Relationship of Prostaglandin Secretion by Rabbit Alveolar Macrophages to Phagocytosis and Lysosomal Enzyme Release

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The phospholipids of rabbit alveolar macrophages were pulse-labelled with [^{14}C]-arachidonic acid, and the subsequent release of labelled prostaglandins was measured. Resting macrophages released measurable amounts of arachidonic acid, the prostaglandins E_2 , D_2 and $\text{F}_{2\alpha}$ and 6-oxoprostaglandin $\text{F}_{1\alpha}$. Phagocytosis of zymosan increased the release of arachidonic acid and prostaglandins to 2.5 times the control value. In contrast, phagocytosis of inert latex particles had no effect on prostaglandin release. Indomethacin inhibited the release of prostaglandin, and, at high doses (20 $\mu\text{g}/\text{ml}$), increased arachidonic acid release. Analysis of the cellular lipids showed that after zymosan stimulation the proportion of label was decreased in phosphatidylcholine, but not in other phospholipids or neutral lipids. Cytochalasin B, at a dose of 2 $\mu\text{g}/\text{ml}$, inhibited the phagocytosis induced by zymosan but increased prostaglandin synthesis to 3.4 times the control. These data suggest that the stimulation of prostaglandin synthesis by zymosan is not dependent on phagocytosis. Exposure to zymosan also resulted in the release of the lysosomal enzyme, acid phosphatase. Furthermore, cytochalasin B augmented the zymosan-stimulated release of acid phosphatase at the same dose that stimulated prostaglandin synthesis. However, indomethacin, at a dose that completely inhibited prostaglandin synthesis, failed to block the lysosomal enzyme release. Thus despite some parallels between the release of prostaglandins and lysosomal enzymes, endogenous prostaglandins do not appear to mediate the release of lysosomal enzymes. The prostaglandins released from the macrophages may function as humoral substances affecting other cells.

There is evidence that prostaglandins are mediators in inflammatory reactions (Willis, 1969; Williams & Morley, 1973). They are synthesized by human and rabbit polymorphonuclear leucocytes (Higgs & Youtlen, 1972; Higgs *et al.*, 1975; Tolone *et al.*, 1977; Northover, 1977). In chronic inflammation, macrophages may be a major source. Prostaglandin E-like activity has been demonstrated by radioimmunoassay in macrophage-rich peritoneal exudate cell preparations from guinea pigs (Bray *et al.*, 1974; Gordon *et al.*, 1976; Bray & Gordon, 1976) and mice (Humes *et al.*, 1977; Glatt *et al.*, 1977; Farzad *et al.*, 1977; Kurland & Bockman, 1978; Weidmann *et al.*, 1978). Mouse peritoneal macrophages also release 6-oxoprostaglandin $\text{F}_{1\alpha}$, prostaglandin $\text{F}_{2\alpha}$ (Humes *et al.*, 1977; Farzad *et al.*, 1977; Weidmann *et al.*, 1978) and thromboxane (Weidmann *et al.*, 1978; Brune *et al.*, 1978) in response to inflammatory stimuli such as zymosan. Prostaglandin synthesis by pulmonary macrophages has never been reported.

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The role of lysosomal enzymes as mediators of the inflammatory reactions is well documented (Weissman, 1965; Cochrane, 1968; Becker & Henson, 1973). Endocytic stimuli result in increased lysosomal enzyme activity in mouse peritoneal macrophages (Weissman, 1965; de Duve & Wattiaux, 1966; Weissman *et al.*, 1971) and polymorphonuclear leucocytes (Weissman, 1965; de Duve & Wattiaux, 1966). However, the mechanisms for lysosomal enzyme induction are poorly understood. Since prostaglandins are also mediators of inflammation (Willis, 1969; Williams & Morley, 1973) and are produced in response to phagocytic stimuli, it is important to examine the relationship between their production and lysosomal enzyme release.

The principal objectives of the present investigation are to identify the various prostaglandins secreted by rabbit alveolar macrophages and to examine their relationship to phagocytosis. Cytochalasin B was used in an effort to dissect the process of phagocytic stimulation into stages, since this drug inhibits the interiorization of particles, but does not interfere with the attachment of the particle to the macrophage

membrane (Allison *et al.*, 1971; Axline & Reaven, 1974). We have also examined that relationship between prostaglandin production and lysosomal enzyme release.

Prostaglandins are not stored preformed in cells. Intracellular concentrations of free fatty acids, including the precursors of prostaglandins, are very low (Piper & Vane, 1971). Consequently, the synthesis of prostaglandins requires the activation of cell lipases to release the precursor, arachidonic acid, from bound tissue lipid, predominantly phospholipid (Flower & Blackwell, 1976; Bills *et al.*, 1976). We have previously developed methods to incorporate [^{14}C]arachidonic acid into the tissue lipid pool in both isolated organs (Isakson *et al.*, 1976) and tissue culture (Jakschik *et al.*, 1978) and to measure the free arachidonic acid release by using fatty acid-free bovine serum albumin to bind the released free fatty acid and prevent its use for reacylation (Isakson *et al.*, 1977). By labelling the precursor pool of macrophages and by measuring the subsequent release of radioactive arachidonic acid and prostaglandins, we obtain measures of both lipase activity and cyclo-oxygenase activity in these cells.

Materials and Methods

Chemicals

[^{14}C]Arachidonic acid (sp. radioactivity 55 mCi/mmol; Amersham/Searle, Arlington Heights, IL, U.S.A.) was prepared as the sodium salt in 0.9% NaCl by adjusting the pH to 9 with 0.1 M-NaOH. Fatty acid-poor bovine serum albumin was obtained from Calbiochem. The albumin was extracted twice with an equal volume of diethyl ether before use to remove the residual fatty acid. The residual ether was evaporated from the aqueous phase in a stream of N_2 or a vacuum evaporator. Cytochalasin B was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. 4-Methylumbelliferyl phosphate was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Dulbecco's modified Eagle's medium, heat-inactivated foetal calf serum and penicillin/streptomycin solution (5000 i.u. of penicillin G and 5000 μg of streptomycin/ml) were purchased from Grand Island Biological Co. (GIBCO), Grand Island, NY, U.S.A. Prostaglandin standards were generously contributed by Dr. John Pike of the Upjohn Co., Kalamazoo, MI, U.S.A.

Preparation and incubation of alveolar macrophages

Adult male New Zealand rabbits weighing 2–3 kg were anaesthetized with pentobarbital sodium (30 mg/kg), and exsanguinated.

The thoracic cavity was opened and the lung was lavaged six times *in situ* through a tracheotomy with 50 ml of sterile 0.15 M-NaCl containing 2.6 mM-EDTA.

Use of EDTA to chelate the bivalent cations Ca^{2+} and Mg^{2+} increases the cell yield (Brain & Frank, 1973), and prevents the cells from adhering to the tube. The wash fluid was pooled and centrifuged at 800g for 10 min. Small samples were taken for viability testing by Trypan Blue dye exclusion, and smears were prepared and fixed in ethanol for histological examination with haematoxylin and eosin stains. The collected cells were approx. 90% macrophages by morphological examination, and over 95% were viable. The cells were counted in a haemocytometer, diluted, and 1 ml portions containing 6×10^6 cells were plated in 35 mm Petri dishes in Dulbecco's modified Eagle's medium containing 15% (v/v) foetal calf serum and 100 i.u. of penicillin/ml and 100 μg of streptomycin/ml. The serum concentration (15%) is known to provide optimal proliferation of mouse and hamster macrophages (Lin *et al.*, 1975). The cells were cultured overnight to allow macrophages to attach to the plates, and the medium was replaced by serum-free Dulbecco's modified Eagle's medium containing the same concentrations of penicillin and streptomycin and 0.2 g of lactalbumin hydrolysate/100 ml. After overnight culturing, 70–80% of the cells attached to the plates; 96–99% of the attached cells were macrophages, and more than 95% excluded Trypan Blue. Of the remaining cells, the majority were lymphocytes (1–3%). Granulocytes comprised less than 1% of the cells in all cultures. [^{14}C]Arachidonic acid (sp. radioactivity 55 mCi/mmol) was added to the cultures as the sodium salt, at a concentration of 2×10^6 – 3×10^6 c.p.m./ml. The cultures were incubated for 1 h to label the cell lipids. The cells were then washed with Dulbecco's modified Eagle's medium to remove the excess free radioactive arachidonate, and the medium and washes pooled and analysed for labelled prostaglandins. The washed cells were then incubated for 90 min in Dulbecco's modified Eagle's medium containing penicillin, streptomycin, 0.2 g of lactalbumin hydrolysate/100 ml and fatty acid-free bovine serum albumin (2 mg/ml final concentration) with or without added drugs or particles. As phagocytic stimuli, zymosan (1 mg/ml) or 1.1 μm latex particles (0.1 mg/ml) were added to some cultures. Indomethacin (2 μg /ml or 20 μg /ml) was used in some cultures to inhibit prostaglandin synthesis. Cytochalasin B was used initially in an attempt to separate effects due to the attachment phase of phagocytosis (not inhibited by cytochalasin B) from those due to particle uptake (inhibited by cytochalasin B) (Allison *et al.*, 1971). Two different concentrations of cytochalasin B, 0.5 μg /ml and 2 μg /ml, were used in most experiments. A stock solution of cytochalasin B (1 mg/ml) in dimethyl sulphoxide was diluted with Dulbecco's modified Eagle's medium to give the desired concentration. The highest concentration of dimethyl sulphoxide in the cultures was 0.2% (v/v) which, by itself, did not affect prostaglandin secretion.

Determination of cell lipids, prostaglandins and phagocytic activity

At the end of the experimental period, usually 90 min, the medium was collected, acidified to pH 3.5 with formic acid, extracted twice with an equal volume of ethyl acetate, dried and applied to thin-layer plates coated with silica gel G. The solvent system usually employed for the separation of prostaglandins was the organic phase from ethyl acetate/acetic acid/trimethylpentane/water (11:2:5:10, by vol.) [solvent system 'A-9' of Hamberg & Samuelsson (1966)]. This system separated 6-oxo-prostaglandin $F_{1\alpha}$, prostaglandins $F_{2\alpha}$, E_2 and D_2 and arachidonic acid. In some experiments, the solvent system 'BDA' (benzene/dioxan/acetic acid; 20:10:1, by vol.) was used to separate prostaglandins $F_{2\alpha}$, E_2 and D_2 , thromboxane B_2 and arachidonic acid. Zones corresponding to the prostaglandin and arachidonic acid standards were scraped off and counted for radioactivity. In most experiments, duplicate cultures were used for prostaglandin extraction. The cells from one culture dish were scraped off the dish into 0.5 ml of 0.15 M-NaCl with a rubber 'policeman', and cell lipids were extracted with 10 ml of chloroform/methanol (2:1, v/v). The lipid extracts were washed with water, dried, applied to thin-layer plates and developed in chloroform/methanol/aq. NH_3 (13:7:1, by vol.) for separation of phospholipids. The cells from the other culture dish were either tested for viability or fixed *in situ* in 70% (v/v) ethanol and stained by the periodate/Schiff base reaction, which colours zymosan particles intensely magenta. The percentage of cells containing two or more zymosan particles was recorded.

In early experiments, to compare our results with published data (Humes *et al.*, 1977), we also examined prostaglandin production by mouse peritoneal macrophages, using cells collected 4 days after thio-glycollate injection into the peritoneal cavity.

Enzyme determinations

Acid phosphatase activity was determined in both medium and cells by modification of the procedure of Leaback (1974). To determine the intracellular enzyme content the cells were scraped off the plates into 0.3 ml of 0.1% (v/v) Triton X-100 in water with a rubber 'policeman'. Portions of the cell lysates were diluted 10-fold with 0.2 M-sodium acetate buffer (pH 5) for assay. Then 30 μ l of the medium or diluted cell lysate was added to 70 μ l of 4-methylumbelliferyl phosphate (0.03 mg/ml) in acetate buffer and incubated at 37°C for 15 min. The enzyme reaction was terminated by adding 1.9 ml of 0.02 M-sodium phosphate buffer (pH 10.7). The fluorescence was measured in a Farrand fluorimeter with a Corning 5860 primary filter and Corning 3387 and 5543 secondary filters.

Lactate dehydrogenase was assayed as described by Lowry & Passonneau (1972): 20 μ l of undiluted supernatant or cell lysate was incubated in 2 ml of substrate consisting of 1 mM-sodium pyruvate and 2 μ M-NADH in 1 mM-sodium phosphate buffer, pH 7.0. The native fluorescence of the NADH was measured at 15 s intervals for 2 min with the Farrand fluorimeter equipped with a Corning 5860 primary filter and Corning 4303 and 3387 secondary filters.

Results

Incorporation of [^{14}C]arachidonic acid into cell lipids and release of [^{14}C]prostaglandins after stimulation

Approx. 40% of the radioactivity was incorporated into cell lipids of rabbit alveolar macrophages in 1 h under our incubation conditions; 65–70% of the incorporated radioactivity was in phospholipids, most in phosphatidylcholine ($41.1 \pm 1.2\%$ of total radioactivity in cells) and phosphatidylethanolamine ($16.7 \pm 1.7\%$) (Fig. 1). Only $11.1 \pm 2.2\%$ of the intracellular label was in neutral lipid, and very little free arachidonic acid ($1.9 \pm 0.4\%$ of total intracellular radioactivity) was detected in the cell lipid extract. A comparison of the cell lipids of rabbit alveolar macrophages and mouse peritoneal macrophages showed similar patterns of incorporation, except for the presence of an unidentified peak (X; $15.4 \pm 1.3\%$ of intracellular radioactivity) migrating between fatty acid and neutral lipid in alveolar macrophages (Fig. 1). This unidentified peak was absent from lipids of mouse peritoneal macrophages, rabbit heart and kidney tissues when extracted by the same techniques and solvent systems. It remained at the origin in the solvent system [hexane/diethyl ether/acetic acid (30:70:1, by vol.)] used for separation of neutral lipids.

Analysis of the medium after zymosan stimulation with mouse peritoneal macrophages after thio-glycollate injection confirmed previous observations that prostaglandin E_2 was the single predominant prostaglandin produced by stimulated mouse peritoneal macrophages (Humes *et al.*, 1977). In contrast, rabbit alveolar macrophages released a mixture of prostaglandins E_2 , D_2 and $F_{2\alpha}$ and 6-oxoprostaglandin $F_{1\alpha}$ (the stable metabolite of prostaglandins I_2) after zymosan stimulation. Typical radiochemical scans of the medium after zymosan stimulation from mouse peritoneal macrophages and rabbit alveolar macrophages are shown in Fig. 1. The proportion of each prostaglandin released by rabbit alveolar macrophages in response to zymosan is shown in Table 1. No thromboxane could be detected after zymosan stimulation by the radiochemical techniques used in these experiments. The pattern of prostaglandin production in response to exogenous arachidonic acid (i.e. [^{14}C]prostaglandin release during incorporation of [^{14}C]arachidonic acid) was

Fig. 1. Radiochemical scans of extracts of media and cells from typical experiments with rabbit alveolar macrophages (b) and mouse peritoneal macrophages (a)

(i) [^{14}C]Prostaglandin release during labelling period, i.e. prostaglandin release in response to exogenous [^{14}C]arachidonic acid. The medium was collected after 1 h incubation with [^{14}C]arachidonic acid, extracted and applied on thin-layer plates. The solvent system used was 'A-9'. Abbreviation: AA, arachidonic acid. (ii) Incorporation of [^{14}C]arachidonic acid into cell lipid. The cells were extracted with 20 vol. of chloroform/methanol (2:1, v/v) at the end of experimental period (90 min), applied on thin-layer plates, and was developed in chloroform/methanol/aq. NH_3 (13:7:1, by vol.). Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, PI, phosphatidylserine and phosphatidylinositol; FA, fatty acid; NL, neutral lipids. (iii) Release of [^{14}C]prostaglandin after zymosan stimulation (90 min). The medium was collected, extracted, applied on thin-layer plates and developed in solvent system 'A-9'. Abbreviation: AA, arachidonic acid.

Table 1. Zymosan-induced prostaglandins production in rabbit alveolar macrophages

Zymosan was added to cultures as described in the Materials and Methods section, and after 90 min the media were collected, extracted, applied to thin-layer plates and developed in solvent system 'A-9'. Zones corresponding to various prostaglandin standards were made visible in iodine vapour, scraped off, counted for radioactivity and the results expressed as percentage of total prostaglandin released (sum of 6-oxoprostaglandin $\text{F}_{1\alpha}$ + prostaglandin $\text{F}_{2\alpha}$ + prostaglandin E_2 + prostaglandin D_2) (means $\pm$ S.E.M. for 10 separate experiments).

Total [^{14}C]prostaglandin (c.p.m.)	Percentage of total prostaglandin released			
	6-Oxo $\text{F}_{1\alpha}$	$\text{F}_{2\alpha}$	E_2	D_2
4220 $\pm$ 730	18.3 $\pm$ 0.7	23.2 $\pm$ 0.8	38.2 $\pm$ 2.6	24.3 $\pm$ 0.9

very similar to the pattern of prostaglandins production in response to endogenous arachidonic acid (after zymosan stimulation) (Fig. 1).

Phagocytosis of zymosan by alveolar macrophages did not affect cell viability, as judged by dye exclusion

or release of lactate dehydrogenase (Table 2). It did result in a 2.5 times increase in prostaglandin synthesis (Fig. 2). This increase could be inhibited by indomethacin (2 $\mu\text{g}/\text{ml}$). Indomethacin did not exert any effect on phagocytosis (Table 2).

Table 2. Effects of cytochalasin B and indomethacin on the phagocytosis of zymosan and enzyme release by cultured alveolar macrophages

Duplicate cell cultures were used. At the end of the experimental period, the media were saved for prostaglandin determination and enzyme assay (see the Materials and Methods section). One culture dish was fixed *in situ* with 70% (v/v) ethanol and stained by the periodate/Schiff base reaction, which colours zymosan particles intensely magenta. The percentage of cells containing two or more zymosan particles was recorded. The cells from the other culture dish were scraped off and lysed and lactate dehydrogenase and acid phosphatase activities were determined. The results are expressed as percentage of total activity in the cultures that was extracellular (means $\pm$ S.E.M., $n = 3$).

Treatment	Phagocytosis (%)	Lactate dehydrogenase (% of extracellular)	Acid phosphatase (% of extracellular)
Control	—	23.2 $\pm$ 2.6	8.9 $\pm$ 0.5
Zymosan	89.5 $\pm$ 3.5	20.2 $\pm$ 4.8	13.2 $\pm$ 1.1
Zymosan+indomethacin (2 μ g/ml)	86.3 $\pm$ 4.1	17.4 $\pm$ 3.8	11.7 $\pm$ 0.9
Zymosan+cytochalasin B (0.5 μ g/ml)	91.5 $\pm$ 2.5	20.4 $\pm$ 3.8	16.6 $\pm$ 1.4
Zymosan+cytochalasin B (2 μ g/ml)	64.7 $\pm$ 4.9	22.2 $\pm$ 5.3	17.0 $\pm$ 2.2
Zymosan+cytochalasin B (0.5 μ g/ml) + indomethacin (2 μ g/ml) *	91.0 $\pm$ 1.0	18.2 $\pm$ 6.8	16.7 $\pm$ 0.9

* Prostaglandin production was completely inhibited at this dose in these experiments.

Fig. 2. Change in total [¹⁴C]prostaglandin production by cultured alveolar macrophages in response to various stimuli The media were extracted at the end of the experimental period (90 min), applied on the thin-layer plates and developed in solvent system 'A-9'. Zones corresponding to various prostaglandin standards were scraped off and counted for radioactivity. The total prostaglandin release (sum of 6-oxo F_{1α} + F_{2α} + E₂ + D₂) was calculated and expressed as the percentage of the control value (unstimulated cells). The following abbreviations are used: Z, zymosan (1 mg/ml); CB_(0.5), cytochalasin B (0.5 μ g/ml); CB₍₂₎, cytochalasin B (2 μ g/ml); I, indomethacin (20 μ g/ml); L, latex particles (0.1 mg/ml). The numbers in circles are the numbers of experiments. Results are means $\pm$ S.E.M.

Effect of cytochalasin B on phagocytosis and prostaglandin release

Addition of a low dose of cytochalasin B ($0.5\mu\text{g}/\text{ml}$) to zymosan-stimulated alveolar macrophages increased the total prostaglandin release to 3.4 times the value of unstimulated cells (Fig. 2), a value significantly above that for zymosan alone ($P < 0.01$; Student's *t* test). Microscopic examination of the cells showed that cytochalasin B at this dose does not affect phagocytosis (Table 2). The prostaglandin-stimulating effect was mediated via activation of the cell lipases (probably phospholipase), as indicated by a parallel increase in arachidonic acid release into the medium (Fig. 3). Cytochalasin B at doses of $2\mu\text{g}/\text{ml}$ and above inhibited phagocytosis (Table 2). At $2\mu\text{g}/\text{ml}$, cytochalasin B enhanced further the release of prostaglandins and arachidonic acid (Figs. 2 and 3). However, increasing the dose of cytochalasin B to

$5\mu\text{g}/\text{ml}$ and $20\mu\text{g}/\text{ml}$ failed to increase prostaglandin production further, despite an additional increment in deacylation (results not shown). Cytochalasin B without zymosan, or in combination with latex, had no effect on prostaglandin production or arachidonic acid release (Figs. 2 and 3). Indomethacin ($20\mu\text{g}/\text{ml}$) also had a facilitating effect on zymosan-induced arachidonic acid release (Fig. 3). This effect was barely noticeable with $2\mu\text{g}$ of indomethacin/ml. In the absence of zymosan or cytochalasin B, indomethacin had no effect on fatty acid release.

Effects of zymosan and cytochalasin B on lysosomal enzyme release

Phagocytosis of zymosan by alveolar macrophages resulted in a 50% increase in acid phosphatase release (Table 2). This increase was further augmented to 185% of the control value by the addition of cyto-

Fig. 3. Changes in [^{14}C]arachidonic acid release after various stimuli in rabbit alveolar macrophages

The medium was extracted at the end of the experimental period, applied on thin-layer plates and developed in solvent system 'A-9'. The zones corresponding to arachidonic acid standard were scraped off, counted for radioactivity and expressed as the percentage of the control value (unstimulated cells). The following abbreviations are used: Z, zymosan ($1\text{ mg}/\text{ml}$); CB_(0.5), cytochalasin B ($0.5\mu\text{g}/\text{ml}$); CB₍₂₎, cytochalasin B ($2\mu\text{g}/\text{ml}$); I, indomethacin ($20\mu\text{g}/\text{ml}$); L, latex particles ($0.1\text{ mg}/\text{ml}$). The numbers within circles are the numbers of experiments. The results are means $\pm$ S.E.M.

Fig. 4. Change in [^{14}C]arachidonic acid in various lipid classes in rabbit alveolar macrophages after stimuli

The cells were extracted with 20 vol. of chloroform/methanol (2:1, v/v), the extracts applied on thin-layer plates, and developed in chloroform/methanol/aq. NH_3 (13:7:1, by vol.). Zones corresponding to various phospholipid standards were scraped off, counted for radioactivity and the percentage of label in each lipid class was calculated. The values expressed are the changes in percentage of total c.p.m. in a given lipid class compared with the value for unstimulated cells. The following abbreviations were used: Z, zymosan (1 mg/ml); CB, cytochalasin B (0.5 $\mu\text{g}/\text{ml}$); I, indomethacin (20 $\mu\text{g}/\text{ml}$), L, latex particles (0.1 mg/ml). Numbers within circles are the numbers of experiments. The results are means $\pm$ S.E.M.

chalasin B (0.5 $\mu\text{g}/\text{ml}$ or 2 $\mu\text{g}/\text{ml}$). Neither cytochalasin B alone nor latex particles increased lysosomal enzyme release (results not shown). Indomethacin at a dose that completely inhibited prostaglandin synthesis (2 $\mu\text{g}/\text{ml}$) did not significantly alter the acid phosphatase release.

Phospholipid source of released arachidonic acid

Analysis of the cell lipids showed that the percentage of labelled arachidonic acid in phosphatidylcholine was decreased by $4 \pm 0.7\%$ with zymosan stimulation (Fig. 4). This loss increased to $7 \pm 0.7\%$ with the addition of cytochalasin B in the presence of zymosan (Fig. 4). The loss of radioactivity from phospholipid was roughly equivalent to the amount of radioactive prostaglandins and arachidonic acid released into the media. No decrease in labelled phosphatidylethanolamine, acidic phospholipid, neutral lipid, or X peak was detected after stimulation with either

zymosan or zymosan with cytochalasin B (Fig. 4). Thus the main source of the arachidonic acid substrate for prostaglandin synthesis in stimulated macrophages appeared to be deacylation of phosphatidylcholine. Indomethacin, at the dose of 20 $\mu\text{g}/\text{ml}$, caused further loss of radioactivity from phosphatidylcholine (Fig. 4). This effect was barely noticeable at the dose of 2 $\mu\text{g}/\text{ml}$.

Discussion

It has been shown that polymorphonuclear leucocytes and mouse peritoneal macrophages produce prostaglandins during phagocytosis (Higgs & Youtlen, 1972; Bray *et al.*, 1974; Higgs *et al.*, 1975; Bray & Gordon, 1976; Gordon *et al.*, 1976; Tolone *et al.*, 1977; Glatt *et al.*, 1977; Humes *et al.*, 1977; Northover, 1977; Brune *et al.*, 1978). The present study shows that rabbit alveolar macrophages also

produce prostaglandins, including D_2 , E_2 , $F_{2\alpha}$ and I_2 (prostaglyclin), when stimulated. Prostaglandin formation was stimulated by zymosan, an inflammatory stimulus, but not by latex, an inert particle (Fig. 2), suggesting that prostaglandins may be involved in the inflammatory response to zymosan. The presence of thromboxane could not be demonstrated by the radiochemical techniques applied in this study. In other studies, however, when alveolar macrophage homogenates were incubated with radioactive prostaglandin H_2 , thromboxane was generated, indicating the presence of thromboxane synthetase in these cells (W. Hsueh, unpublished work).

As with other cells, the rate-limiting factor in prostaglandin production by alveolar macrophages appeared to be the availability of free arachidonic acid. When exogenous [^{14}C]arachidonic acid was supplied, unstimulated alveolar macrophages produced labelled prostaglandins. Zymosan stimulation was accompanied by activation of lipase, as indicated by the release of labelled arachidonate from the cells accompanying the release of prostaglandins. The reduction in labelled arachidonic acid in phosphatidylcholine relative to the other lipid classes after phagocytosis of zymosan suggests that phosphatidylcholine is the main source of arachidonic acid for prostaglandin synthesis in macrophages (Fig. 4). These observations differ from those on platelets (Bills *et al.*, 1977), in which both phosphatidylcholine and phosphatidylinositol were reported to be sources of deacylation for prostaglandin and thromboxane synthesis. We believe that this change in the proportion of label in the various lipid classes was related to prostaglandin synthesis and did not result simply from interiorization of the endocytic membrane during particle ingestion, with subsequent redistribution of the membrane lipids, since interiorization of latex particles did not cause either a change in the distribution of label between lipid classes or in prostaglandin synthesis.

We also observed that indomethacin, especially at a high dose (20 $\mu g/ml$), augmented arachidonic acid release from stimulated alveolar macrophages. Possible mechanisms of this augmentation include (a) induction of phospholipase, thus facilitating deacylation, or (b) inhibition of reacylation of phospholipids and reutilization of arachidonic acid. The latter explanation appears to be more plausible, since indomethacin without phagocytic stimuli had no effect on arachidonic acid release. In some experiments, the use of indomethacin resulted in complete inhibition of prostaglandin synthesis in zymosan-treated macrophages, i.e. the prostaglandin secretion under those circumstances was actually lower than in the control group. However, complete inhibition was not achieved every time; thus the mean value for the indomethacin-treated group was comparable with that for the control group. The possible

explanations are: (1) the bovine serum albumin in the culture medium binds indomethacin; (2) indomethacin is unstable at neutral pH, and thus gradually loses its effect; (3) indomethacin is slowly degraded by cells. This loss of inhibitory effect of indomethacin on prostaglandin synthesis in cultured macrophages was more apparent in long-term (24h) incubation (results not included in Results), in which a considerable amount of prostaglandin could be detected despite the addition of indomethacin.

Endocytosis itself may not be critical to the process whereby zymosan stimulates prostaglandin production. Although cytochalasin B at a dose of 2 $\mu g/ml$ decreased the uptake of zymosan, it paradoxically increased the amount of prostaglandin secretion that the zymosan provoked. Similar results were obtained with mouse peritoneal cells (Brune *et al.*, 1978). Goldstein *et al.* (1978) have also observed that cytochalasin B inhibited zymosan uptake by polymorphonuclear leucocytes, without diminishing the stimulation of prostaglandin and thromboxane synthesis. In the polymorphonuclear leucocyte, however, cytochalasin B did not increase prostaglandin secretion above that produced by zymosan alone. The observations with both cell types suggest that it is the interaction of the zymosan with the membrane of the phagocyte or the resultant metabolic changes (Schnyder & Baggiolini, 1978), rather than the uptake of the zymosan, that is the stimulus to secretion.

Cytochalasin B stimulates macrophages to increase secretion not only of prostaglandins, but also of elastase, collagenase (Gordon & Werb, 1976) and lysosomal enzymes (Davies *et al.*, 1973; Ackerman & Beebe, 1975). In contrast with our finding that cytochalasin B at low doses required zymosan to increase release of lysosomal enzymes, with the larger doses and longer incubation used in the above studies, cytochalasin B stimulated lysosomal enzyme release even in the absence of phagocytosis.

Our initial observations raised the possibility of a direct relationship between lysosomal enzyme release and secretion of prostaglandins. Both processes were evoked by the inflammatory stimulus, zymosan, but not by inert latex particles. Both prostaglandin release and lysosomal enzyme release were stimulated by the addition of cytochalasin B to zymosan-treated cells, whereas cytochalasin B alone had no effect. We can exclude a regulatory role of prostaglandins on lysosomal enzyme release, since inhibition of prostaglandin synthesis by indomethacin did not alter lysosomal enzyme release and since Ackerman & Beebe (1975) had previously found that exogenous prostaglandins (E_1 , E_2 , $F_{1\alpha}$ and $F_{2\alpha}$) had no effect on the release of β -glucuronidase from guinea-pig alveolar macrophages. During the preparation of this paper, Bonney *et al.* (1978) also reported the lack of effect of indomethacin on the

release of lysosomal acid hydrolases in mouse peritoneal macrophages. The converse, that lysosomal enzymes influence prostaglandin synthesis, is more difficult to exclude. Alveolar macrophage lysosomal granules do contain phospholipase A₁ and A₂ (Franson & Waite, 1973). Hence it is possible that during the redistribution of lysosomal enzymes that accompanies zymosan or zymosan and cytochalasin B stimulation, lysosomal phospholipase A₂ has access to a lipid pool from which it is otherwise separated.

Further studies have provided evidence for a variety of secretory functions for macrophages (Unanue, 1976, 1977). Macrophages have been reported to secrete mitogenic factors that influence granulopoiesis (Golde *et al.*, 1972), B- and T-lymphocyte function (Unanue *et al.*, 1976), fibroblast proliferation (Leibovich & Ross, 1975, 1976) and angiogenesis (Polverini *et al.*, 1977). Several investigations have pointed to prostaglandins being among the mediators that are responsible for the interactions between macrophages and other cells. For instance, macrophage prostaglandins have been suggested to depress myelopoiesis induced by colony-stimulating factor (Kurland *et al.*, 1978), to depress lymphokine production (Gordon *et al.*, 1976), to depress the plaque-forming response to autologous erythrocyte antigen (Zimecki & Webb, 1977), to prevent enhancement of B-lymphocyte colony formation by macrophages (Kurland *et al.*, 1977) and to depress the mitogenic response of T-lymphocytes in Hodgkin's disease (Goodwin *et al.*, 1977). The present investigation has shown that alveolar macrophages can be stimulated to synthesize and release a variety of prostaglandins. These products may help regulate the activities of lymphocytes and other target cells.

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