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# High Bactericidal Efficiency of Type IIA Phospholipase $A_2$ against *Bacillus anthracis* and Inhibition of Its Secretion by the Lethal Toxin[1]

**Alejandro Piris Gimenez,* Yong-Zheng Wu,† Miguel Paya,† Christophe Delclaux,‡ Lhousseine Touqui,† and Pierre L. Goossens[2]***

**There is a considerable body of evidence supporting the role of secretory type II-A phospholipase $A_2$ ($sPLA_2$-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 $sPLA_2$-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 $sPLA_2$-IIA in vitro. In contrast, nongerminated spores were resistant. This bactericidal effect was correlated to the ability of $sPLA_2$-IIA to hydrolyze bacterial membrane phospholipids. Guinea pig alveolar macrophages, the major source of $sPLA_2$-IIA in an experimental model of acute lung injury, released enough $sPLA_2$-IIA to kill extracellular *B. anthracis*. The production of $sPLA_2$-IIA was significantly inhibited by *B. anthracis* lethal toxin. Human bronchoalveolar lavage fluids from acute respiratory distress syndrome patients are known to contain $sPLA_2$-IIA; bactericidal activity against *B. anthracis* was detected in a high percentage of these samples. This anthracidal activity was correlated to the levels of $sPLA_2$-IIA and was abolished by an $sPLA_2$-IIA inhibitor. These results suggest that $sPLA_2$-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 $sPLA_2$-IIA by inhibiting the production of this enzyme.** ***The Journal of Immunology,*** **2004, 173: 521–530.**

Phospholipases $A_2$ ($PLA_2$s)[3] 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 $PLA_2$s can be divided into two major classes according to their molecular mass and location: intracellular $PLA_2$ and secreted $PLA_2$ ($sPLA_2$). The 10 distinct members identified to date share <50% identity (for review, see Refs. 2 and 3). Group IIA $sPLA_2$ ($sPLA_2$-IIA), the best-known enzyme of this group, is involved in the pathogenesis of various inflammatory diseases (4–6). Secretory $PLA_2$-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 $PLA_2$-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 $PLA_2$s play a more direct role in the host defense reaction against bacteria, especially toward Gram-positive bacteria. Indeed, $sPLA_2$-IIA displays bactericidal activity toward several strains of bacteria (10–14). The mode of action of $sPLA_2$-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 $sPLA_2$-IIA against bacterial infections was highlighted recently in $sPLA_2$-IIA$^{-/-}$ mice (15). The absence of $sPLA_2$-IIA in these mice affects their antibacterial response to *Staphylococcus aureus* infection, leading to a higher death rate compared with mice overexpressing $sPLA_2$-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:

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[3] Abbreviations used in this paper: $PLA_2$, phospholipase $A_2$; AM, alveolar macrophage; ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; BALF, BAL fluid; BHI, brain heart infusion; EF, edema factor; LF, lethal factor; LeTx, lethal toxin; OA, oleic acid; PA, protective Ag; PC, phosphatidylcholine; PE, phosphatidyl-ethanolamine; PG, phosphatidylglycerol; rgp-$sPLA_2$-IIA, recombinant guinea pig secreted $PLA_2$ type IIA; $sPLA_2$-IIA, secreted $PLA_2$ type IIA; rh-$sPLA_2$-IIA, recombinant human secreted $PLA_2$ type IIA; $sPLA_2$-I, secreted $PLA_2$ type I.

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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-D-glutamic acid, contributes to pathogenicity through its antiphagocytic properties, thus enabling the bacteria to evade the host's immune defenses and provoking septicemia (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* (anthracidal) have been partially purified from anthrax cutaneous lesions (22). Defensins (23) and PLA$_2$ 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 ability of human sPLA$_2$-IIA to kill *B. anthracis* and to determine whether this enzyme can be involved in the anthracidal activity of human bronchoalveolar lavage (BAL) fluids (BALF). We also examined the effect of *B. anthracis* on the production of sPLA$_2$-IIA by alveolar macrophages (AM), a major pulmonary source of this enzyme.

## Materials and Methods

### *Recombinant sPLA$_2$-IIA*

Purified human recombinant sPLA$_2$-IIA (rh-sPLA$_2$-IIA) was a gift from C. Mounier (Unité Défense Innée et Inflammation, Institut Pasteur, Paris, France). Recombinant guinea pig sPLA$_2$-IIA (rgp-sPLA$_2$-IIA) was prepared and purified in our laboratory as previously described (24). PLA$_2$-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 derivatives: RPLC2, which carries point mutations affecting the catalytic sites of EF and LF (25); RPA500, which carries a nonpolar spectinomycin resistance marker within the *pag* gene (same construction as the 9602P strain described in Ref. 25); and RPG1, RPLC2 derivative containing pXO2 (this work).

These strains were used at different stages of differentiation. Nongerminated spores were prepared and purified on Radioselectan 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 sPLA$_2$-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% NaHCO$_3$ 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 RPG1 strains were incubated with rh-sPLA$_2$-IIA in 100 μl of PBS with 1 mM Ca$^{2+}$ at 37°C for various time periods. The spectinomycin-resistant strain RPA500 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 spectinomycin-resistant CFU were detected in these fluids. When required, LY311727, an sPLA$_2$-IIA inhibitor (a generous gift of Lilly Corporate Center, Indianapolis, IN), was incubated with the samples containing sPLA$_2$ for 30 min at 37°C before the bactericidal assay. The concentration of germinated spores used in the assays was 5.44 ± 0.27 log$_{10}$ 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 PLA$_2$-IIA binding assay*

Nongerminated or germinated spores (10$^7$) were incubated in 100 μl of PBS-1 mM Ca$^{2+}$ in the presence or the absence of 20 ng of rh-sPLA$_2$-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-sPLA$_2$-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 × 10$^7$ spores/ml) were labeled with 1 μCi/ml [$^3$H]oleic acid ([$^3$H]OA; 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 sPLA$_2$ assay medium (see above) supplemented with 0.25% delipidated BSA to bind the released free [$^3$H]OA. After 120 min in the presence or the absence of sPLA$_2$-IIA (2 μg/ml), bacterial suspensions were centrifuged and washed three times in 0.15 M NaCl. The lipids from the supernatants and bacterial pellets were extracted with chloroform/methanol/acetic acid (50/40/10, v/v/v) and separated by TLC using H$_2$O/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 475 × *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 × 10$^6$/well) were incubated in a 24-well tissue culture plate for 1 h at 37°C in a CO$_2$ incubator (5% CO$_2$). 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 sPLA$_2$-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 sPLA$_2$-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).

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*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 $\mu$g/ml) and LF (1 $\mu$g/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 (Créteil, 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 Societé 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 $sPLA_2$ enzymatic assay.

*Secretory $PLA_2$-IIA enzymatic assay*

The fluorescent phospholipid (1-hexadecanoyl-2-(1-pyrene-decanoyl)-sn-glycero-3-phosphoglycerol; phosphatidyl-glycerol ((PG)) was used as a substrate. The measurements were performed with a Jobin & Yvon JY3D spectrofluorometer 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 $\mu$l of the substrate solution with 10 $\mu$l of 10% fatty acid-free BSA in a cuvette and adding 50 $\mu$l of the sample. Reactions were then initiated by adding 10 $\mu$l of $CaCl_2$ at a 10-mM final concentration and measuring fluorescence as described previously (32).

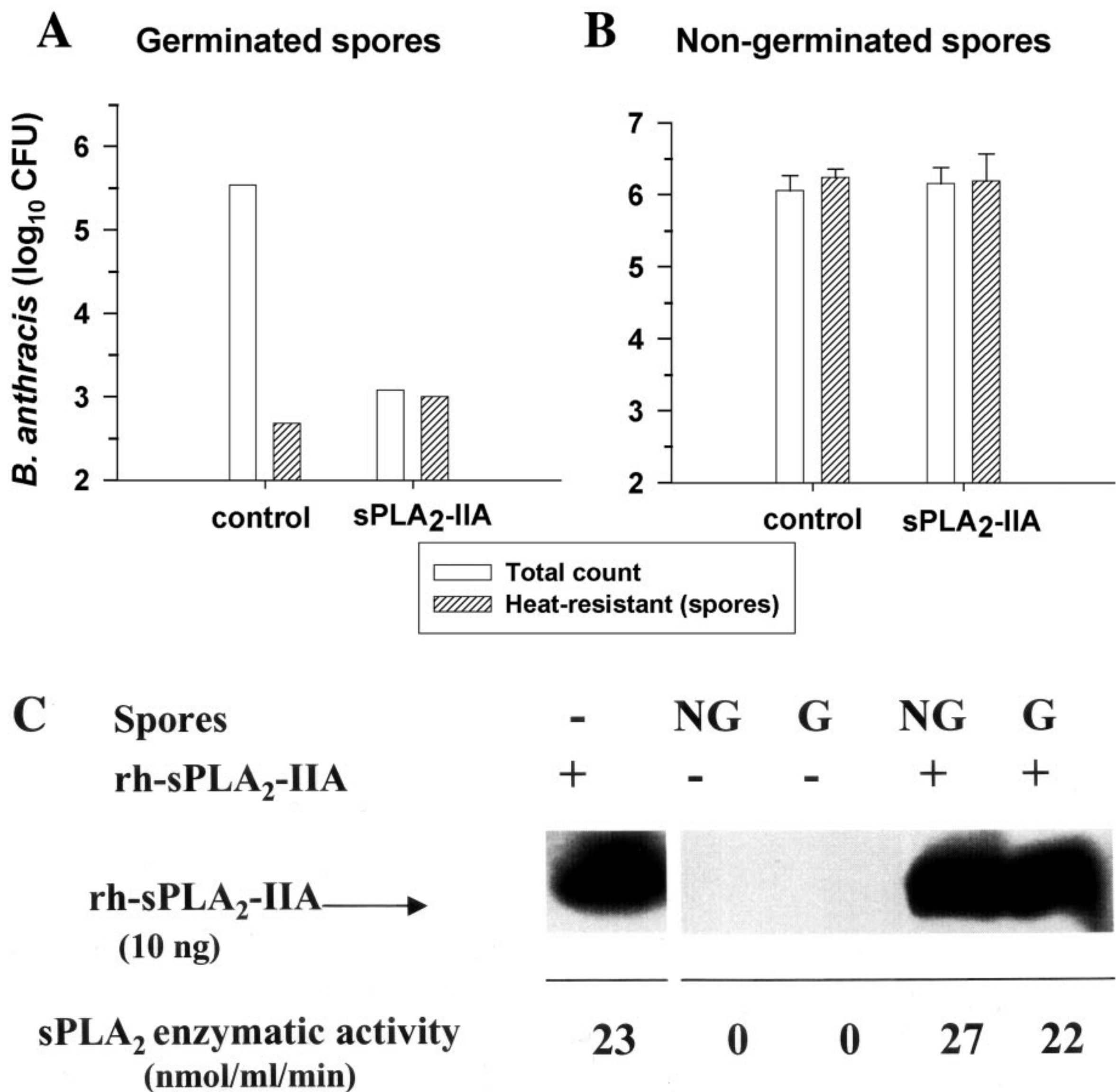


**FIGURE 1.** Recombinant human $sPLA_2$-IIA is bactericidal for *B. anthracis* germinated spores, but not for nongerminated spores. *A*, After 45 min in BHI medium, germinated spores were incubated with purified rh-$sPLA_2$-IIA (1 $\mu$g/ml) for 15 min. Results are expressed as total CFU counts (□) and CFU counts after heating at 65°C for 30 min (▨), i.e., nongerminated spores. *B*, Nongerminated spores were incubated with purified rh-$sPLA_2$-IIA (1 $\mu$g/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-$sPLA_2$-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.

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## 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. 1*A*). 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. 1*B*). 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. 1*C*).

The bactericidal effect against germinated spores was directly proportional to rh-sPLA$_2$-IIA concentrations (Fig. 2*A*); 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-γ-D-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. 2*B*); 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

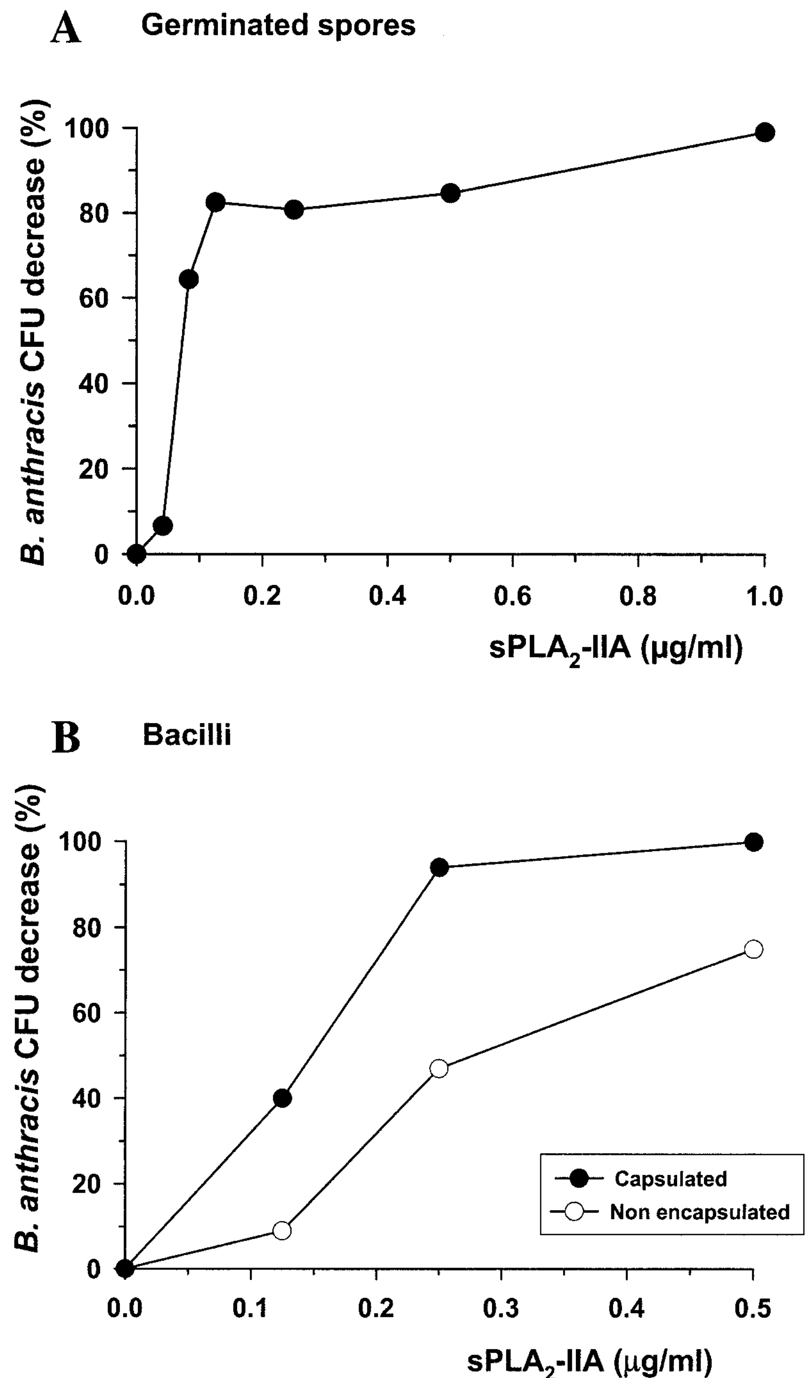


**FIGURE 2.** Dose-effect curve of rh-sPLA$_2$-IIA bactericidal activity on germinated *B. anthracis* spores (*A*) and bacilli (*B*). *A*, Germinated *B. anthracis* spores were incubated for 45 min with increasing concentrations of rh-sPLA$_2$-IIA. Results are expressed as the percentage of CFU decrease for germinated spores, calculated after differential counts of heat-sensitive and heat-resistant bacterial forms. *B*, Equivalent concentrations of encapsulated and nonencapsulated *B. anthracis* ($1.4 \times 10^7$ CFU/ml) were incubated with increasing concentrations of rh-sPLA$_2$-IIA for 30 min. Results are expressed as the percentage of CFU decrease. These data are representative of three independent experiments.

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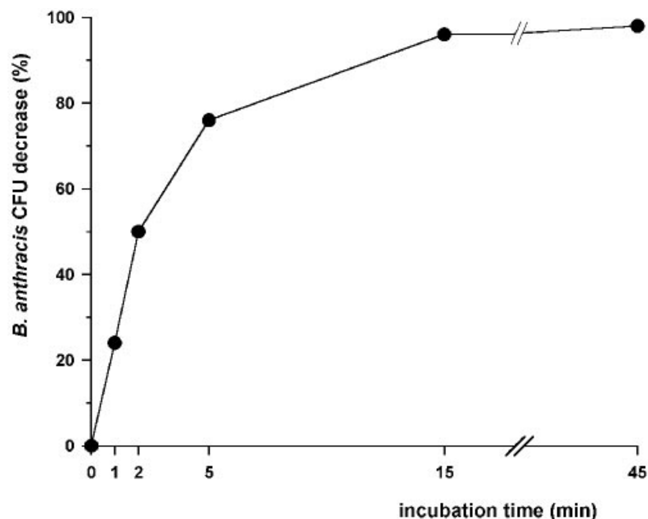


**FIGURE 3.** Rapid destruction of germinated *B. anthracis* spores by rh-sPLA$_2$-IIA. Germinated spores were incubated with sPLA$_2$-IIA (0.5 $\mu$g/ml) for increasing time periods. Results are expressed as the percentage of CFU decrease, calculated after differential counts of heat-sensitive and heat-resistant bacterial forms. These data are representative of at least two independent experiments.

germination (27). Our results showed that spores treated in this way were sensitive to sPLA$_2$-IIA (84.5 and 94.5% decrease in CFU in the presence and the absence of chloramphenicol, respectively; mean of two independent experiments), thus indicating that protein synthesis was not required for this process.

*Importance of sPLA$_2$-IIA enzymatic activity for the anthracidal effect*

Recombinant human sPLA$_2$-IIA was able to hydrolyze the membrane phospholipids of *B. anthracis*. Labeling of *B. anthracis* with [$^3$H]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 sPLA$_2$-IIA, leading to a decrease in the amount of labeled PG and PE in the bacterial membranes (Fig. 4*B*) and the concomitant release of [$^3$H]OA into the medium (Fig. 4*A*).

The rh-sPLA$_2$-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 sPLA$_2$-IIA released by guinea pig AM and its modulation by* B. anthracis *LeTx*

Guinea pig AM, the major pulmonary source of sPLA$_2$-IIA in an experimental model of ARDS (32), secrete this enzyme in vitro (29, 33). We first ensured that purified recombinant gp-sPLA$_2$-IIA had the same bactericidal effect against *B. anthracis*, and the same enzymatic activity against known substrates as recombinant human sPLA$_2$-IIA (data not shown). We then tested whether guinea pig AM in those experimental conditions released enough sPLA$_2$-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 sPLA$_2$-IIA inhibitor, LY311727. This suggested that this enzyme was the major *B. anthracis* bactericidal component released by AM.

*B. anthracis* LeTx (PA+LF) plays a crucial role in anthrax pathogenicity (16). The PA receptor is a ubiquitous protein (16). Macrophages have been reported to be one of the main targets. LeTx reduces their ability to release cytokines and NO in response to LPS stimuli (34, 35). We thus tested whether LeTx could also modulate in guinea pig AM the release of sPLA$_2$-IIA induced by LPS, which was used as a bacterial stimulus. These cells were resistant to the cytotoxic activity of LeTx, whereas the RAW 264.7 macrophage cell line was susceptible, as expected (Fig. 7*B*). Guinea pig AM were pretreated with LeTx for 2 h and then incubated overnight with LPS. The levels of extracellular and cell-associated sPLA$_2$-IIA were decreased by LeTx in a concentration-dependent manner (Fig. 7, *A* and *C*). When only the LF enzymatic moiety of LeTx was added, the levels of sPLA$_2$-IIA remained unchanged. The decrease in sPLA$_2$-IIA levels were paralleled by a decrease in the bactericidal activity of these supernatants (Fig. 8).

*Anthracidal activity of human BALF*

Increased levels of sPLA$_2$ have been reported in BALF of ARDS patients (36). BALF from such patients were thus tested for their sPLA$_2$ content and their potential bactericidal activity against germinated *B. anthracis* spores. Bactericidal activity was detected in a large proportion of the BALF samples: 10 of 19 were highly bactericidal (>80% CFU decrease), six of 19 were moderately bactericidal (24–65% CFU decrease), and three of 19 presented no significant bactericidal activity (<10%; Table I). We tested whether this bactericidal activity was correlated with sPLA$_2$ enzymatic activity. The highest sPLA$_2$ activities (>730 pmol/ml/min) were found mainly in the BALF samples that had the strongest bactericidal activity. Low or no sPLA$_2$ activity could be detected in some BALF displaying a significant bactericidal activity (BALF 10 and 2, in particular), suggesting the existence of bactericidal effectors other than sPLA$_2$.

We further analyzed the 10 BALF samples that were highly bactericidal (>80%) for *B. anthracis*. In eight cases (group I, Table II), the bactericidal effect was essentially linked to sPLA$_2$-IIA, as it was completely inhibited by LY 311727, a competitive inhibitor of this enzyme. In the two remaining cases (group II, Table II), although LY 311727 significantly inhibited sPLA$_2$ enzymatic activity, no decrease was observed in bactericidal activity, suggesting the existence of bactericidal effectors other than sPLA$_2$-IIA.

## Discussion

In this study we showed that purified rh-sPLA$_2$-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 sPLA$_2$-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 sPLA$_2$-IIA. Thus, the capsule cannot prevent sPLA$_2$-IIA from reaching the cytoplasmic membrane phospholipids of *B. anthracis*, indicating that sPLA$_2$-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 $\mu$mol of $COO^-$/mg of capsule in *B. licheniformis,* the

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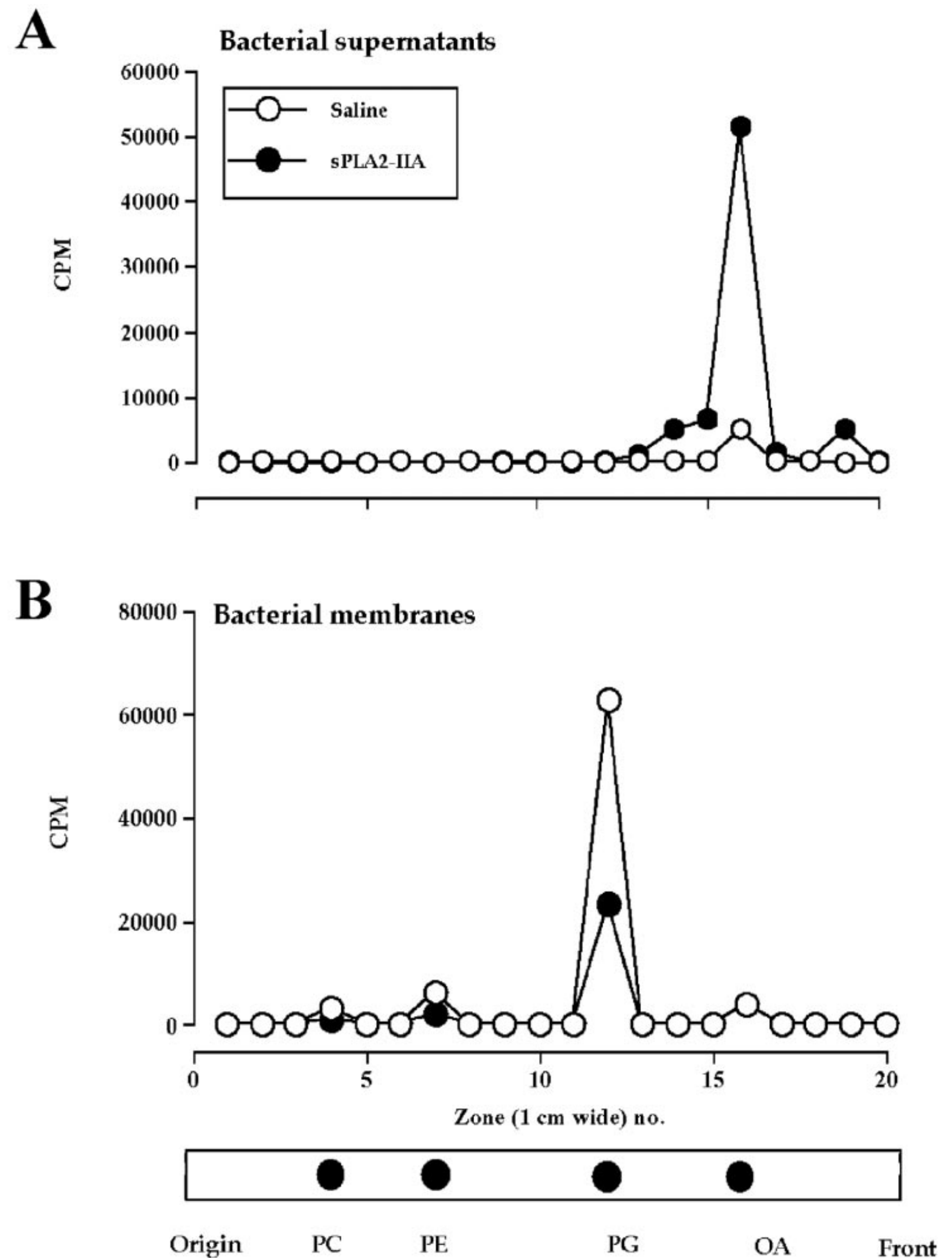


**FIGURE 4.** Secretory PLA$_2$-IIA hydrolyzes *B. anthracis* phospholipids and releases [$^3$H]OA into the medium. Germinated *B. anthracis* spores (3.5 × $10^7$ spores/ml) were labeled with 1 $\mu$Ci/ml [$^3$H]OA. The labeled spores were incubated for 2 h with (2 $\mu$g/ml; ●) or without sPLA$_2$-IIA (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.

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 $\mu$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

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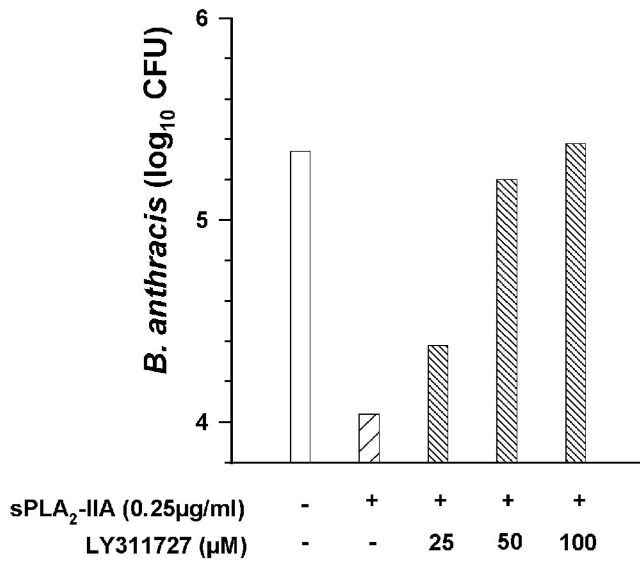


**FIGURE 5.** Anthracidal activity of $sPLA_2$-IIA depends on a functional catalytic enzyme. Germinated *B. anthracis* spores were incubated with or without the $sPLA_2$-IIA inhibitor, LY311727 (30 min at the indicated concentrations), before the bactericidal assay. The spores were incubated with $sPLA_2$-IIA (0.25 $\mu$g/ml) for 30 min. Results are expressed as $\log_{10}$ *B. anthracis* CFU.

$sPLA_2$-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 $sPLA_2$-IIA in this process. As reported by Kim et al. (36), increased levels of $sPLA_2$ activity were indeed found in the BALF samples from ARDS patients. Western blot analysis showed that $sPLA_2$-IIA was the main $sPLA_2$ 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 $sPLA_2$-IIA, as this activity was strongly inhibited by pretreating the BALF with $sPLA_2$-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 $sPLA_2$-IIA (29). In the present study we show that guinea pig AM spontaneously secreted enough $sPLA_2$-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 $sPLA_2$-IIA enzymatic activity was totally inhibited, and another BALF sample exhibited anthracidal activity and no $sPLA_2$ 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, $sPLA_2$-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 $sPLA_2$-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 $PLA_2$ 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 $sPLA_2$-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 $sPLA_2$-IIA in the guinea pig AM model. Our

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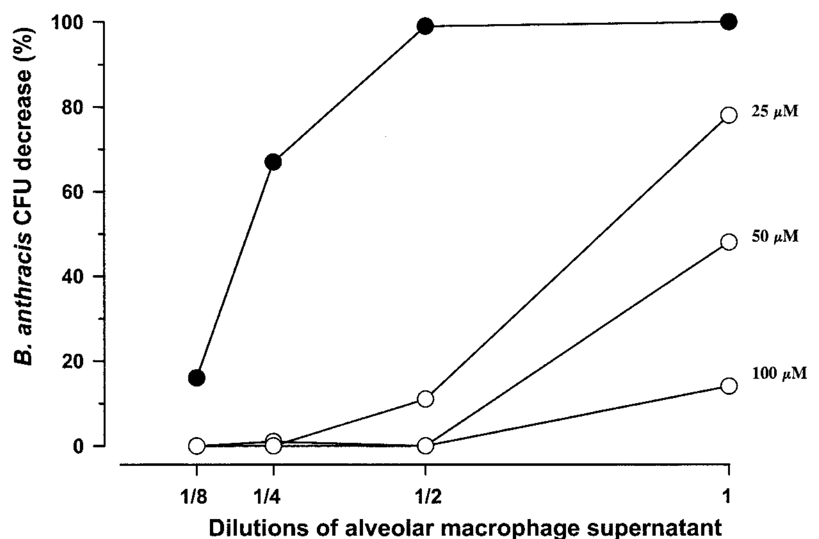


**FIGURE 6.** Anthracidal activity of $sPLA_2$-IIA released by unstimulated guinea pig AM. Guinea pig AM ($1.5 \times 10^6$ cells/well) were incubated overnight in RPMI 1640-3% FCS medium, and the supernatant was recovered. Undiluted (1) and various dilutions (1/2 to 1/8) of the supernatant were incubated for 1 h with germinated *B. anthracis* spores, either directly or after a 30-min incubation with different concentrations of the $sPLA_2$-IIA inhibitor LY311727 (25–100 $\mu$M). Results are expressed as the percent decrease in CFU number. These data are representative of four independent experiments.

**FIGURE 7.** *B. anthracis* LeTx reduces extracellular and intracellular accumulation of sPLA$_2$-IIA in LPS-stimulated guinea pig AM. *A*, Guinea pig AM ($1.5 \times 10^6$ 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 $\mu$g/ml) and LF (1 ng/ml to 1 $\mu$g/ml)) or with PA (1 $\mu$g/ml) or LF (10 ng/ml to 1 $\mu$g/ml) alone, as indicated. The supernatants and the cell pellets were recovered, and sPLA$_2$ 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 $\mu$g/ml) and LF (1 $\mu$g/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 sPLA$_2$-IIA was analyzed by Western blot.

results show that *B. anthracis* LeTx reduced both intracellular and extracellular levels of sPLA$_2$-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 sPLA$_2$-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 sPLA$_2$-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 sPLA$_2$-IIA release could thus represent an adaptive mechanism that allows *B. anthracis* to escape from the innate immune response. Indeed, the decrease in sPLA$_2$-IIA levels was paralleled by a decrease in the bactericidal activity of these supernatants. Furthermore, PLA$_2$ 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 sPLA$_2$-IIA released by the recruited inflammatory cells, but would also inhibit the local inflammatory reaction at the infected site; this would promote local multiplica-

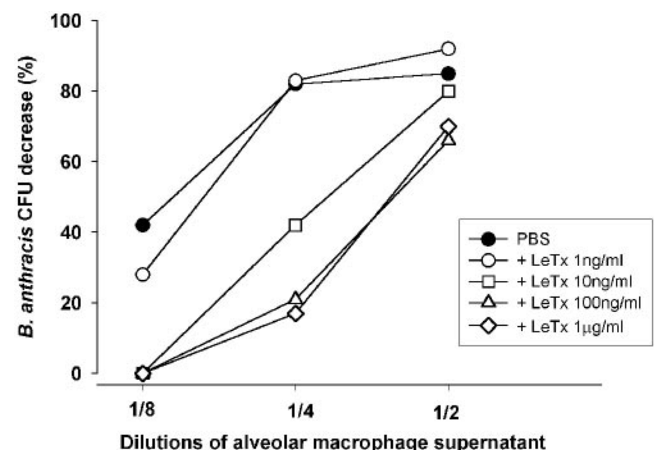


**FIGURE 8.** *B. anthracis* LeTx decreases the bactericidal effect of LPS-stimulated guinea pig AM supernatants. Guinea pig AM ($1.5 \times 10^6$ cells/well) were incubated in the presence (PA (1 $\mu$g/ml) and LF (1 ng/ml to 1 $\mu$g/ml)) or the absence of the LeTx, as indicated in Fig. 7. Various 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.

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Table I. *Anthracidal activity in human BALF is correlated with $sPLA_2$ enzymatic activity*[a]

| Patient | Bactericidal Effect (% CFU decrease) | $sPLA_2$ Activity (pmol/ml/min) |
|---|---|---|
| 1 | 99 | 2722 |
| 2 | 98 | 124 |
| 3 | 98 | 577 |
| 4 | 96 | 4907 |
| 5 | 95 | 1608 |
| 6 | 91 | 1629 |
| 7 | 86 | 742 |
| 8 | 82 | 176 |
| 9 | 82 | 268 |
| 10 | 81 | ≤21 |
| 11 | 65 | 50 |
| 12 | 55 | 730 |
| 13 | 53 | 101 |
| 14 | 33 | 25 |
| 15 | 24 | 50 |
| 16 | 24 | 82 |
| 17 | 5 | ≤21 |
| 18 | 0 | 103 |
| 19 | 0 | ≤21 |

[a] Bactericidal activity was assayed on germinated RPA500 spores after a 1-h incubation with undiluted 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 $sPLA_2$ enzymatic activity was 21 pmol/ml/min.

tion and spreading of *B. anthracis* within the infected host. The magnitude of the local inflammatory response is indeed correlated with the in situ control of *B. anthracis* multiplication in resistant and susceptible hosts (21).

In conclusion, we report in this study that $sPLA_2$-IIA exhibits anti-*B. anthracis* activity on both germinated spores and capsulated bacilli. This bactericidal effect was correlated to the ability of $sPLA_2$-IIA 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. 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*[a]

| | Bactericidal Activity[b] | | $sPLA_2$ Activity[b] | |
|---|---|---|---|---|
| Patient No. | CFU decrease (%) | LY311727 inhibition (%)[c] | pmol/ml/min | LY311727 inhibition (%)[c] |
| Group I | | | | |
| 1 | 98 | 69 | 8137 | 97 |
| 4 | 96 | 81 | 9829 | 97 |
| 5 | 99 | 100 | 2606 | 89 |
| 6 | 99 | 95 | 4571 | 97 |
| 7 | 89 | 91 | 1966 | 97 |
| 8 | 99 | 63 | 183 | 100 |
| 9 | 82 | 95 | 503 | 83 |
| Group II | | | | |
| 2 | 100 | 1 | 229 | 63 |
| 3 | 98 | 2 | 1463 | 92 |

[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.

[c] 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.

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