High Bactericidal Efficiency of Type IIA Phospholipase A2 against Bacillus anthracis and Inhibition of Its Secretion by the Lethal Toxin

Alejandro Piris Gimenez, Yong-Zheng Wu, Miguel Paya, Christophe Delclaux, Lhousseine Touqui and Pierre L. Goossens

*J Immunol* 2004; 173:521-530; doi: 10.4049/jimmunol.173.1.521

http://www.jimmunol.org/content/173/1/521

**References**

This article cites 47 articles, 16 of which you can access for free at: http://www.jimmunol.org/content/173/1/521.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
High Bactericidal Efficiency of Type IIA Phospholipase A2 against *Bacillus anthracis* and Inhibition of Its Secretion by the Lethal Toxin

Alejandro Piris Gimenez,* Yong-Zheng Wu,† Miguel Paya, † Christophe Delclaux, † Lhoussaine Touqui, † and Pierre L. Goossens2*  

There is a considerable body of evidence supporting the role of secretory type II-A phospholipase A₂ (sPLA₂-IIA) as an effector of the innate immune response. This enzyme also exhibits bactericidal activity especially toward Gram-positive bacteria. In this study we examined the ability of sPLA₂-IIA to kill *Bacillus anthracis*, the etiological agent of anthrax. Our results show that both germinated *B. anthracis* spores and encapsulated bacilli were sensitive to the bactericidal activity of recombinant sPLA₂-IIA in vitro. In contrast, nongerminated spores were resistant. This bactericidal effect was correlated to the ability of sPLA₂-IIA to hydrolyze bacterial membrane phospholipids. Guinea pig alveolar macrophages, the major source of sPLA₂-IIA in an experimental model of acute lung injury, released enough sPLA₂-IIA to kill extracellular *B. anthracis*. The production of sPLA₂-IIA was significantly inhibited by *B. anthracis* lethal toxin. Human bronchoalveolar lavage fluids from acute respiratory distress syndrome patients are known to contain sPLA₂-IIA; bactericidal activity against *B. anthracis* was detected in a high percentage of these samples. This anthracidal activity was correlated to the levels of sPLA₂-IIA and was abolished by an sPLA₂-IIA inhibitor. These results suggest that sPLA₂-IIA may play a role in innate host defense against *B. anthracis* infection and that lethal toxin may help the bacteria to escape from the bactericidal action of sPLA₂-IIA by inhibiting the production of this enzyme. *The Journal of Immunology*, 2004, 173: 521–530.

Phospholipases A₂ (PLA₂) are a family of enzymes that catalyze the hydrolysis of phospholipids at the sn-2 position, generating lysophospholipids and free fatty acids, especially arachidonic acid (1). Mammalian PLA₂ can be divided into two major classes according to their molecular mass and location: intracellular PLA₂ and secreted PLA₂ (sPLA₂). The 10 distinct members identified to date share <50% identity (for review, see Refs. 2 and 3). Group IIA sPLA₂ (sPLA₂-IIA), the best-known enzyme of this group, is involved in the pathogenesis of various inflammatory diseases (4–6). Secretory PLA₂-IIA is secreted by various inflammatory cells, including macrophages and neutrophils (for review, see Refs. 7 and 8).

The production of lipid mediators by a PL₂-dependent pathway is an integral component of the inflammatory reaction and thus plays, although indirectly, a major role in protecting the host against invading pathogens (9). However, some PLA₂ play a more direct role in the host defense reaction against bacteria, especially toward Gram-positive bacteria. Indeed, sPLA₂-IIA displays bactericidal activity toward several strains of bacteria (10–14). The mode of action of sPLA₂-IIA depends on whether bacteria are Gram-negative or Gram-positive, but it always involves the hydrolysis of the phospholipids in bacterial membranes (11). The protective role of sPLA₂-IIA against bacterial infections was highlighted recently in sPLA₂-IIA−/− mice (15). The absence of sPLA₂-IIA in these mice affects their antibacterial response to *Staphylococcus aureus* infection, leading to a higher death rate compared with mice overexpressing sPLA₂-IIA.

*Bacillus anthracis*, the etiological agent of anthrax, is a Gram-positive, spore-forming bacterium (16). Dormant spores are highly resistant to adverse environmental conditions and can survive for long periods of time in contaminated soils. Anthrax is primarily a disease of herbivores, but all mammals, including humans, are susceptible. Human infection can occur via the cutaneous, gastrointestinal, or respiratory route. Whatever the infection route, spores are thought to be taken up by macrophages and to migrate to the draining lymph nodes (17, 18). The infection then spreads to successive nodes, and the encapsulated bacilli enter the blood compartment and disseminate within the whole organism. Despite appropriate therapy, all these forms of infection may progress to fatal systemic anthrax, which is characterized by shock-like symptoms, sepsis, and respiratory failure (19).

Fully virulent strains of *B. anthracis* carry two large plasmids, pXO1 and pXO2, which encode the primary virulence factors:
lethal and edema toxins, and the proteins required for capsule synthesis, respectively (16). The toxins are composed of three secreted proteins: protective Ag (PA), lethal factor (LF), and edema factor (EF). These proteins act in pairs (16), leading to the lethal toxin (LeTx; PA plus LF) and the edema toxin (PA plus EF). The capsule, a linear homopolymer of γ-glutamic acid, contributes to pathogenicity through its antiphagocytic properties, thus enabling the bacteria to evade the host’s immune defenses and provoking septicaemia (20).

However, little is known about the innate immune response that is triggered upon infection by *B. anthracis* spores. The toxic effects of LeTx on the immune system have led to conflicting reports (16). A local inflammatory reaction is induced in the first hours of a cutaneous anthrax infection (21); in addition, there is a correlation between the magnitude of the in situ recruitment of leukocytes and the ability of relatively resistant host species to control anthrax infection (21). Bactericidal substances for *B. anthracis* (anthrachnicidal) have been partially purified from anthrax cutaneous lesions (22). Defensins (23) and PLA2 are first-line effectors from the innate immune system that may potentially be involved in killing *B. anthracis* in the infected host.

The aims of this study were to investigate the activity of human sPLA2-IIA to kill *B. anthracis* and to determine whether this enzyme can be involved in the anthrachnicidal activity of human bronchialveolar lavage (BAL) fluids (BALF). We also examined the effect of *B. anthracis* on the production of sPLA2-IIA by alveolar macrophages (AM), a major pulmonary source of this enzyme.

**Materials and Methods**

**Recombinant sPLA2-IIA**

Purified human recombinant sPLA2-IIA (rh-sPLA2-IIA) was a gift from C. Mounier (Unité Défense Innée et Inflammation, Institut Pasteur, Paris, France). Recombinant guinea pig sPLA2-IIA (gp-sPLA2-IIA) was prepared and purified in our laboratory as previously described (24). PLA2-I from porcine pancreas was obtained from Sigma-Aldrich (St. Louis, MO).

**Bacterial strains and growth conditions**

The *B. anthracis* strains used in this study were the pXO1* - Sterne derivates: RPLC2, which carries point mutations affecting the catalytic sites of PLA2-I; and RFG1, RPLC2 derivative containing pXO2 (this work).

These strains were used at different stages of differentiation. Nongerminated spores were prepared and purified on Radioslectan 76% (Schering, Berlin, Germany) as previously described (26). For germinated spores, germination was triggered by incubating spores in liquid brain heart infusion (BHI) (Difco, Detroit, MI) for 15 min at 37°C: samples were then centrifuged (13,000 × g for 2 min at 4°C), and the pellet was recovered in the incubation medium used for the bactericidal assay; >90% of spores germinated. When specified, chloramphenicol (250 μg/ml) was added to the BHI for 30 min to inhibit protein synthesis during germination. When chloramphenicol is added to the germination medium, germination is blocked at an early step (27). After three washes with 0.15 M NaCl, the spores were resuspended in sPLA2-IIA incubation medium. Preliminary experiments have shown that germination resumes after removal of the antibiotic and plating on BHI (data not shown). To obtain encapsulated and nonencapsulated bacilli, germinated spores were incubated in R medium supplemented with 0.6% NaHCO3, for 2–3 h at 37°C with agitation (28). The presence of the capsule was checked by light microscopy, using India ink coloration. Bacilli concentrations in both samples (encapsulated and nonencapsulated) were determined by measuring the OD of the bacterial cultures at 600 nm and by microscopic enumeration. Bacterial concentrations were confirmed by CFU counts on BHI plates.

**Bactericidal assay**

The RPLC2 and RFG1 strains were incubated with rh-sPLA2-IIA in 100 μl of PBS with 1 mM Ca2+ at 37°C for various time periods. The spectino-

mycin-resistant strain RP500 was used to avoid contamination with the resident flora recovered during the BALs when human BALF or guinea pig AM supernatants (prepared as described below) were assayed; no spectino-

mycin-resistant CFU were detected in these fluids. When required, LY311727, an sPLA2-IIA inhibitor (a generous gift of Lilly Corporate Research, Indianapolis, IN), was incubated with sPLA2 for 30 min at 37°C before the bactericidal assay. The concentration of germinated spores used in the assays was 5.44 ± 0.27 log10 CFU/ml (mean ± SD; n = 18). *B. anthracis* were counted by plating serial 10-fold dilutions on solid BHI. The number of nongerminated spores was determined after heating samples for 30 min at 65°C. Results are expressed as the percentage of destruction of heat-sensitive vegetative forms.

**Secretory PLA2-IIA binding assay**

Nongerminated or germinated spores (107) were incubated in 100 μl of PBS-1 mM Ca2+ in the presence or the absence of 20 ng of rh-sPLA2-IIA for 30 min at 37°C. Spores were collected by centrifugation at 13,000 × g for 2 min at 4°C. Spore pellets were washed twice in PBS, and bound rh-sPLA2-IIA was extracted and detected by Western blot as well as by enzymatic assay, as described below.

**Radiolabeling of B. anthracis membrane and analysis of bacterial phospholipid degradation**

Germinated *B. anthracis* spores (3.5 × 107 spores/ml) were labeled with 1 μCi/ml 3Hjoleic acid ([3H]JOA; NEN, Boston, MA; 14 Ci/mmol) in the presence of BHI for 3 h at 37°C. After a 30-min chase period, labeled bacteria were washed three times with 0.15 M NaCl and resuspended in the sPLA2 assay medium (see above) supplemented with 0.25% delipidated BSA to bind the released free [3H]JOA. After 120 min in the presence or the absence of sPLA2-IIA (2 μg/ml), bacterial suspensions were centrifuged and washed three times in 0.15 M NaCl. The lipid from the supernatants and bacterial pellets were extracted with chloroform/methanol/acetic acid (50/40/10, v/v/v) and separated by TLC using H2O/acetic acid/methanol/ chloroform (1/3/45/65) as the solvent system. Phospholipids were then localized using corresponding standards, and the spots were scraped and placed into scintillation vials containing 5 ml of scintillation counting liquid BCS (Amersham, Little Chalfont, U.K.).

**Guinea pig AM**

Guinea pig BALs were performed with PBS as previously described (29), using spectinomycin (100 μg/ml) to avoid contamination with resident bacteria. After centrifugation at 450 × g for 10 min, the cell pellets were resuspended in RPMI 1640 culture medium (Life Technologies, Gaithersburg, MD) containing spectinomycin (100 μg/ml) and 3% FCS. Cells (1.5 × 107/well) were incubated in a 24-well tissue culture plate for 1 h at 37°C in a CO2 incubator (5% CO2). After removing the nonadherent cells by washing with RPMI 1640, 95–99% of the remaining cells were identified as macrophages. The plates were further incubated in medium containing 3% FCS for 20 h. Cell viability was checked by the trypan blue dye exclusion test and was always >90%. When indicated, AM were pretreated with LeTx (1 μg/ml PA and increasing concentrations of LF (1 ng/ml to 1 μg/ml) or each of its components, i.e., PA or LF, for 2 h before overnight incubation with LPS from *Pseudomonas aeruginosa* (50 ng/ml). After incubation, supernatants were harvested and centrifuged to remove detached cells. The adherent macrophages were washed twice in PBS and disrupted as described below. Both supernatants and disrupted cells were stored at −20°C until the sPLA2-IIA enzymatic assay.

**Protein extraction and Western blot analysis**

Proteins from spores or AM (treated as described above) were extracted in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 3 mM EDTA, 100 μM leupeptin, 100 μM aprotinin, 1 μM soybean trypsin inhibitor, 5 mM NEM, 1 mM PMSF, 5 mM benzamidine, and 1% Triton X-100, pH 7.4) and electrophoresed under nonreducing conditions according to the procedure described by Laemmli (47). Proteins were transferred onto polyvinylidene difluoride membranes by semidry transfer. Nonspecific binding sites were blocked overnight with 5% nonfat dry milk in 20 mM Tris-HCl (pH 7.6), 140 mM NaCl, and 0.1% Tween 20. Blots were probed for 1 h with rabbit polyclonal anti-human sPLA2-IIA (1/1,000 dilution). After washing, the immunoreactive bands were visualized using a peroxidase-conjugated, goat anti-rabbit IgG (1/10,000 dilution) and an ECL Plus Western Blotting Detection System (Amersham).
Analysis of LeTx toxicity

The susceptibility of guinea pig AM to the lethal effect of LeTx was evaluated by measuring MTT reduction after incubation with PA (1 and 10 μg/ml) and LF (1 μg/ml) as previously described (25). Absorbance at 540 nm was measured and expressed as a percentage of the control cells incubated without toxin. The positive control of LeTx toxicity was conducted on the sensitive macrophage cell line, RAW 264.7.

Patient selection and BALF sample collection

BALF were collected from patients suffering from acute respiratory distress syndrome (ARDS) in the medical intensive care unit of the Henri Mondor Hospital (Creteil, France). ARDS was defined according to the recommendations of the international American-European consensus conference (30). Patients scheduled for BAL to evaluate suspected ventilator-associated pneumonia were eligible for the study. The study was approved by the ethics committee of the Société de Réanimation de Langue Française.

Nineteen ARDS patients were studied (12 males and seven females; age, 54 ± 18 years, mean ± SD. BAL was performed as previously described (31). Briefly, three 50-ml aliquots of sterile, pyrogen-free, 0.15 M NaCl were instilled and recovered using gentle suction. The fluid recovered after the first 50-ml instillation was discarded. BALF was filtered through moistened coarse gauze to remove mucus, centrifuged at 300 × g for 7 min immediately after collection, and divided into aliquots before being frozen at −80°C until the sPLA2 enzymatic assay.

Secretory PLA2-IIA enzymatic assay

The fluorescent phospholipid (1-hexadecanoyl-2-(1-pyrene-decanoyl)-sn-glycero-3-phosphoglycerol; phosphatidylylglycerol ([PG]) was used as a substrate. The measurements were performed with a Jobin & Yvon JY3D spectrophuorometer equipped with a xenon lamp and monitored using excitation and emission wavelengths of 345 and 398 nm, respectively, with a slit width of 4 nm. In brief, substrate buffer was prepared by mixing the 0.2 mM ethanol solution of the fluorescent phospholipid with a solution containing 50 mM Tris-HCl, 500 mM NaCl, and 1 mM EGTA (pH 7.5). Assays were performed by mixing 960 μl of the substrate solution with 10 μl of 10% fatty acid-free BSA in a cuvette and adding 50 μl of the sample. Reactions were then initiated by adding 10 μl of CaCl2 at a 10-mM final concentration and measuring fluorescence as described previously (32).

FIGURE 1. Recombinant human sPLA2-IIA is bactericidal for B. anthracis germinated spores, but not for nongerminated spores. A, After 45 min in BHI medium, germinated spores were incubated with purified rh-sPLA2-IIA (1 μg/ml) for 15 min. Results are expressed as total CFU counts (□) and CFU counts after heating at 65°C for 30 min (◼), i.e., nongerminated spores. B, Nongerminated spores were incubated with purified rh-sPLA2-IIA (1 μg/ml) for 45 min, and results are expressed as described in A. C, After a 30-min incubation of nongerminated (NG) or germinated (G) spores with or without rh-sPLA2-IIA (20 ng), bound enzyme was extracted and analyzed by Western blot, and its enzymatic activity was measured as described in Materials and Methods. The results are representative of two independent experiments conducted in duplicate.
Results

Bactericidal effect of rh-sPLA$_2$-IIA on B anthracis: importance of germination

Incubation of B. anthracis spores with 1 $\mu$g/ml rh-sPLA$_2$-IIA, after initiation of germination in BHI, led to a >99% decrease in CFU (Fig. 1A). The remaining CFU were from nongerminated spores, as shown by their heat resistance properties. Indeed, nongerminated spores were confirmed to be resistant to rh-sPLA$_2$-IIA (Fig. 1B). This resistance was not due to insufficient binding of rh-sPLA$_2$-IIA, because the enzyme was readily associated with the nongerminated spores after a 30-min incubation (Fig. 1C).

The bactericidal effect against germinated spores was directly proportional to rh-sPLA$_2$-IIA concentrations (Fig. 2A); the calculated ED$_{50}$ was close to 50 ng/ml rh-sPLA$_2$-IIA. One of the major virulence factors of B. anthracis is a poly-$\gamma$-$\delta$-glutamic acid capsule that surrounds the vegetative form. This capsule possesses antiphagocytic properties and leads to septicemia in the infected host (16, 20). We tested whether the B. anthracis capsule could prevent rh-sPLA$_2$-IIA from reaching the membrane phospholipids (Fig. 2B); both encapsulated and nonencapsulated bacilli were sensitive to sPLA$_2$-IIA. The bactericidal effect mediated by rh-sPLA$_2$-IIA occurred very rapidly (Fig. 3), as a 24% decrease in CFU was already observed after 1 min; the bactericidal effect was maximal at 15 min (96% CFU decrease).

To determine at which step of germination the spores became sensitive to sPLA$_2$-IIA, germination was initiated in the presence of chloramphenicol. In these conditions, protein synthesis is inhibited, and the spores are blocked in the very first stages of germination.
Germinated spores were incubated with sPLA2-IIA (0.5 μg/ml) for increasing time periods. Results are expressed as the percentage of CFU decrease, calculated after differential counts of heat-sensitive and heat-resistant bacterial forms. These data are representative of at least two independent experiments.

Recombinant human sPLA2-IIA was able to hydrolyze the membrane phospholipids of B. anthracis. Labeling of B. anthracis with [3H]OA led to its incorporation into membrane phospholipids. The relative proportion of each labeled phospholipid was: PG (86%), phosphatidylethanolamine (PE; 9%) and phosphatidylcholine (PC; 5%). B. anthracis phospholipids were hydrolyzed by sPLA2-IIA, leading to a decrease in the amount of labeled PG and PE in the bacterial membranes (Fig. 4B) and the concomitant release of [3H]OA into the medium (Fig. 4A).

The rh-sPLA2-IIA enzymatic activity was implicated in the bactericidal effect on B. anthracis (Fig. 5), as LY 311727 significantly inhibited the decrease in B. anthracis CFU, showing a direct relationship between enzymatic activity and the bactericidal effect.

Bactericidal activity of sPLA2-IIA released by guinea pig AM and its modulation by B. anthracis LeTx

Guinea pig AM, the major pulmonary source of sPLA2-IIA in an experimental model of ARDS (32), secrete this enzyme in vitro (29, 33). We first ensured that purified recombinant gp-sPLA2-IIA had the same bactericidal effect against B. anthracis, and the same enzymatic activity against known substrates as recombinant human sPLA2-IIA (data not shown). We then tested whether guinea pig AM in those experimental conditions released enough sPLA2-IIA to produce a bactericidal effect on germinated B. anthracis spores (Fig. 6). Incubation with increasing concentrations of AM supernatant led to a 100% decrease in B. anthracis CFU number, and the effect was inhibited by the sPLA2-IIA inhibitor, LY311727. This suggested that this enzyme was the major B. anthracis bactericidal component released by AM.

Discussion

In this study we showed that purified rh-sPLA2-IIA exerted a significant bactericidal effect against B. anthracis in vitro. This bactericidal effect was observed as soon as germination occurred, because spores were sensitive even when germination was arrested in the earliest steps by inhibiting protein synthesis (27). In contrast, nongerminated spores were resistant to sPLA2-IIA, and this was not due to lack of binding of the enzyme. This resistance may be related to the well-known high resistance of nongerminated spores to a variety of physical, chemical, and enzymatic agents (16) due to its particular structure. Indeed, spores are in a dehydrated state and possess a tight structure made of many crystalline layers impermeable to many potentially aggressive compounds.

The encapsulated vegetative form of B. anthracis was also sensitive to sPLA2-IIA. Thus, the capsule cannot prevent sPLA2-IIA from reaching the cytoplasmic membrane phospholipids of B. anthracis, indicating that sPLA2-IIA is a potentially important host defense factor against this extracellular bacterium. The B. anthracis capsule is a homopolymer of γ-D-glutamic acid. This capsule is anionic due to the high density of carboxylate groups (37). The number of negatively charged motifs available has been estimated to be 8.2 μmol of COO−/mg of capsule in B. licheniformis, the...
biochemical structure of which is the same as that of B. anthracis (38). Mammalian sPLA$_2$-IIA contains a cluster of basic residues that are required for interaction with the anionic bacterial cell wall, promoting initial interactions and penetration of the cell wall and allowing the catalytic domain of the enzyme access to the phospholipids in the bacterial membrane (39). The negative charges present on the B. anthracis capsule may thus enhance the interaction of sPLA$_2$-IIA with the bacilli surface and thus stabilize the enzyme to gain access to the bacterial membrane. In contrast, sPLA$_2$-I had no bactericidal effect on B. anthracis, even at high concentrations (up to 10$^{-9}$ g/ml; data not shown). This is in agreement with previous studies of Gram-positive bacteria, such as S. aureus and B. subtilis (12). Previous reports have shown that the failure of sPLA$_2$-I to be bactericidal is not due to low enzymatic activity, but to a low ability to bind to the bacterial cell wall compared with sPLA$_2$-IIA (12).

The bactericidal activity on germinated B. anthracis spores was maximal within 15 min. This is in agreement with the time course observed with S. aureus, where ~80% cell death is obtained within 15 min (12). The ED$_{50}$ for germinated spores (~50 ng/ml) was similar to that reported for S. aureus (15–80 ng/ml), but higher than that for B. subtilis (0.2–0.8 ng/ml) (12, 40). The ED$_{50}$ of sPLA$_2$-IIA for the encapsulated vegetative form of B. anthracis was slightly higher than that for germinated spores. However, B. anthracis bacilli grow in chains, and the numbers of CFU detected in these conditions might underestimate the actual number of bacilli that were unable to grow after the action of sPLA$_2$-IIA. The concentrations of rh-sPLA$_2$-IIA used in this in vitro study were compatible with the concentrations found in human biological fluids. Indeed, the sPLA$_2$-IIA concentration in normal human serum is close to 1.7 ng/ml and is dramatically increased (500-fold) in patients with severe acute diseases (13). High sPLA$_2$-IIA concentrations (up to 30 $\mu$g/ml) have also been reported in normal human tears (40).

The respiratory tract is one of the most lethal routes of infection by B. anthracis in humans. We used two pulmonary models of sPLA$_2$-IIA production ex vivo and in vivo to examine whether

---

**FIGURE 4.** Secretory PLA$_2$-IIA hydrolyzes B. anthracis phospholipids and releases [$^3$H]OA into the medium. Germinated B. anthracis spores (3.5 × $10^7$ spores/ml) were labeled with 1 $\mu$Ci/ml [$^3$H]OA. The labeled spores were incubated for 2 h with (2 $\mu$g/ml; ○) or without sPLA$_2$-IIA (control; □) in medium containing 0.25% delipidated BSA to bind the released free [$^3$H]OA. The lipids were then extracted from the supernatants and bacterial pellets and separated by TLC, and their radioactivity was determined. Phospholipids and OA were localized by use of corresponding standards.
sPLA₂-IIA could potentially be anthracidal. In the first model we investigated the ability of human BALF samples from ARDS patients to be bactericidal for *B. anthracis* and the possible implication of sPLA₂-IIA in this process. As reported by Kim et al. (36), increased levels of sPLA₂ activity were indeed found in the BALF samples from ARDS patients. Western blot analysis showed that sPLA₂-IIA was the main sPLA₂ present in these BALF samples (L. Touqui and C. Delclaux, manuscript in preparation). Our results showed that these BALF samples exhibit potent anthracidal activity, which was mainly mediated by sPLA₂-IIA, as this activity was strongly inhibited by pretreating the BALF with sPLA₂-IIA inhibitor, LY311727. The second model is the model of acute lung injury in the guinea pig, where AM are the major pulmonary source of sPLA₂-IIA (29). In the present study we show that guinea pig AM spontaneously secreted enough sPLA₂-IIA to exert a significant anthracidal activity in our in vitro experimental conditions.

What is the relevance of these observations for a *B. anthracis* infection through the pulmonary tract? In an elegant histological study, Ross (18) has shown that germination occurred rapidly upon entry in the lung (35–60 min), and that the spores were mainly found inside the AM. The actual place where germination might occur in vivo or in vitro is still under debate; evidence for the presence of germinated spores inside macrophages has been reported, but actual germination intracellularly after phagocytosis is still lacking. In contrast, extracellular germination without host cell contact could occur in an in vivo model of a diffusion chamber in the guinea pig peritoneum (A. Piris, A. Fouet, P. L. Goossens, M. Mock, and J. C. Sirard, manuscript in preparation). Germination can thus occur in the lung, and the resulting germinated spores could be exposed to antibacterial compounds present in lung tissue. Interestingly two BALF samples examined in the present study had strong anthracidal activity even after sPLA₂-IIA enzymatic activity was totally inhibited, and another BALF sample exhibited anthracidal activity and no sPLA₂ enzymatic activity. These observations strongly suggest that other bactericidal components were present in these inflammatory fluids. Among these components, defensins (23) and surfactant (41) have been reported to display significant bactericidal action against various Gram-positive and Gram-negative bacteria. Further studies of the effectors of the innate immune system and their potential roles in the control of the first steps of *B. anthracis* infection are thus needed.

Nevertheless, sPLA₂-IIA is found in many tissues and at sites of inflammation, and it may thus play a role in controlling *B. anthracis* infection at the various sites where the spores and encapsulated bacteria will spread. One may hypothesize that at an infected site, secreted sPLA₂-IIA could have a direct antibacterial effect against encapsulated extracellular *B. anthracis* that are resistant to phagocytosis by macrophage. Another mechanism could also be involved. Weiss et al. (42) have reported that the PLA₂ secreted by polymorphonuclear leukocytes can bind to extracellular *Escherichia coli* and lead to its death inside the cells after phagocytosis has occurred. If a similar mechanism occurs with *B. anthracis*, the binding of sPLA₂-IIA to extracellular spores would lead to decreased intracellular early survival of *B. anthracis*.

Finally, we investigated whether *B. anthracis* was able to modulate the release of sPLA₂-IIA in the guinea pig AM model. Our

---

**FIGURE 5.** Anthracidal activity of sPLA₂-IIA depends on a functional catalytic enzyme. Germinated *B. anthracis* spores were incubated with or without the sPLA₂-IIA inhibitor, LY311727 (30 min at the indicated concentrations), before the bactericidal assay. The spores were incubated with sPLA₂-IIA (0.25 μg/ml) for 30 min. Results are expressed as log₁₀ *B. anthracis* CFU.

**FIGURE 6.** Anthracidal activity of sPLA₂-IIA released by unstimulated guinea pig AM. Guinea pig AM (1.5 × 10⁶ cells/well) were incubated overnight in RPMI 1640-3% FCS medium, and the supernatant was recovered. Undiluted (1) and various dilutions (1/2 to 1/8) of the supernatant were incubated for 1 h with germinated *B. anthracis* spores, either directly or after a 30-min incubation with different concentrations of the sPLA₂-IIA inhibitor LY311727 (25–100 μM). Results are expressed as the percent decrease in CFU number. These data are representative of four independent experiments.
results show that *B. anthracis* LeTx reduced both intracellular and extracellular levels of sPLA2-IIA, probably by inhibiting its synthesis. This inhibitory effect was not due to a cytotoxic action of LeTx, because guinea pig AM were found to be resistant to the lethal effect of LeTx. Thus, guinea pig AM belong to the group of macrophages that are resistant to the lytic action of LeTx (43), but responsive to its action, as detected by inhibition of sPLA2-IIA production. This extends previous reports showing that sublytic doses of LeTx inhibit the release of NO and cytokines in macrophages (34, 35) and dendritic cells (44). Keeping in mind that sPLA2-IIA is found in many tissues and at sites of inflammation, and that *B. anthracis* toxins are synthesized shortly after germination (45), the control of sPLA2-IIA release could thus represent an adaptive mechanism that allows *B. anthracis* to escape from the innate immune response. Indeed, the decrease in sPLA2-IIA levels was paralleled by a decrease in the bactericidal activity of these supernatants. Furthermore, PLA2 plays a key role in the production of lipid mediators (i.e., eicosanoids and platelet-activating factor) that have proinflammatory activities (8, 46). The inhibitory effect of LeTx would thus not only lead to a decrease in bactericidal activity associated with sPLA2-IIA released by the recruited inflammatory cells, but would also inhibit the local inflammatory reaction at the infected site; this would promote local multiplica-

**FIGURE 7.** *B. anthracis* LeTx reduces extracellular and intracellular accumulation of sPLA2-IIA in LPS-stimulated guinea pig AM. A, Guinea pig AM (1.5 × 10⁶ cells/well) were incubated overnight in RPMI 1640-3% FCS medium with LPS (50 ng/ml) in the presence or the absence of the LeTx (PA (1 μg/ml) and LF (1 ng/ml to 1 μg/ml)) or with PA (1 μg/ml) or LF (10 ng/ml to 1 μg/ml) alone, as indicated. The supernatants and the cell pellets were recovered, and sPLA2 enzymatic activity was measured; inhibition with LY311727 was >94%. The results shown are representative of two independent experiments. B, The susceptibility of guinea pig AM to the lethal effect of LeTx was evaluated by measuring MTT reduction after incubation with PA (1 and 10 μg/ml) and LF (1 μg/ml). The macrophage cell line, RAW 264.7, was used as a positive control of LeTx toxicity. Absorbance at 540 nm was measured and is expressed as a percentage of the control cells incubated without toxin. C, Same experimental conditions as in A. The proteins were extracted from the cell pellets, and the presence of sPLA2-IIA was analyzed by Western blot.

**FIGURE 8.** *B. anthracis* LeTx decreases the bactericidal effect of LPS-stimulated guinea pig AM supernatants. Guinea pig AM (1.5 × 10⁶ cells/well) were incubated in the presence (PA (1 μg/ml) and LF (1 ng/ml to 1 μg/ml)) or the absence of the LeTx, as indicated in Fig. 7. Various dilutions (1/2 to 1/8) of the supernatants were incubated for 1 h with germinated *B. anthracis* spores. The bactericidal effect results are expressed as the percent decrease in the number of germinated spores CFU. These data are representative of three independent experiments.
tion and spreading of \( B. \) \textit{anthracis} within the infected host. The magnitude of the local inflammatory response is indeed correlated with the in situ control of \( B. \) \textit{anthracis} multiplication in resistant and susceptible hosts (21).

In conclusion, we report in this study that sPLA\(_2\)-IIA exhibits anti-\( B. \) \textit{anthracis} activity on both germinated spores and capsulated bacilli. This bactericidal effect was correlated to the ability of \( B. \) \textit{anthracis} to hydrolyze bacterial membrane phospholipids. We showed that the sPLA\(_2\)-IIA present in human BALF or secreted by \( B. \) \textit{anthracis} is due to a 14-kD phospholipase A\(_2\).

Based on these findings, we propose that sPLA\(_2\)-IIA may play a role in the defense mechanism against \( B. \) \textit{anthracis} infection in vivo. Further studies are needed to investigate the potential therapeutic use of sPLA\(_2\)-IIA in experimental anthrax.

**Acknowledgments**

We gratefully acknowledge Michèle Mock for her constant scientific support, Agnès Fouet for helpful discussions and critical reading of the manuscript, Anne Moir for stimulating discussions on spore germination and Dominique Leduc for technical help with Western blot analysis. We also thank Dr. A. Demoule for performing the BAL in the human patients. We are grateful to Dr. Carine Mounier (Institut Pasteur, Paris, France) for the gift of human recombinant sPLA\(_2\)-IIA and polyclonal sPLA\(_2\)-IIA Ab.

**References**


**Table I. Anthracidal activity in human BALF is correlated with sPLA\(_2\) enzymatic activity**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Bactericidal Effect (%) CFU decrease</th>
<th>sPLA(_2) Activity (pmol/ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99</td>
<td>2722</td>
</tr>
<tr>
<td>2</td>
<td>98</td>
<td>124</td>
</tr>
<tr>
<td>3</td>
<td>98</td>
<td>577</td>
</tr>
<tr>
<td>4</td>
<td>96</td>
<td>4907</td>
</tr>
<tr>
<td>5</td>
<td>95</td>
<td>1608</td>
</tr>
<tr>
<td>6</td>
<td>91</td>
<td>1629</td>
</tr>
<tr>
<td>7</td>
<td>86</td>
<td>742</td>
</tr>
<tr>
<td>8</td>
<td>82</td>
<td>176</td>
</tr>
<tr>
<td>9</td>
<td>82</td>
<td>268</td>
</tr>
<tr>
<td>10</td>
<td>81</td>
<td>(\leq 21)</td>
</tr>
<tr>
<td>11</td>
<td>65</td>
<td>50</td>
</tr>
<tr>
<td>12</td>
<td>55</td>
<td>730</td>
</tr>
<tr>
<td>13</td>
<td>53</td>
<td>101</td>
</tr>
<tr>
<td>14</td>
<td>33</td>
<td>25</td>
</tr>
<tr>
<td>15</td>
<td>24</td>
<td>50</td>
</tr>
<tr>
<td>16</td>
<td>24</td>
<td>82</td>
</tr>
<tr>
<td>17</td>
<td>5</td>
<td>(\leq 21)</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>103</td>
</tr>
<tr>
<td>19</td>
<td>0</td>
<td>(\leq 21)</td>
</tr>
</tbody>
</table>

\(^{a}\) Bactericidal activity was assayed on germinated RPA500 spores after a 1-h incubation with unfertilized BALF. The values for the bactericidal effect are expressed as percentages of CFU decrease in germinated spores compared with spores incubated in assay medium alone (without BALF). The detection threshold of sPLA\(_2\)-IIA enzymatic activity was 21 pmol/ml/min.

\(^{b}\) sPLA\(_2\)-IIA activity on both germinated spores and capsulated bacilli. This bactericidal effect was correlated to the ability of \( B. \) \textit{anthracis} to hydrolyze bacterial membrane phospholipids. We showed that the sPLA\(_2\)-IIA present in human BALF or secreted by guinea pig AM was anthracidal ex vivo. The release of sPLA\(_2\)-IIA by AM, the major source of this enzyme during acute lung injury, was down-regulated by \( B. \) \textit{anthracis} LeTx. Further studies in in vivo models are clearly needed to test the actual implication of sPLA\(_2\)-IIA in experimental anthrax.

**Table II. Anthracidal activity in the highly bactericidal human BALF samples is essentially mediated by sPLA\(_2\)**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Bactericidal Activity(^{b})</th>
<th>sPLA(_2) Activity(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>98</td>
<td>8137</td>
</tr>
<tr>
<td>4</td>
<td>96</td>
<td>9829</td>
</tr>
<tr>
<td>6</td>
<td>99</td>
<td>2606</td>
</tr>
<tr>
<td>7</td>
<td>89</td>
<td>4571</td>
</tr>
<tr>
<td>8</td>
<td>99</td>
<td>1966</td>
</tr>
<tr>
<td>9</td>
<td>82</td>
<td>183</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>229</td>
</tr>
<tr>
<td>3</td>
<td>98</td>
<td>1463</td>
</tr>
</tbody>
</table>

\(^{a}\) BALF samples exhibiting \(>80\)% bactericidal activity (see Table I) were assayed for their bactericidal and sPLA\(_2\) enzymatic activities with or without the sPLA\(_2\)-IIA inhibitor, LY311727. BALF samples were divided into two groups according to whether LY311727 inhibited bactericidal activity.

\(^{b}\) Bactericidal and sPLA\(_2\) enzymatic activities were assayed as described in Table I.

**Table III. Anthracidal activity is modulated by sPLA\(_2\)-IIA**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Bactericidal Activity(^{b})</th>
<th>sPLA(_2) Activity(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>98</td>
<td>8137</td>
</tr>
<tr>
<td>4</td>
<td>96</td>
<td>9829</td>
</tr>
<tr>
<td>6</td>
<td>99</td>
<td>2606</td>
</tr>
<tr>
<td>7</td>
<td>89</td>
<td>4571</td>
</tr>
<tr>
<td>8</td>
<td>99</td>
<td>1966</td>
</tr>
<tr>
<td>9</td>
<td>82</td>
<td>183</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>229</td>
</tr>
<tr>
<td>3</td>
<td>98</td>
<td>1463</td>
</tr>
</tbody>
</table>

\(^{a}\) BALF samples were incubated with the sPLA\(_2\)-IIA inhibitor, LY311727 (100 \(\mu\)M), for 30 min before the bactericidal and enzymatic assays. The results are expressed as the percent inhibition of bactericidal and enzymatic activities.


