollate-stimulated ($\blacktriangle$) and non-stimulated mice ($\bullet$), cultured and labelled for 4 h with [^3H]arachidonate as described in the Experimental section. The cells were washed and fresh medium containing zymosan ($50 \mu\text{g/ml}$) was added to non-stimulated cells and $300 \mu\text{g}$ of zymosan/ml to stimulated cells. Control cultures ($\triangle$ and $\circ$) without added zymosan were also incubated for 24 h. Media and cells were harvested after various times of incubation and prostaglandin and enzyme-activity measurements made. The results are the mean $\pm$ s.d. for three determinations.

evidence that the identity of peak 1 was 6-oxoprostaglandin $\text{F}_{1\alpha}$ was obtained from studies where zymosan-stimulated macrophages had been prelabelled with one of three ^3H -labelled fatty acids, namely

Fig. 4. Radioscan of released arachidonate oxygenation products

Non-stimulated macrophages were labelled with [^3H]arachidonic acid for 4 h. The medium was removed and the cells were washed twice. Fresh medium was added containing the following supplements: (a) no addition; (b) 50 μg of zymosan/ml; (c) 50 μg of zymosan plus 10 μg of indomethacin/ml. Folch extracts were prepared after 4 h of incubation from the combined media of three or four plates and the entire amounts chromatographed in system 3 with the indicated authentic standards. Abbreviations: 6KF, 6-oxoprostaglandin $\text{F}_{1\alpha}$; PGF, prostaglandin $\text{F}_{2\alpha}$; PGE, prostaglandin E_2 ; TXB, thromboxane B_2 ; PGD, prostaglandin D_2 ; HETE, 12-hydroxy-eicosatetraenoate; AA, arachidonate.

Fig. 5. Radioscan of zymosan-induced products from [^3H]arachidonate-, [^3H]eicosatrienoate- and [^3H]oleate-labelled macrophages treated with zymosan. Culture medium was collected from non-stimulated macrophages labelled with one of three fatty acids and then subjected to treatment with 100 μg of zymosan/ml for 4 h. Folch extracts were prepared from the combined media of four plates and the entire extract was chromatographed in system 3 with the indicated authentic standards. (a) Arachidonate; (b) eicosatrienoate; (c) oleate. For abbreviations see the legend to Fig. 4.

arachidonate, eicosa-8,11,14-trienoate or oleate. As expected, cells prelabeled with [^3H]arachidonate synthesized and released a radioactive product co-chromatographic with 6-oxoprostaglandin $\text{F}_{1\alpha}$ (Fig. 5a). Since eicosatrienoic acid lacks a double bond between positions C-5 and C-6, cultures labelled with this fatty acid would not be expected to generate 6-oxoprostaglandin $\text{F}_{1\alpha}$. Fig. 5(b) shows that this is the case. [^3H]Prostaglandin E_1 (peak 2) was found to be the exclusive product of cells labelled with [^3H]eicosatrienoate. Cells labelled with [^3H]oleate and stimulated with zymosan did not release radioactive products in excess of the concentrations observed in control cultures (Table 2). None of this radioactivity

was found to be associated with prostaglandins (Fig. 5c).

Peak 2. This product has been identified as prostaglandin E_2 by the following experiments. (1) This material is co-chromatographic with authentic prostaglandin E_2 in the three solvent systems described in the Experimental section. (2) The material can be converted into prostaglandin B_2 by alkaline dehydration. The component(s) were extracted from the chromatogram and treated with 0.5 M-NaOH in 50% methanol. In four separate experiments, the majority of this zymosan-induced radioactive component ($75 \pm 6\%$) was converted into a product with

Table 2. Uptake of arachidonate, eicosatrienoate and oleate by non-stimulated macrophages and subsequent release after zymosan treatment

Cells were incubated with 1 μ Ci of the various 3 H-labelled fatty acids for 4 h as described in the Experimental section. The culture media were collected and the amounts of the fatty acids incorporated by the cells determined. Fresh medium containing zymosan (100 μ g/ml) was then added and the cells were incubated for an additional 4 h. The culture media were again collected and the percentage of radioactivity that was released was calculated. Results are the means $\pm$ s.d. for three determinations.

Fatty acid	Uptake (pmol)	Radioactivity released (%)	
		No additions	Zymosan
Arachidonate	9.3 $\pm$ 0.2	3.7 $\pm$ 0.3	19.3 $\pm$ 1.8
Eicosatrienoate	25.5 $\pm$ 1.8	5.0 $\pm$ 0.3	10.7 $\pm$ 0.7
Oleate	31.7 $\pm$ 3.9	13.3 $\pm$ 1.1	9.9 $\pm$ 0.3

identical chromatographic mobility with prostaglandin B₂, thereby confirming that at least this proportion of the component was prostaglandin E₂. (3) The material reacts with antisera to prostaglandin E. In a separate series of experiments the effect of zymosan on the stimulation of prostaglandin synthesis and release was evaluated by radioimmunoassay. Cultures containing 5×10^6 non-stimulated macrophages released 6.1 ± 2.2 ng of prostaglandin E into their culture medium over a 4 h period. Similar cultures incubated with zymosan over the same period of time released 392 ± 23 ng of prostaglandin E into their culture medium, representing a stimulation of release of greater than 60-fold. Culture medium containing 50 μ g of zymosan/ml, but not exposed to cells, contained 2.9 ± 0.1 ng of prostaglandin E. Assays for prostaglandin F in the same experiments showed less than 0.5 ng/ml in cultures not exposed to zymosan and 1.3 ± 0.2 ng in cultures exposed to zymosan. Since culture medium not exposed to cells contained 1.0 ± 1.3 ng of prostaglandin F it is clear that under these experimental conditions non-stimulated peritoneal macrophages do not release prostaglandin F into their culture medium.

Source of arachidonate utilized for prostaglandin synthesis by macrophages

We have shown (Table 1) that the majority of the [3 H]arachidonate incorporated by non-stimulated macrophages is located in phosphatidylcholine and phosphatidylethanolamine. Since the addition of zymosan causes large increases in the release of 3 H-labelled oxygenation products of arachidonate, we questioned whether or not this fatty acid was being released from a specific phospholipid. As shown in Table 1, zymosan causes a disproportionate loss of radioactivity from phosphatidylcholine with respect to the other two components. In addition, the increase in the percentage of label found in triacylglycerols after zymosan treatment suggests that the released arachidonate is utilized for prostaglandin synthesis or reacylated into triacylglycerols.

Comparison of the release of arachidonate oxygenation products and lysosomal acid hydrolase by macrophages stimulated with different stimuli

We have shown that the addition of the inflammatory particle zymosan to cultured macrophages stimulates the release of large amounts of prostaglandins under identical conditions to those under which the selective release of lysosomal acid hydrolases occurs (Figs. 2 and 3). It has been reported that particles of similar size such as latex beads, which are readily phagocytosed by macrophages (Michl *et al.*, 1976), are not inflammatory in mice and do not cause the selective release of lysosomal acid hydrolases from cultured macrophages (Schorlemmer *et al.*, 1977). We now show that addition of latex to non-stimulated macrophages also fails to stimulate the release of prostaglandins (Fig. 6). As expected, the addition of 50 μ g of zymosan to the medium caused the release of 6-oxoprostaglandin F_{1 α} , prostaglandin E₂ and β -glucuronidase. In contrast the addition of 320 μ g of latex beads under identical conditions did not elicit these responses. The latex was not toxic to the cells as determined by the retention of cytoplasmic lactate dehydrogenase.

Indomethacin inhibits the synthesis of prostaglandin E₂ and 6-oxoprostaglandin F_{1 α} by macrophages. In contrast, indomethacin did not inhibit the zymosan stimulated release of lysosomal acid hydrolases (Fig. 6). Macrophages do not synthesize and release prostaglandins when stimulated with agents such as foetal calf serum or bradykinin, which are both known to stimulate the formation of these products by fibrosarcoma cells (Hong & Levine, 1976a). Table 3 shows that although both zymosan and foetal calf serum cause considerable increases in the [3 H]arachidonate release from macrophages into culture medium only the former stimulates the synthesis of prostaglandin E₂ and 6-oxoprostaglandin F_{1 α} . This difference is emphasized by the finding that more arachidonate was released into serum-containing cultures; this may be accounted for in part by binding of [3 H]arachidonate to albumin, as shown

by Hong & Levine (1976b). Bradykinin caused only slight increases in [^3H]arachidonate release and no significant increases in ^3H -labelled prostaglandin E_2 and 6-oxoprostaglandin $\text{F}_{1\alpha}$ synthesis and release.

Discussion

The fatty acids of macrophage phospholipids contain an unusually high proportion of arachi-

donate (Mason *et al.*, 1972; Stossel *et al.*, 1974). Since the oxygenation products of this fatty acid have a wide variety of biological activities (Kuehl, 1974), it was pertinent to determine whether such substances were synthesized by macrophages under certain conditions. Indeed, prostaglandins of the E and F series had already been detected by radioimmunoassay in the culture medium of human macrophages residing on interuterine contraceptive devices (Myatt *et al.*, 1975) and in the medium of guinea-pig peritoneal exudate cells stimulated with lymphokines (Gordon *et al.*, 1976).

Hong & Levine (1976a,b) have shown that synthesis of arachidonate oxygenation products can be detected readily in cultures of fibroblasts that have been pre-labelled with [^3H]arachidonate and subsequently exposed to various stimuli. In the present studies we have utilized this technique and have also used radioimmunoassays to measure prostaglandin synthesis and release by cultured mouse peritoneal macrophages.

Macrophages lavaged from the peritoneum of untreated mice and from thioglycollate-stimulated mice incorporated [^3H]arachidonate at approximately the same rate (Fig. 1). The major labelled phospholipids in both cases were found to be phosphatidylcholine and phosphatidylethanolamine (Table 1). However, there was a significant decrease in the ratio of labelled phosphatidylcholine to labelled triacylglycerols in stimulated cells compared with unstimulated cells (Table 1). Others have shown that [^3H]arachidonate is incorporated into phospholipids of human platelets (Bills *et al.*, 1976), methylcholanthrene-transformed Balb/3T3 cells (Pong & Levine, 1976) and heart tissue (Isakson *et al.*, 1976). Non-stimulated macrophages also incorporated eicosatrienoate, the substrate for prostaglandins of the 1-series, under similar conditions. Oleate, which is not a substrate for prostaglandins, was also incorporated by these cells. On treatment with zymosan, [^3H]arachidonate-labelled cells released 5 times the radioactivity of control cultures. The major constituents of the released radioactive material were

Fig. 6. Effect of indomethacin on the zymosan-induced release of prostaglandins and acid hydrolases by macrophages

Macrophages were isolated from non-stimulated mice, cultured and labelled with [^3H]arachidonate as described in the Experimental section. Various additions were made as indicated and the cells incubated for 4 h. The media and cells were harvested for subsequent prostaglandin and enzyme activity measurements. The results are the averages $\pm$ s.d. for three determinations. ■, 6-Oxo[^3H]prostaglandin $\text{F}_{1\alpha}$; □, [^3H]prostaglandin E_2 ; ▨, β -glucuronidase.

Table 3. Lack of effect of foetal calf serum and bradykinin on prostaglandin synthesis and release from macrophages. Macrophages were isolated from non-stimulated mice, cultured and labelled with [^3H]arachidonate as described in the Experimental section. Various additions were made as indicated and the cells incubated for 4 h. The media were harvested for prostaglandin measurements. The results are the averages for two determinations $\pm$ deviation.

Addition	Release of radioactivity (c.p.m.)		
	[^3H]Prostaglandin E_2	6-Oxo[^3H]prostaglandin $\text{F}_{1\alpha}$	[^3H]Arachidonate
None	204 $\pm$ 8	115 $\pm$ 1	1760 $\pm$ 50
Zymosan (50 $\mu\text{g}/\text{ml}$)	4010 $\pm$ 290	1166 $\pm$ 63	2770 $\pm$ 104
Foetal calf serum (10%)	344 $\pm$ 2	145 $\pm$ 0	22000 $\pm$ 790
Bradykinin (0.4 $\mu\text{g}/\text{ml}$)	199 $\pm$ 26	102 $\pm$ 1	1410 $\pm$ 290
Bradykinin (10 $\mu\text{g}/\text{ml}$)	219 $\pm$ 13	115 $\pm$ 2	1620 $\pm$ 90

shown to be [^3H]prostaglandin E_2 and 6-oxo-[^3H]prostaglandin $\text{F}_{1\alpha}$. [^3H]Eicosatrienoate-labelled cells exposed to zymosan released 2 times the radioactivity of the controls and the major component was [^3H]prostaglandin E_1 . No radioactive 6-oxoprostaglandin $\text{F}_{1\alpha}$ was released from eicosatrienoate-labelled cells. Oleate-prelabelled cells did not release an increased amount of radioactivity when challenged with zymosan. The arachidonate release stimulated by zymosan occurred mainly from phosphatidylcholine (Table 1).

The synthesis of prostaglandins by unstimulated macrophages exposed to zymosan coincides with the selective release of lysosomal acid hydrolases (Figs. 2 and 3). The release of the two classes of products differed by their sensitivity to inhibition by indomethacin. The synthesis of prostaglandins was completely inhibited by indomethacin at a concentration of $10\text{ }\mu\text{g/ml}$. On the other hand this concentration of indomethacin gave no significant inhibition of the release of lysosomal acid hydrolases (Fig. 6).

The [^3H]prostaglandin E_2 component released into the medium after zymosan treatment of non-stimulated macrophages was initially identified by its identical mobility to authentic prostaglandin E_2 in three separate chromatographic systems. This component, eluted from the paper chromatogram (Fig. 4), was dehydrated in alkali and greater than 75% of the radioactive product was converted into prostaglandin B_2 (Bergström *et al.*, 1962). G.l.c. and mass-spectral analysis of the trimethylsilylmethyl ester of the prostaglandin E_2 material showed predominantly *m/e* ions identical with prostaglandin E_2 (Nicosia & Galli, 1976). Finally, purified prostaglandin E_2 from zymosan-treated macrophages was detected by radioimmunoassay. This confirms our finding that [^3H]prostaglandin E_2 was released by the zymosan treatment from cells prelabelled with [^3H]arachidonate.

The identity of the other major peak was determined to be 6-oxoprostaglandin $\text{F}_{1\alpha}$ and not prostaglandin $\text{F}_{2\alpha}$. The 6-oxo-[^3H]prostaglandin $\text{F}_{1\alpha}$ component, the stable metabolite of prostaglandin I_2 (Moncada *et al.*, 1976), had identical mobility with authentic 6-oxoprostaglandin $\text{F}_{1\alpha}$ in three chromatographic systems. Although the separation of 6-oxoprostaglandin $\text{F}_{1\alpha}$ from prostaglandin $\text{F}_{2\alpha}$ is difficult, solvent system 2 clearly showed that the second major [^3H]labelled peak co-migrated with the former and not the latter component. No radioactive component was found to co-chromatograph with prostaglandin $\text{F}_{2\alpha}$ in this system. Furthermore, the only major oxygenated product released from [^3H]eicosatrienoate-labelled cells was prostaglandin E_1 , with no prostaglandin F -like material being observed. Eicosatrienoic acid lacks the double bond between C-5 and C-6 and cannot be cyclized to 6-oxoprostaglandin $\text{F}_{1\alpha}$. In agreement with this result, radioimmunoassay

with antibody prepared against prostaglandin $\text{F}_{2\alpha}$ did not detect any significant concentration of this prostaglandin in the macrophage culture media.

The release of lysosomal hydrolases and prostaglandins by macrophages depends on the nature of the stimulus. Zymosan particles have been shown to enhance the selective release of lysosomal acid hydrolase, whereas latex beads of similar size phagocytosed by the cells did not (Allison & Davies, 1975; Schorlemmer *et al.*, 1977). We confirm this finding in the present paper and further show that zymosan, but not latex, also stimulates the release of prostaglandins from non-stimulated macrophages. In contrast with its effect on platelets (Bills *et al.*, 1976) and fibroblasts (Hong & Levine, 1976a), bradykinin did not stimulate the release of prostaglandins from macrophages. In addition, unlike fibroblasts, macrophages did not release prostaglandins in response to serum (Table 3) despite the fact that increased amounts of [^3H]arachidonate were found in the serum-containing medium.

Macrophages from untreated mice were far more responsive to zymosan treatment than macrophages from thioglycollate-stimulated mice. The release of prostaglandins from stimulated cells exposed to $300\text{ }\mu\text{g}$ of zymosan was less than the release from non-stimulated cells exposed to $50\text{ }\mu\text{g}$. In addition, there was little or no release of the lysosomal hydrolases from stimulated cells exposed to zymosan. This observation cannot be explained by a lack of labelled arachidonate available for conversion into prostaglandin. In Fig. 1, it is shown that stimulated cells took up more of the [^3H]arachidonate than non-stimulated cells. Preliminary experiments measuring the amount of zymosan-induced release of arachidonate into the medium in the presence of indomethacin showed that stimulated and non-stimulated cells responded equally, indicating that the diminished capacity of the former cells to synthesize arachidonate oxygenation products is not due to a lack of substrate.

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