The selective release of phospholipase A₂ by resident mouse peritoneal macrophages

Paul D. WIGHTMAN, Mary Ellen DAHLGREN, Philip DAVIES and Robert J. BONNEY
*Department of Immunology, Merck Institute for Therapeutic Research, P.O. Box 2000,
Rahway, NJ 07065, U.S.A.*

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Resident mouse peritoneal macrophages have three phospholipase activities: a phospholipase A₂ active at pH 4.5, a Ca²⁺-dependent phospholipase A₂ active at pH 8.5 and a phosphatidylinositol-specific phospholipase C activity. When macrophages are exposed to zymosan in culture, the cellular activity of pH-4.5 phospholipase A₂ is diminished in a manner dependent on zymosan concentration and time of exposure, whereas the cellular activities of pH-8.5 phospholipase A₂ and phospholipase C remain unchanged. The depletion of pH-4.5 phospholipase A₂ activity from the cell is paralleled by a quantitative recovery of this activity in the culture medium in a manner similar to the cellular depletion and extracellular recovery of two lysosomal enzymes. This release is specifically elicited by an inflammatory substance such as zymosan, since macrophages incubated with 6 µm latex spheres retain pH-4.5 phospholipase A₂ activity and lysosomal enzyme activities intracellularly.

Cells of the mononuclear phagocyte system synthesize and release a number of inflammatory mediators into their pericellular environment on appropriate stimulation with inflammatory substances (Davies & Bonney, 1979). These include oxygenated products of arachidonic acid derived from both the cyclo-oxygenase and lipoxygenase pathways. Large amounts of prostaglandins E₂ and I₂ are synthesized by peritoneal macrophages responding to inflammatory stimuli such as antigen-antibody complexes (Bonney *et al.*, 1979), lymphokines (Gordon *et al.*, 1976), phorbol myristate acetate (Bonney *et al.*, 1980) and zymosan (Humes *et al.*, 1977). Products of the lipoxygenase pathway synthesized by macrophages include 12-hydroxyeicosa-5,8,10,14-tetraenoic acid (Rigaud *et al.*, 1979) and slow-reacting substance of anaphylaxis (Bach & Brashler, 1978; Orange *et al.*, 1980), including one characterized as leukotriene C (Bach *et al.*, 1980; Rouzer *et al.*, 1980). The formation of many of these lipid-derived mediators is initially dependent on the activity of phospholipases. Until recently only one such enzyme, a phospholipase A₂, optimally active at acid pH, has been described in mononuclear phagocytes (Franson & Waite, 1973). Studies in this laboratory have established the presence in resident mouse peritoneal macrophages of two phospholipase A₂ activities with pH optima of 4.5 and 8.5 respectively

(Wightman *et al.*, 1981a) and a phospholipase C (Wightman *et al.*, 1981c). At this time there is no clear indication which of these enzymes, alone or in concert, participate in the macrophage's response to inflammatory stimuli. In the present study we have measured the activity of these enzymes in macrophages after exposure to zymosan in culture. We report that, whereas the cellular activities of the pH-8.5 phospholipase A₂ and phospholipase C remain unchanged, the cellular activities of the pH-4.5 phospholipase A₂ are depleted from the cell in a manner linearly dependent on the amount of zymosan added and the time of exposure to this stimulus and are quantitatively recovered in the culture medium.

Materials and methods

Macrophages were collected from male Swiss mice by peritoneal lavage with M199 containing 1% heat-inactivated porcine serum, 20 units of heparin/ml, 100 units of penicillin/ml and 100 µg of streptomycin/ml. The cells were plated at 5×10^6 – 6×10^6 per 50 mm culture dish and allowed to adhere for 2 h at 37°C in an atmosphere of CO₂/air (1:19). The non-adherent cells were removed by washing the cell sheet in 5 vol. of phosphate-buffered saline. The adherent cells, which we have previously demonstrated by morphological and functional criteria to

be >95% macrophages, were maintained in serum-free medium M199 supplemented with 100 µg of gentamycin/ml.

Cultures maintained in 2 ml of serum-free medium M199 were incubated in the presence of the indicated amounts of zymosan particles or 6 µm latex spheres for 4 h (Humes *et al.*, 1977). The culture medium was then removed and the cell sheet scraped into 2 ml of 0.01 M-Tris-buffered saline, pH 7.0. The suspension was briefly sonicated on ice and assayed for phospholipase activities with phospholipid substrates extracted from cultured mouse LM fibroblasts that had incorporated radioactive precursors of phospholipid (Wightman *et al.*, 1981a). The phospholipase A_2 activities were assayed at their respective pH optima by methods previously described (Wightman *et al.*, 1981a,b). Briefly, the pH-4.5 phospholipase A_2 activities were determined in a final volume of 80 µl of 0.1 M-acetate buffer, pH 4.5, containing 1 mM-EDTA, and were incubated at 37°C for 1 h. The pH-8.5 phospholipase A_2 activities were measured in a final volume of 80 µl of 0.1 M-Tris/HCl buffer, pH 8.5, containing 2 mM- Ca^{2+} , and were incubated at 37°C for 18 h. The phospholipase A_2 reactions contained 6 nmol of [^{14}C]arachidonic acid-labelled phospholipid added as a sonicated suspension and 10 µl of culture medium or sonicated cell suspension. The reactants and products were extracted (Bligh & Dyer, 1959) and purified on silica gel GF t.l.c. plates

developed in chloroform/methanol/acetic acid/water (25:15:2:1, by vol.). [^{14}C]Arachidonic acid hydrolysed was quantified by liquid-scintillation counting. Phospholipase C was assayed by measuring the release of phospho[3H]inositol from [3H]phosphatidylinositol. Enzyme activity was determined in a final volume of 80 µl of 0.1 M-Tris/HCl buffer, pH 7.0, containing 1 mM- Ca^{2+} . The reaction contained 8 nmol of [3H]phosphatidylinositol and 1–2 µg of macrophage homogenate protein. The reactions were terminated by extraction (Bligh & Dyer, 1959) and a portion of the aqueous phase was counted for released 3H . The phospholipase activity per culture is expressed as the number of nmol of [^{14}C]arachidonic acid released (for phospholipases A_2) or [3H]phosphorylinositol released (for phospholipase C) per h.

N-Acetyl- β -D-glucosaminidase was assayed by the method of Woolen *et al.* (1961) and β -glucuronidase by the method of Talalay *et al.* (1946). Lactate dehydrogenase was assayed by determining the rate of oxidation of NADH at 340 nm.

Results

Mouse peritoneal macrophages incubated in the presence of zymosan or latex spheres were assayed for their phospholipase activities. Fig. 1 shows that although the cellular pH-8.5 phospholipase A_2 and phospholipase C activities remain unchanged

Fig. 1. Concentration-dependent depletion of pH-4.5 phospholipase A_2 from mouse peritoneal macrophages treated with zymosan

(a) shows cellular pH-4.5 phospholipase A_2 ; (b) shows cellular pH-8.5 phospholipase A_2 ; (c) shows cellular phospholipase C. * indicates those cellular enzyme activities that are significantly less than the untreated controls ($P < 0.005$).

Fig. 2. Quantitative recovery in the culture medium of cellular pH-4.5 phospholipase A₂ depleted from macrophages after zymosan exposure

The cellular (●), medium-associated (■) and total (○) pH-4.5 phospholipase A₂ activity per culture is expressed in nmol of [¹⁴C]arachidonic acid released/h. Values shown are means for three determinations. Coefficients of variation are less than 10%. (a) Concentration-dependence of zymosan-induced pH-4.5 phospholipase A₂ depletion. Cultures maintained in 2 ml of medium were incubated in the presence of the indicated amounts of zymosan for 4 h. (b) Time dependence of zymosan-induced pH-4.5 phospholipase A₂ depletion. Cultures maintained in 2 ml of medium were exposed to 100 μg of zymosan.

whether exposed to zymosan or latex, the cellular pH-4.5 phospholipase A₂ activity is depleted in a concentration-dependent manner from cells treated with zymosan. Those cultures incubated with latex retain their pH-4.5 phospholipase A₂ activity intracellularly with no indication of extracellular release. Latex spheres are efficiently phagocytosed by macrophages but, unlike zymosan particles, they are not inflammatory (Humes *et al.*, 1977).

The culture media from the untreated cells and from those cells that had been exposed to zymosan were assayed for pH-4.5 phospholipase A₂ activity. Fig. 2(a) shows that, as the pH-4.5 phospholipase A₂ is depleted from cells responding to zymosan, it is quantitatively recovered in the culture medium. The extent of this depletion and recovery are dependent on the amount of zymosan with which the macrophages were treated. Fig. 2(b) shows that this depletion from the cells and the accumulation in the culture medium are also dependent on the duration of exposure to zymosan.

The zymosan-induced release of pH-4.5 phospholipase A₂ from macrophages was then compared with the release of two lysosomal enzymes, *N*-acetyl-β-D-glucosaminidase and β-glucuronidase in the same cultures. Lactate dehydrogenase, a cytoplasmic enzyme, was also assayed in these cultures. Fig. 3 shows that the zymosan-induced

Fig. 3. The parallel release from macrophages of pH 4.5 phospholipase A₂ and of two lysosomal enzymes after exposure to zymosan

(a) shows concentration-dependence, (b) shows time-dependence. The results are expressed as the percentage of the total activity of each culture (cellular plus medium-associated) that was present in the medium. Symbols: ●, pH-4.5 phospholipase A₂; ■, β-glucuronidase; ▲, *N*-acetyl-β-D-glucosaminidase; ○, lactate dehydrogenase. Values shown are means for three determinations. Coefficients of variation are less than 10%.

release of pH-4.5 phospholipase A₂ is paralleled by the release of the two lysosomal enzymes. Lactate dehydrogenase, however, is retained in the cell and is not released in response to this inflammatory stimulus. Both the intracellular and medium-associated lactate dehydrogenase activities remained constant regardless of exposure to zymosan or concentration of zymosan used, indicating a conservation of cell density throughout the course of the experiments. Therefore the translocation of lysosomal enzyme activities to the medium is one of specific release from the cell rather than selective detachment of cells as a result of ingesting zymosan. The zymosan-induced release of these enzymes is, therefore, selective and occurs in a manner dependent on the amount of zymosan added (Fig. 3a) and the duration of exposure (Fig. 3b) and suggests that the pH-4.5 phospholipase A₂ is of lysosomal origin.

Discussion

This significance of the release of pH-4.5 phospholipase A₂ by macrophages responding to inflammatory stimuli to the pathogenesis of inflammation remains to be resolved. Phospholipase A₂ activity is, however, known to accumulate in fluids associated with inflammatory exudates (Thouvenot *et al.*, 1974; Sahu & Lynn, 1977). Owens and his colleagues (Owens *et al.*, 1980) have reported that a granulocyte phospholipase A₂ efficiently hydrolyses sarcolemma phospholipids,

producing, predominantly, non-esterified arachidonic acid and lysophosphatides. Both of these products can play a role in inflammatory processes. Arachidonic acid is the precursor for products of the lipoxygenase and cyclo-oxygenase pathways, and lysophosphatides are cytotoxic substances that can cause inflammation when applied locally (Phillips *et al.*, 1965) or intraperitoneally (Muder *et al.*, 1979). Sahu & Lynn (1977) have reported large amounts of non-esterified fatty acids and lysophosphatidylcholine in the airway secretions of patients with asthma, cystic fibrosis and alveolar proteinosis. Furthermore, they have isolated large amounts of phospholipase A₂ from these secretions and propose that this enzyme is significant in the pathogenesis of these diseases. Since alveolar macrophages are surrounded by surfactant composed of dipalmitoylglycerophosphocholine, this may be an important target for any pH-4.5 phospholipase A₂ released by alveolar macrophages responding to inflammatory stimuli.

Phospholipase A₂ activity has been described in glycogen-induced rabbit peritoneal exudate fluids (Franson *et al.*, 1978) and its release has been characterized in polymorphonuclear leucocytes phagocytosing zymosan-complement (Authi & Traynor, 1979). Both of these activities are ascribed to the polymorphonuclear leucocyte and are similar in their pH optima (pH 6.0) and requirement for Ca²⁺ (10 mM).

We have characterized the phospholipase A₂ selectively released by resident mouse peritoneal macrophages exposed to zymosan. This activity is very different from those of the polymorphonuclear leucocyte (Authi & Traynor, 1979; Franson *et al.*, 1974) in that it does not require Ca²⁺ for activity and is optimal at pH 4.5. The findings that we present complement those of other investigators (Muder *et al.*, 1970; Authi & Traynor, 1979; Sirois *et al.*, 1980), who have described phospholipase A₂ activities secreted under inflammatory conditions and that may be involved in the pathogenesis of these processes. The amount of pH-4.5 phospholipase A₂ secreted by the macrophage in response to zymosan is sufficient to produce 5 nmol of non-esterified arachidonic acid/h per culture. Macrophages under the same conditions produce 0.25 nmol of prostaglandins/h per culture (Bonney *et al.*, 1978). However, whether the released pH-4.5 phospholipase A₂ can function in providing arachidonic acid to its oxygenation pathways or only complements the battery of macrophage enzymes that can function in tissue destruction remains to be elucidated.

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