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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 antracidal 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 (PLA₂s) 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 PLA₂s can be divided into two major classes according to their molecular mass and localization: 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 PLA₂-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 PLA₂s 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:

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methicillin-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 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 [3H]oleic acid ([3H]OEA; 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% depleted BSA to bind the released free [3H]OEA. 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/ammonia (13/45/65) 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 5% 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 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 (1/10,000 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; 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 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 µg/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-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. 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.

FIGURE 3. Rapid destruction of germinated B. anthracis spores by rh-sPLA₂-IIA. 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.

Importance of sPLA₂-IIA enzymatic activity for the anthracidal effect
Recombinant human sPLA₂-IIA was able to hydrolyze the membrane phospholipids of B. anthracis. Labeling of B. anthracis with [³H]OEA 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]OEA 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 these 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.

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 sPLA₂-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. 7B). Guinea pig AM were pretreated with LeTx for 2 h and then incubated overnight with LPS. The levels of extracellular and cell-associated sPLA₂-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 sPLA₂-IIA remained unchanged. The decrease in sPLA₂-IIA levels were paralleled by a decrease in the bactericidal activity of these supernatants (Fig. 8).

Anthracidal activity of human BALF
Increased levels of sPLA₂ have been reported in BALF of ARDS patients (36). BALF from such patients were thus tested for their sPLA₂ 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 sPLA₂ enzymatic activity. The highest sPLA₂ activities (>730 pmol/ml/min) were found mainly in the BALF samples that had the strongest bactericidal activity. Low or no sPLA₂ 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 sPLA₂.

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 sPLA₂-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 sPLA₂ enzymatic activity, no decrease was observed in bactericidal activity, suggesting the existence of bactericidal effectors other than sPLA₂-IIA.

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 α-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 sPLA₂-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 sPLA₂-IIA with the bacilli surface and thus stabilize the enzyme to gain access to the bacterial membrane. In contrast, sPLA₂-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 sPLA₂-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 sPLA₂-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 sPLA₂-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 sPLA₂-IIA. The concentrations of rh-sPLA₂-IIA used in this in vitro study were compatible with the concentrations found in human biological fluids. Indeed, the sPLA₂-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 sPLA₂-IIA concentrations (up to 30 µg/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 sPLA₂-IIA production ex vivo and in vivo to examine whether...
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 $B. \text{anthracis}$ 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 sPLA2-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 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 $B. \text{anthracis}$ 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 $B. \text{anthracis}$ 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 $B. \text{anthracis}$ 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 $B. \text{anthracis}$ 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 $B. \text{anthracis}$, the binding of sPLA$_2$-IIA to extracellular spores would lead to decreased intracellular early survival of $B. \text{anthracis}$.

Finally, we investigated whether $B. \text{anthracis}$ was able to modulate the release of sPLA$_2$-IIA in the guinea pig AM model. Our
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](http://www.jimmunol.org/)

**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](http://www.jimmunol.org/)

**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.
Table I. Anthracidal activity in human BALF is correlated with sPLA₂-IIA enzymatic activity

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<tr>
<td>18</td>
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<td>103</td>
</tr>
<tr>
<td>19</td>
<td>0</td>
<td>≤21</td>
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</table>

* Bacterial activity was assayed on germinated RPAS000 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 sPLA₂ enzymatic activity was 21 pmol/ml/min.

Table II. Anthracidal activity in the highly bactericidal human BALF samples is essentially mediated by sPLA₂

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>CFU decrease (%)</th>
<th>LY311727 inhibition (%)</th>
<th>sPLA₂ Activity pmol/ml/min</th>
<th>LY311727 inhibition (%)</th>
<th>sPLA₂ Activity pmol/ml/min</th>
</tr>
</thead>
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<tr>
<td>Group I</td>
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<td>97</td>
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<td></td>
</tr>
</tbody>
</table>

* BALF samples exhibiting >80% bactericidal activity (see Table I) were assayed for their bactericidal and sPLA₂ enzymatic activities with or without the sPLA₂-IIA inhibitor, LY311727. BALF samples were divided into two groups according to whether LY311727 inhibited bactericidal activity.

* Bacterial and sPLA₂ enzymatic activities were assayed as described in Table I.

* BALF samples were incubated with the sPLA₂-IIA inhibitor, LY311727 (100 μM), for 30 min before the bactericidal and enzymatic assays. The results are expressed as the percent inhibition of bactericidal and enzymatic activities.

Acknowledgments

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References