



Alpha-1 antitrypsin is markedly decreased following pulmonary *F. tularensis* challenge

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Neutrophils form the first line of defense during infection and are indispensable in this function. The neutrophil elastase is a key effector molecule of the innate immune system with potent antimicrobial activity against Gram-negative bacteria, spirochaetes, and fungi. However, the release of neutrophil elastase during bacterial infection must be checked otherwise its release in the extracellular milieu will result in damage to surrounding tissues. Alpha-1 antitrypsin is a small glycoprotein clade A serpine serine protease inhibitor and has been shown to increase in humans following bacterial and viral infection. *Francisella tularensis* is a Gram-negative facultative intracellular bacterium and the causative agent of tularemia. Type A strains are the most virulent with an infectious dose as low as 10 colony forming units and a mortality rate of 30–60% among untreated cases of pneumonic tularemia. We report here significant reduction of this major inhibitor of the neutrophil elastase in plasma of *F. tularensis* LVS and *F. tularensis* (type A) SCHU S4 infected animals following pulmonary challenge. Associated with an imbalance of protease–antiprotease function at the alveolar level in lungs of infected animals, increased elastase activity was observed in lung lavage fluids accompanied by decrease lung function, i.e., loss of lung elastance with concomitant increase of pulmonary hysteresivity. Consistent with a competent acute phase response following *F. tularensis* LVS and *F. tularensis* (type A) SCHU S4 pulmonary challenge and proposed up-regulation of plasma haptoglobin during the course of the acute phase reaction, haptoglobin was observed significantly increased. These data suggest that unchecked neutrophil serine protease activity may arise from *F. tularensis* targeted reduction of plasma α_1 -antitrypsin promoting lung tissue damage facilitating increased dissemination of this bacterium in infected animals.

Keywords: antiprotease, neutrophil, elastase, plasma, lung lavage, *Francisella*

INTRODUCTION

Neutrophils are one of the first immune cells to arrive at the site of inflammation. These cells efficiently degrade microorganisms via the action of proteases and oxidant species restricting pathogen replication and spreading at multiple levels, e.g., direct cleavage of virulence factors (Weinrauch et al., 2002), degradation of cytokines (Mezyk-Kopec et al., 2005), modulation of production of chemokines (Geraghty et al., 2007), and tissue breakdown allowing direct contact between immune cells and pathogens preventing bacterial escape (Kielty et al., 1993).

The neutrophil protease elastase is a key effector molecule of the innate immune system with potent antimicrobial activity against Gram-negative bacteria (Belaouaj et al., 1998), spirochaetes (Garcia et al., 1998), and fungi (Tkalcevic et al., 2000). Because neutrophil elastase has been shown to digest virtually every type of matrix protein, including several types of collagens, fibronectin, proteoglycans, heparin, and cross-linked fibrin (Travis, 1988), tissue digestion is its best-known extracellular manifestation which has its roots in hereditary α_1 -antitrypsin

deficiency. Thus, elastase is synthesized as a zymogen and requires cleavage by cathepsin-C to become active and is subsequently stored within cytoplasmic azurophilic granules until extruded into phagolysosomes or out of the cell (Methot et al., 2007). Once released, active elastase is held in check by the protease inhibitor α_1 -antitrypsin.

Although named α_1 -antitrypsin, the physiologic target is neutrophil elastase rather than trypsin and for this reason it is commonly referred to as α_1 -proteinase inhibitor. Alpha-1 antitrypsin is a small glycoprotein (52 kDa), clade A serpine serine protease inhibitor which is constitutively made in the liver and released into the blood stream. The archetype of the serpin family and present at highest concentration in plasma, α_1 -antitrypsin is the major inhibitor of extracellular neutrophil elastase (Heutinck et al., 2010). The acute phase response to infection and injury is characterized by changes in serum protein composition. Such changes may give rise to specific response patterns and thus provide information as to the etiology of disease. Alpha-1 antitrypsin and haptoglobin are acute phase reactants, the

concentration of which typically increase during the acute phase response.

Francisella tularensis is a Gram-negative facultative intracellular bacterium and the causative agent of tularemia. Type A strains are the most virulent with an infectious dose as low as 10 colony forming units (CFU) and a mortality rate of 30–60% among untreated cases of pneumonic tularemia (Stuart and Pullen, 1945). The basis of the very infectious nature of inhaled *Francisella* remains unclear. Neutrophil influx and release of neutrophil-chemotactic chemokines have been observed in the lungs including monkeys, rabbits, rats, and mice (Schricker et al., 1972; Dunaeva and Shlygina, 1975) following respiratory infection. Using an *in vitro* approach, Moreland et al. (2009) studied the interaction of *F. tularensis* LVS with pulmonary microvascular endothelial cells. Although *F. tularensis* LVS interacted with pulmonary cells in a fashion that promoted PMN transmigration, these stimulated PMN did not elicit proinflammatory cytokine release nor was NADPH oxidase activity which generates pathogen reactive neutralizing oxygen species compromised. This is in contrast to earlier studies indicating disruption of normal PMN oxidative function following ingestion of *Francisella* (Lofgren et al., 1983 and Proctor et al., 1975). In a recent *in vivo* study, neutrophils were recruited in a dose-dependent fashion; however, neither neutrophil depletion nor enhancement of early recruitment affected either bacterial burdens in the mouse lung or survival times following *F. tularensis* (type A) SCHU S4 infection with NADPH oxidase playing only a minor role in host defense (KuoLee et al., 2011). These findings are in contrast to those of Conlan and Lindgren indicating neutrophils as well as the NADPH dependent phagocyte oxidase to be critical for host defense against systemic *F. tularensis* LVS infection (Conlan et al., 2002; Lindgren et al., 2004).

Although examination of the orchestrated shaping of the host defense immune response to *Francisella* has led to conflicting reports, the interaction of neutrophil elastase, α_1 -antitrypsin, and possible involvement of other proteinases is consistent with the theme of unchecked elastase activity, i.e., α_1 -antitrypsin reduction/clearance. For example, in response to neutrophil elastase, macrophages produce IL-8, matrix metalloproteinase-2, and cathepsin B; proteins that typically promote chemotaxis (Geraghty et al., 2007). Using an *in vivo* model of acute lung injury, Ferry et al. (1997) suggested neutrophil elastase to be a physiological activator of matrix metalloproteinase-9. Subsequently, Malik et al. (2007) demonstrated matrix metalloproteinase-9 activity to enhance host susceptibility to pulmonary infection with types A and B strains of *F. tularensis*. This finding was consistent with an earlier observation by Liu et al. (2000) indicating α_1 -antitrypsin was recognized as a degradative substrate by gelatinase B metalloproteinase-9, cleavage of which indirectly enhances further neutrophil elastase activity generating α_1 -antitrypsin peptide fragments that have neutrophil-chemotactic activity.

Both α_1 -antitrypsin and haptoglobin are major participants in the evolutionarily conserved acute phase response. In this report, we describe significant reduction of α_1 -antitrypsin, the major inhibitor of extracellular neutrophil elastase in plasma of *F. tularensis* LVS and *F. tularensis* (type A) SCHU S4 strain infected

animals with a concomitant increase in haptoglobin, the major acute phase hemoglobin-binding protein in the plasma of most vertebrates and all mammals (Wicher and Fries, 2006) supporting the competent nature of the acute phase response in infected animals. We suggest that following infection by *F. tularensis*, plasma α_1 -antitrypsin is preferentially reduced leading to directly and/or indirectly via possible involvement of other proteases (1) clearance of α_1 -antitrypsin in the plasma and (2) unchecked lung lavage fluid neutrophil elastase activity thus promoting lung tissue damage and increased dissemination of this bacterium from the lungs to other tissues.

MATERIALS AND METHODS

BACTERIAL STRAINS

Studies described in this report utilized *F. tularensis* LVS and *F. tularensis* subsp. *tularensis* (type A) SCHU S4 strain. Bacteria were grown at 37°C in trypticase soy broth (TSB) or on trypticase soy agar (TSA) each supplemented with 0.1% cysteine for 24–48 h.

MICE

Four to six-week-old female C57BL/6 mice were obtained from Charles River Laboratories (Frederick, MD, USA). Mice were housed at the University of Texas at San Antonio animal facility and all experimental procedures were performed in compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines.

INTRANASAL (I.N.) CHALLENGE

Mice were first anesthetized with 3% isoflurane using a rodent anesthesia system (Harvard Apparatus, Holliston, MA, USA) followed by inoculation i.n. with either 5000 CFU *F. tularensis* LVS, 50 CFU *F. tularensis* (type A) SCHU S4, or mock (PBS-treated) control.

PREPARATION OF PLASMA

Mice (5) in each respective group, i.e., Mock (PBS-treated) control, *F. tularensis* LVS, and *F. tularensis* (type A) SCHU S4 were bled 72 h post i.n. challenge and plasma prepared using plasma collection tubes containing lithium heparin and polymer gel (BD Bioscience, NJ, USA). Respective plasma samples were centrifuged for 5 min at 5000 rpm and supernatant (plasma) was removed and stored at –20°C until used. The respective plasmas were assayed individually.

PREPARATION OF LUNG LAVAGE AND ELASTASE DETERMINATION

For collection of bronchoalveolar lavage fluid, five mice (5-day post challenge) from all three groups, i.e., Mock (PBS-treated) control, *F. tularensis* LVS, and *F. tularensis* (type A) SCHU S4 infected animals were sacrificed and the trachea intubated using a 0.58 mm (outside diameter) polyethylene catheter (Becton Dickinson, Sparks, MD, USA). Lungs were lavaged twice with Hanks balanced salt solution (Invitrogen, Carlsbad, CA, USA). Recovered fluid (1 ml) was centrifuged at 9,500 × g for 7 min at 5°C and the supernatant was stored at –70°C until used. Each lavage fluid sample was assayed fluorometrically in triplicate for elastase activity using 0.1 mM suc-ala-ala-ala-MCA substrate (Peptide Institute, Inc., Osaka, Japan) dissolved in 0.05 M Tris-HCl

buffer, pH 7.5 in a final reaction volume of 1 ml for 48 h at 37°C. Purified elastase (Sigma Chemical Company, St. Louis, MO, USA) served as control. Hydrolysis of substrate was extrapolated to a 7-amino-4-methylcoumarin standard curve. Purified elastase (1 Unit) was observed to liberate 1.84 μ moles/ml/h. Lavage elastase activity is expressed as nmoles/ml lavage material/h.

DETERMINATION OF PLASMA ALPHA-1 ANTITRYPSIN CONTENT

The concentration of α_1 -antitrypsin in the plasma of each animal of the respective groups was determined by ELISA using a mouse α_1 -antitrypsin ELISA Kit (Kamiya Biochemical Company) per manufacturer's instructions.

ASSESSMENT OF RESPIRATORY MECHANICS

Lung tissue hysteresivity and elastance were assessed 5-days post i.n. challenge. Briefly, mice were anesthetized and given an intraperitoneal injection of muscle relaxant (pancuronium bromide). Mock (PBS-treated) controls and *F. tularensis* LVS infected animals were intubated and mechanically ventilated by a computer controlled piston ventilator (FlexiVent, SCIREQ, Montreal, QC, Canada). Lung function analyses were carried out using pressure-volume loops by inflating mice at various increments of pressure until a maximum pressure of 33 mm Hg (25 cm H₂O). Deflation was achieved by step-wise decrements of pressure at corresponding intervals. Anesthetized mice were mechanically ventilated with a tidal volume of 10 ml/kg and a frequency of 2.5 Hz. Resistance data were collected using a single compartment model and the derived data plotted following normalization to percent base line resistance/compliance.

TWO DIMENSIONAL PAGE AND MASS SPECTROMETRY ANALYSIS

Two dimensional PAGE analyses were carried out on different plasmas of each respective group using BioRad Ready Prep Reagents/gels (Hercules, CA, USA) per manufacturer's instructions. Respective mouse plasmas (150 μ l) were separated in the first dimension using pH 4–7 Ready Strip IPG strips and in the second dimension on 8–16% precast SDS Ready Gels. Gels were stained with SYPRO Ruby (Invitrogen/Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer's rapid staining protocol. Gel spots of interest were excised, digested *in situ* with trypsin (Trypsin Profile IGD Kit; Sigma Chemical Co., St. Louis, MO, USA) and the resulting peptides analyzed by capillary HPLC–electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS) on a Thermo Fisher LTQ mass spectrometer. The tandem mass spectra were searched against the Swiss-Prot database by means of Mascot (Matrix assessment of probabilities of peptide and protein assignments by Scaffold, Proteome Software, Portland, OR, USA). The significance thresholds for peptide and protein assignments were 95 and 99%, respectively with a minimum of two peptides required for protein identification.

STATISTICAL ANALYSES

Data were evaluated using the Student's *t*-test (SigmaStat statistical software program) and are presented as the mean value \pm SD. The number of replicates for each experiment is indicated in the respective figure legends.

RESULTS

FRANCISELLA INFECTION RESULTED IN MARKEDLY DECREASED PLASMA α_1 -ANTITRYPSIN ACCOMPANIED BY A CONCOMITANT INCREASE IN HAPTOGLOBIN (β -CHAINS)

Mice are highly susceptible to *Francisella* infection and have been used widely as an animal model to study tularemia disease. In order to study the acute phase response, we challenged mice intranasally with a lethal dose of *F. tularensis* LVS or *F. tularensis* (type A) SCHU S4 strain and collected plasma at 3-days post-challenge for protein profile comparison to a similarly prepared plasma from mock (PBS-treated) mice using two dimensional gel/proteomic analyses. Shown in **Figure 1** are representative images of three different two-dimensional gel electrophoresis experiments. A significantly different protein profile was observed for mock (PBS-treated) plasma compared to *F. tularensis* LVS and *F. tularensis* (type A) SCHU S4 infected plasma exhibiting (1) a significant decrease in a protein of approximate 50 kDa molecular weight, and (2) a significant increase in a protein of approximate 40 kDa molecular weight. These proteins were identified as α_1 -antitrypsin (charge train 1) and haptoglobin β -chains (charge train 2) by tandem mass spectrometry. Charge heterogeneity in both α_1 -antitrypsin and haptoglobin β -chains arises from differing sialic acid content of the *N*-type glycosides (Mega et al., 1980; Park et al., 2010). Haptoglobin like α_1 -antitrypsin is a major participant/member of the evolutionarily conserved set of acute phase plasma proteins.

In order to further corroborate the two dimensional gel analysis, α_1 -antitrypsin content in plasma of mock (PBS-treated) controls and 3-day post challenge *Francisella* infected animals was determined. Although less than the expected almost complete removal of α_1 -antitrypsin shown in **Figure 1**, a 25% reduction

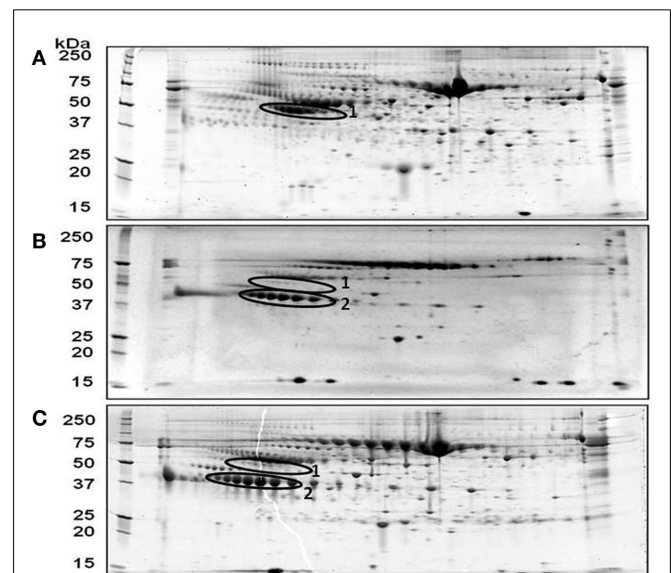


FIGURE 1 | Representative two-dimensional PAGE gel images of mouse plasma proteins following pulmonary *F. tularensis* challenge. Two-dimensional PAGE analysis was carried out as described under Section "Materials and Methods" using 3-day post challenge plasma. **(A)** Mock (PBS-treated) control; **(B)** *F. tularensis* LVS; **(C)** *F. tularensis* (type A) SCHU S4. Encircled area 1, α_1 -antitrypsin, encircled area 2, haptoglobin (β -chains).

in plasma α_1 -antitrypsin level was observed in *F. tularensis* LVS (6.8 ± 1.2 mg/ml) infected animals compared to the mock (PBS-treated) control (9.1 ± 0.5 mg/ml, **Figure 2**). Mice infected with *F. tularensis* (type A) SCHU S4 strain (3.2 ± 0.4 mg/ml) exhibited a 65% reduction of plasma α_1 -antitrypsin, an approximate 2.6-fold greater reduction than that observed for LVS infected animals (**Figure 2**).

LUNG ELASTASE ACTIVITY WAS INCREASED IN *FRANCISELLA* INFECTED ANIMALS

Neutrophil elastase is capable of digesting the underlying elastin structure of the alveolar walls (Janoff, 1985). Normally, neutrophil elastase-mediated damage is suppressed by α_1 -antitrypsin (Carrell, 1986). Since α_1 -antitrypsin was significantly reduced upon *Francisella* infection, we examined elastase activity residing at the primary infection site, i.e., lungs, 3-days after intranasal *F. tularensis* LVS and *F. tularensis* (type A) SCHU S4 challenge. Consistent with decreased levels of plasma and lung lavage α_1 -antitrypsin, a 1.9-fold increase in respiratory lavage fluid elastase activity was observed following *F. tularensis* challenge (**Figure 3**).

LUNG TISSUE ELASTANCE WAS REDUCED IN *FRANCISELLA* INFECTED ANIMALS

Examination of lung function following *F. tularensis* LVS infection indicated lung tissue elastance to be reduced 24% with a concomitant 24% increase in the tissue hysteresivity ratio compared to the mock (PBS-treated) control (**Figure 4**). Assessment of lung function following *F. tularensis* (type A) SCHU S4 challenge could not be performed under required BSL-3 containment conditions.

DISCUSSION

Serpins compose the largest super family of peptidase inhibitors and are well known as regulators of hemostasis and thrombolysis. Acute phase proteins are generally viewed as being relevant in providing protective functions at sites of tissue injury such as containment of tissue degradation, facilitation of wound healing, and

restoration of homeostasis. Alpha-1 antitrypsin, an acute phase protein has been shown to increase in humans following bacterial and viral infection (Bini et al., 1996; Zhang et al., 2010) using both *in vivo* and *in vitro* models (Pineiro et al., 2004). However, to the best of our knowledge, the reduction of the major extracellular serpine, i.e., α_1 -antitrypsin in plasma has not been described.

Comparison of two dimensional gel plasma protein profiles of mock (PBS-treated) control and infected animals indicate a significant reduction of plasma α_1 -antitrypsin following pulmonary challenge with *F. tularensis* LVS and *F. tularensis* (type A) SCHU S4. Haptoglobin also an acute phase protein was observed to increase markedly in intensity (β -chains) following infection supporting

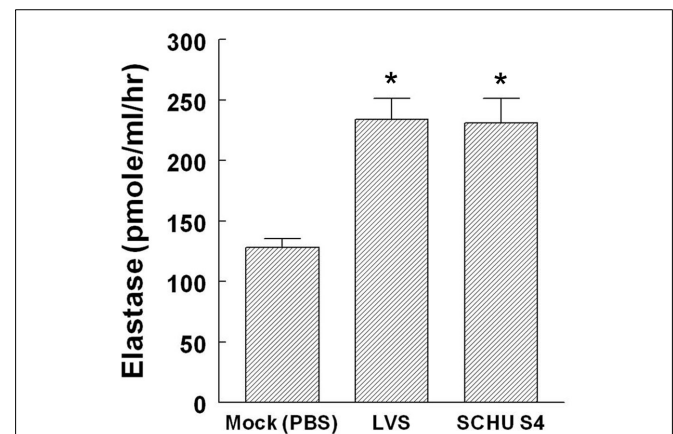


FIGURE 3 | Increased elastase activity in respiratory fluids following pulmonary *F. tularensis* challenge. Bronchoalveolar lavage fluid from mice (5-day post challenge, $n = 5$ /group) from each group, i.e., Mock (PBS-treated) control, *F. tularensis* LVS, and *F. tularensis* (type A) SCHU S4 infected animals was prepared and assayed for elastase activity in triplicate as described under Section "Materials and Methods" ** = $p < 0.05$.

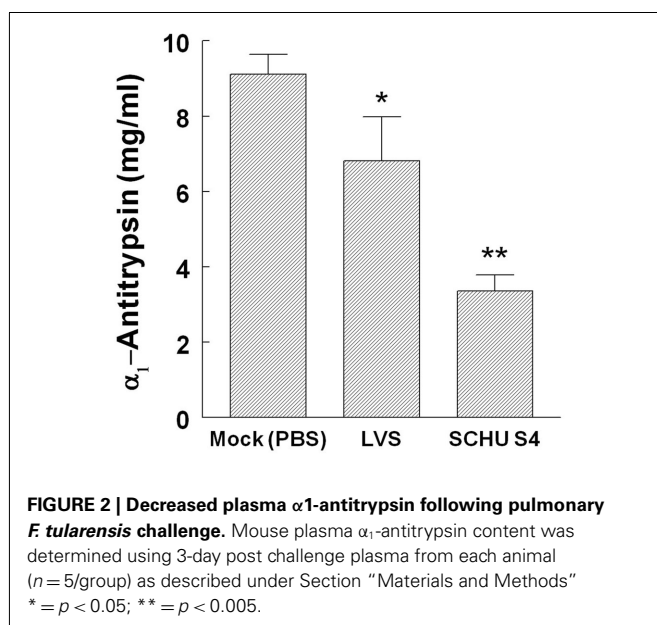


FIGURE 2 | Decreased plasma α_1 -antitrypsin following pulmonary *F. tularensis* challenge. Mouse plasma α_1 -antitrypsin content was determined using 3-day post challenge plasma from each animal ($n = 5$ /group) as described under Section "Materials and Methods" * = $p < 0.05$; ** = $p < 0.005$.

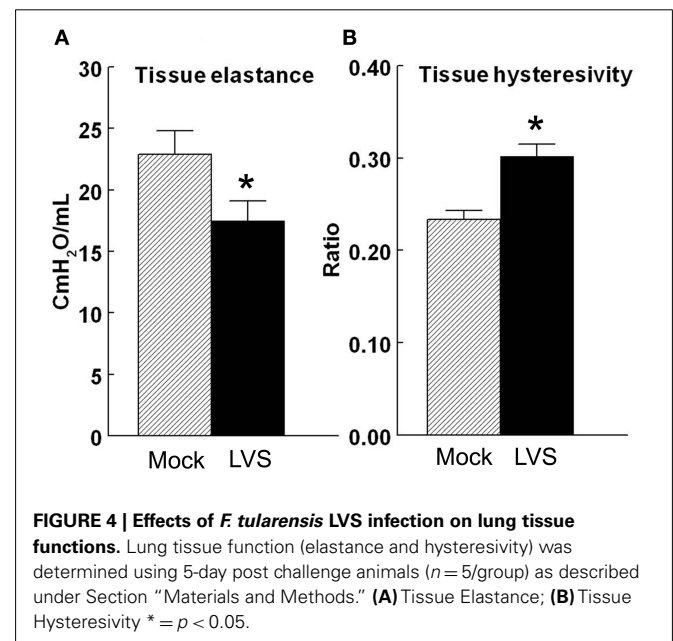


FIGURE 4 | Effects of *F. tularensis* LVS infection on lung tissue functions. Lung tissue function (elastance and hysteresivity) was determined using 5-day post challenge animals ($n = 5$ /group) as described under Section "Materials and Methods." (A) Tissue Elastance; (B) Tissue Hysteresivity * = $p < 0.05$.

the competent nature of the acute phase response in infected animals. Haptoglobin's hepatic expression is strongly induced by inflammatory mediators such as cytokines which are known to be critical for immune-regulation, increasing within 24 h the haptoglobin plasma concentration from as low as $<50 \mu\text{g/ml}$ to $\sim 2.5 \text{ mg/ml}$ (Huntoon et al., 2008). Quantitative ELISA analyses corroborated reduced levels of plasma α_1 -antitrypsin although the percent decrease observed for *F. tularensis* LVS and *F. tularensis* (type A) SCHU S4 infected animals was significantly different. In contrast to the ELISA data, the increase in lung lavage elastase activity for both *F. tularensis* LVS and *F. tularensis* (type A) SCHU S4 was the same, i.e., 1.9-fold following infection. The incongruity between plasma ELISA data and increased elastase enzyme activity of lung lavage fluid in all likelihood arises from partial proteolytic clipping/destruction of anti α_1 -antitrypsin specific epitopes in the respective plasmas which stoichiometrically is unrelated to the lung lavage elastase pool activity. Examination of respective *F. tularensis* LVS and *F. tularensis* (type A) SCHU S4 2D PAGE protein profiles indicate a generalized clearing of the plasma α_1 -antitrypsin consistent with extensive proteolytic degradation. Due to the lung lavage fluid elastase pool being much smaller and restricted in locale, i.e., non-circulating compared to that of plasma α_1 -antitrypsin, the fold increase in lung lavage fluid elastase activity would be expected to exhibit little variation. Additionally, the observed range of plasma α_1 -antitrypsin values for *F. tularensis* (type A) SCHU S4 treated animals is considerably smaller, i.e., tighter (mean value $\pm 0.4 \text{ mg/ml}$, $n = 5$) compared to that of plasma obtained from animals treated with *F. tularensis* LVS (mean $\pm 1.2 \text{ mg/ml}$, $n = 5$). The difference in plasma α_1 -antitrypsin content could arise from the more virulent nature of *F. tularensis* (type A) SCHU S4; however, this remains to be established. Consistent with the primary protective function of α_1 -antitrypsin, imbalance of the protease-antiprotease function at the alveolar level in the lungs of infected animals and concomitant increase in lung lavage fluid elastase activity was associated with decreased lung function, i.e., loss of lung elastance with concomitant increase of pulmonary hysteresivity (Stockley, 1983, 1987).

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