Sequential release of TNFα and phospholipase A2 in a rat model of LPS-induced pleurisy

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The levels of extracellular phospholipase A2 (sPLA2) and TNFα, and cell accumulation were measured in the pleural washings obtained at different times following the induction of Escherichia coli lipopolysaccharide (LPS, 100 µg/cavity) pleurisy in rats. TNFα peaked at 2 hours (3036 ± 160.3 units/ml) and decreased thereafter. Conversely, levels of sPLA2 peaked at 48 hours (1.97 ± 0.64 ng/ml) and were increased further (14.02 ± 4.16 ng/ml) by pretreatment with anti-TNFα antibody. Cell accumulation was not affected by antibody pretreatment. These data indicate that the sPLA2 enzyme is involved in LPS-induced pleurisy. The enzyme seems not to be stimulated by TNFα which may be involved in the down-regulation of sPLA2 in this model of inflammation.

Key words: Endotoxin, Phospholipase A2, Pleurisy, Rat, Tumour necrosis factor α

Introduction

The extracellular form of phospholipase A2 has been shown to be involved in several inflammatory disorders, including those arising from Gram negative bacterial infections.1, 2 Phospholipase A2 (PLA2) activation is a rate limiting step in the generation of arachidonic acid, the substrate necessary for eicosanoid generation. The secreted form of PLA2 (sPLA2 group II) has been found at high levels in the serum and locally during inflammation in experimental animals and humans,3 and there is in vitro evidence that its induction is under cytokine control.4−7 Furthermore, sPLA2 has been proposed to play a role in septic shock. To date, however, the relationship between cytokines and sPLA2 levels in inflammatory exudates and in the serum is still controversial.8, 9 It has been shown that intrapleural injection of LPS into rats elicits an inflammatory response characterized by leucocyte accumulation.9 Furthermore, challenge of isolated guinea pig lung with endotoxin generates TNFα,11 and the presence of TNFα in the bronchoalveolar lavage fluid of experimental animals has been demonstrated following intratracheal administration of LPS.12 It appears that tumour necrosis factor alpha (TNFα) is a cytokine playing a pivotal role in the development of endotoxin-induced inflammation.10

We have examined whether intrathoracic injection of bacterial endotoxin into rats is capable of causing the release of sPLA2 and TNFα, and the potential relationship between this enzyme and this cytokine in this model of inflammation.

Materials and Methods

Pleurisy

Pleurisy was induced in enflurane anaesthetized male Wistar rats (250–300 g; Charles River, Milan, Italy) by intrathoracic injection of E. coli lipopolysaccharide (LPS, 0127:B8; 100 µg/cavity) in a final volume of 200 µl. Control animals received an equivalent volume of phosphate buffered saline (PBS). Different groups of animals were treated with a polyclonal anti-TNF antibody (3 mg/kg i.v.; TAB, London) or control IgG (3 mg/kg i.v.) immediately before the induction of pleurisy. Animals were killed with CO2 at 2, 6, 24, 48, or 96 hours following the injection of LPS or PBS. Groups of animals pretreated with anti-TNF antibody, or control IgG, were killed after 2 and 48 hours, the time required to obtain peak TNFα and sPLA2 levels respectively. Immediately after sacrifice the thoracic cavity was opened and washed with 2 ml of heparinized (5 UI/ml) sterile saline. The fluid volume was collected in graduated tubes and centrifuged at 125 × g for 10−15 min. The
pellet was suspended in 1 ml of PBS and total leucocyte count was evaluated. Supernatant was further centrifuged at 650 × g for 10–15 min, immediately frozen and kept at −20°C until assayed for TNFα and sPLA2.

Leucocyte count
Total leucocyte count was evaluated by optical microscopy in the cell suspension diluted with Turk’s solution. Differential cell count was performed by counting air dried smears stained with Giemsa reagent under an oil immersion objective. Counts are reported as number of cells × 10⁶/cavity.

TNFα determination
TNFα in the cell-free pleural washing was assessed on WEHI-164 cells by a bioassay using recombinant human TNFα (Sigma) as the reference standard, and rabbit anti-murine TNFα antiserum (Genzyme) which cross reacts with rat TNFα to assess the specificity of TNFα dependent cytolytic activity.13 TNFα is expressed as units of TNFα/ml, where one unit is defined as the quantity required to lyse 50% of WEHI-164 cells.

PLA2 assay
A sandwich assay for sPLA2 was run according to the method described by Santos et al.8 with some modifications. Plates were coated overnight with a monoclonal anti-human recombinant sPLA2 antibody (BA11), 10 µg/ml, in 50 mM sodium bicarbonate buffer, pH 9.6. Six cycles of washing with 0.1% w/v Tween-20 in PBS were done at each step and all incubations were at 4°C. The plates were blocked overnight with 10 mg/ml of gelatin in PBS. Human recombinant sPLA2 was diluted in 50 mM Tris- HCl, pH 7.5, 0.1% w/v Tween-20, 1% v/v foetal calf serum (FCS), to obtain the desired dilution, and 0.05 ml were added to the wells at 4°C for 1 hour (low temperature decreases non-specific PLA2 binding). Bound human recombinant sPLA2 was detected by incubation for 90 min with 0.05 ml of a 1:2000 dilution of rabbit anti-human recombinant sPLA2 (n.207) in assay buffer. Plates were washed and 0.1 ml of a solution containing 1 mg/ml of tetramethylbenzidine in DMSO, mixed with 10 ml of acetate buffer (0.1 M, pH 5.0) and 0.125 ml of diluted hydrogen peroxide (0.2 ml 30% hydrogen peroxide and 7 ml of water), were added to each well. After 15–20 min, 0.025 ml of stop solution (H₂SO₄, 2 M) was added and the yellow colour read at 450 nm by an ELISA reader. sPLA2 in the cell-free pleural washing was expressed as ng/ml.

Histological analysis
Lungs from control or LPS-injected animals were removed and fixed in neutral buffered formalin before being embedded in paraffin. Sections (4 µm) were stained with hematoxylin and eosin.

Statistical analysis
Data are expressed as mean ± SEM and analysed with a computerized package for statistical comparison.14 To compare groups of animals, unpaired two tailed Student’s t test was used. Data on TNFα were analysed by the Mann–Whitney test. A P < 0.05 was taken as significant.

Materials
Recombinant human sPLA2 produced in mammalian cells, monoclonal anti-human recombinant sPLA2 antibody (BA11) and rabbit polyclonal anti-human recombinant sPLA2 antibody (n. 217) were a generous gift from Dr J. L. Browning (Biogen). Enflurane was purchased from Abbott (Italy). Lipopolysaccharide from E. coli (serotype 0127:B8), PBS and heparin were purchased from Sigma (Italy). Antibody to human TNFα was a generous gift from TAB London, and has been shown to neutralize rat TNFα in vitro (TAB London, unpublished data).

Results
Leucocytes
Following intrathoracic injection of LPS, total leucocyte count was significantly increased only at 48 and 96 hours, while at 6 hours there was a significant reduction in the leucocyte count compared to control value. Polymorphonuclear count was significantly increased at 24 and 48 hours, significantly reduced at 6 hours and returned to control levels after 96 hours. Monocyte count was significantly increased at 96 hours, and significantly reduced below control levels at 6 and 24 hours (Fig. 1).

TNFα determination
TNFα in the cell-free pleural washing peaked at 2 hours following LPS injection. At the successive time points, TNFα levels were reduced progressively, becoming undetectable at 96 hours (Fig. 2).
PLA₂ determination

Phospholipase A₂ levels in the cell-free pleural washing were detectable at 2 hours, reached their maximum at 48 hours and were stable up to 96 hours (Fig. 2).

Pretreatment with anti-TNFα antibody

Levels of TNFα at 2 hours were significantly reduced by anti-TNFα antibody (118.4 ± 41.4 units/ml vs 3036 ± 1603 units/ml as determined by the Mann–Whitney test, P < 0.01, n = 5). Pretreatment with anti-TNFα antibody significantly increased sPLA₂ levels at 48 hours (Fig. 3). Both total and differential cell analysis were unaffected by antibody pretreatment (data not shown).

Histological analysis

In the histological sections of lungs obtained from either PBS or LPS injected animals there was neither leukocyte infiltration, nor alteration in the cell lining of tissue.

FIG. 1. Time course of cell accumulation in the pleural space following LPS (100 μg/cavity) injection: (A) total leucocytes; (B) polymorphonuclear leucocytes; (C) monocytes. Control, open bars; LPS, filled bars. *P < 0.05; **P < 0.01. Student’s t-test, n = 4–6.

FIG. 2. Time course of sPLA₂ ( ■; n = 4–6) and TNFα ( □; n = 3–7) levels in the cell-free pleural washing following LPS (100 μg/cavity) administration.
Discussion

By using a highly specific immunoenzymatic assay, we have demonstrated the presence of extracellular sPLA₂ group II in the cell-free pleural washing at different time points following LPS injection, providing direct evidence for a role of this enzyme in the pleurisy triggered by LPS. In our model, the appearance in the cell-free pleural washing of increased levels of sPLA₂ was delayed compared to TNFα levels. Indeed, sPLA₂ increased in the cell-free pleural washing only when TNFα levels started to decline, and it reached its maximum at 48 hours, when TNFα levels were very low. At the same time, cells significantly accumulated: polymorphonuclear leucocytes reached their maximum at 48 hours, and at 96 hours the pleural washing consisted almost totally of monocytes. When animals were pretreated with anti-TNFα antibody, TNFα levels were significantly reduced. However, cell accumulation did not parallel the increase in TNFα and pretreatment with anti-TNFα antibody did not affect total and differential cell count, suggesting that resident pulmonary cells, stimulated by LPS, may account for TNFα production and that TNFα is not involved in LPS-induced leucocyte margination in the pleural space. These data are consistent with data reported by others, who demonstrated that IL-1 and not TNFα is mostly responsible for LPS-induced neutrophil migration to an inflammatory site. Moreover, it is known that adhesion molecules contribute greatly to LPS-induced leucocyte margination in the lung and their expression is under the control of multiple cytokines. Other authors report that the ability of TNFα inhibitors to protect animals from flogogen-induced lung injury is not linked to a reduction in PMN sequestration. In the light of this knowledge, it is not unexpected that the inhibition of TNFα alone did not affect leucocyte accumulation.

Surprisingly, when we pretreated animals with the anti-TNFα antibody, we observed a strong increase in sPLA₂ levels in the cell-free pleural washing at 48 hours. Thus, it seems that TNFα does not exercise a positive control on sPLA₂ induction in vivo, rather its inhibition causes sPLA₂ levels to increase further, at least in this experimental model of pleurisy. Systemic administration of the antibody allows us to rule out the hypothesis of any interference of the antibody with ELISA assay reagents. Although there is much evidence that in vitro cytokines are the major inducers of sPLA₂ synthesis and secretion from several different cell types, there is no direct evidence that in vivo sPLA₂ secretion is under cytokine control. A positive correlation has been found between TNFα and sPLA₂ plasma levels both in human and in experimental animals challenged with bacterial endotoxin, as well as an increase in plasma sPLA₂ levels following TNFα injection in volunteers, but this evidence has never been followed by clarification of the in vivo role of the enzyme. In 1993 synthesis and secretion of a 14 kDa sPLA₂ from guinea pig alveolar macrophages was demonstrated and it was found that the enzyme was not involved in the release of arachidonic acid metabolites. More recently the same research group has shown that expression of sPLA₂ in guinea pig alveolar macrophages is downregulated by an inflammatory stimulus such as FMLP and it has been found that the release of arachidonic acid metabolites from guinea pig alveolar macrophages in vivo is reduced below control levels 24 hours after pretreatment with LPS. Up to now, the role of sPLA₂ in vivo has been unresolved; following these more recent findings, it has been suggested that the secreted form of PLA₂ might have a protective role at site of injury.

It is noteworthy that 6 hours following LPS administration there was a significant reduction in leucocyte number in the pleural space, paralleled by a reduction in both granulocyte and monocyte counts. Although there was neutrophil accumulation in the lung at 24 hours, the total leucocyte count did not increase since the monocyte count was still reduced below the control level. This reduction could be explained by an increased cell adhesivity which precedes cell accumulation. Indeed, recent data have demonstrated that injection of
increasing doses of IL-1 or TNFα in to the pleural space of rats causes a bell-shaped cell accumulation curve. This finding has been shown to be linked to the increased cell adhesivity, evoked by high doses of IL-1 and TNFα.22

In conclusion, our data demonstrate that following intrapleural injection of endotoxin into rats, TNFα and sPLA₂ group II appear sequentially in the pleural washings. The early appearance of TNFα is in agreement with data reported by others who describe TNFα as an early response cytokine. The high levels of sPLA₂ in the late phase of the inflammation further suggest a role for the extracellular form of the enzyme in inflammation triggered by bacterial endotoxins. TNFα is probably produced by resident pulmonary cells and does not seem to be directly involved in cell accumulation. TNFα does not stimulate the enzyme induction, rather there is evidence that its inhibition increases sPLA₂ levels, suggesting that this cytokine could participate in a mechanism of downregulation of the enzyme during inflammation; however, this hypothesis requires further clarification.

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