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Release of the antimicrobial LL37 peptide from DNA/F-actin bundles in CF sputum

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Running heads: LL37 peptide in CF sputum

Key words: Cystic fibrosis, LL37 peptide, rhDNase I, gelsolin, poly-Aspartate, LPS.

Abbreviations: BSA, bovine serum albumin; CFU, colony forming units; CF, cystic fibrosis; rhDNase I, recombinant human deoxyribonuclease I; hCAP-18, human cathelicidin; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide (endotoxin) from PA - *Pseudomonas aeruginosae*; poly-ASP, poly-Aspartic acid; RT, room temperature, LL37, LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES peptide; TLRs, Toll-like receptors.

Summary: Cationic antibacterial peptides (ABPs) are secreted in airways and function in the first line of defense against infectious agents. They attack multiple molecular targets to cooperatively penetrate and disrupt microbial surfaces and membrane barriers. Antibacterial properties of ABPs including cathelicidin LL37 are reduced in cystic fibrosis (CF) airways as a result of direct interaction with DNA and F-actin. Microscopic evaluation of a mixed solution of DNA and F-actin after addition of rhodamine B-labeled LL37 peptide reveals the presence of a bundle structure similar to that present in CF sputum. Analysis of CF sputum after centrifugation shows that LL37 is mostly bound to components of the pellet fraction containing DNA, F-actin and cell remnants. Factors that dissolve actin/DNA bundles and fluidize CF sputum such as Dornase alfa (rhDNase I), gelsolin, poly-aspartate or their combinations increase the amount of LL37 peptide detected in the supernatant of CF sputum. The presence of bacterial endotoxin (LPS) in CF sputum and the ability of LPS to inhibit the antibacterial activity of LL37 suggest that inactivation of LL37 function in CF sputum partially results from its interaction with LPS. LL37-LPS interaction was prevented by a lipopolysaccharide-binding protein (LBP)-derived peptide known for its ability to neutralize LPS, whereas LBPW91A a mutant peptide that lacks ability to bind LPS had no effect. A combination of factors that dissolve DNA/F-actin aggregates together with LPS-binding agents may represent a potential for the treatment of chronic infections that occur in CF airways.

Cathelicidin LL37, beta-defensin, lactoferrin and lysozyme expressed in the epithelium of the human lung and secreted to the airway surface fluid (ASF), are key elements in the responses of respiratory airways to the presence of bacteria (1, 2). In cystic fibrosis, ASF antibacterial activity was found to be substantially reduced (3), partially as a result of high ionic strength (3-5) and direct interaction of antimicrobial peptides with DNA and F-actin (6, 7). Defects in the biogenesis of airway secretions secondary to CF transmembrane conductance regulator (CFTR) mutation may also contribute to the compromised antimicrobial activity of CF airways (4). Despite loss of antibacterial function, CF epithelia express bactericidal molecules, and the concentrations of known antibacterial factors in ASF of CF subjects are not reduced when compared to ASF from normal epithelia (3, 8). Overexpression of LL37/hCAP-18 after pulmonary gene transfection in mice was found to inhibit the bacterial load and inflammatory response following pulmonary *Pseudomonas aeruginosa* challenge, whereas systematic gene transfer was shown to protect against endotoxemia, indicating interaction of LL37/hCAP-18 with LPS (9). Recently, it was found that an increased concentration of LL37 peptide in bronchoalveolar fluid correlates with higher severity of CF lung disease (8), but this increase is likely to result from inflammatory reaction. Besides promoting chronic colonization with *Pseudomonas aeruginosa*, inactivation of antimicrobial peptides in CF sputum may also influence specific inflammatory processes such as neutrophil activation and LPS-mediated cytokine production (8).

In addition to altered ionic and hydration states, sputum samples from patients with CF contain large amounts of DNA (10) and filamentous actin (11) that contribute

significantly to sputum viscosity. Both F-actin and DNA are anionic polyelectrolytes, with surface charges sufficiently high that a significant concentration of otherwise soluble cations is sequestered near the surface of those polymers. One result of polyvalent counterion condensation that overcomes the strong electrostatic repulsions between the filaments is the lateral association of such filaments into bundles. Antibacterial peptides including cathelicidin LL37 form heterogeneous bundles of DNA and F-actin (6) which dissolve after addition of rhDNase I, gelsolin or multivalent anions such as polymeric aspartate or glutamate (7, 12, 13). Treatment of CF sputum with gelsolin or poly-ASP lowers the elastic moduli of the samples and reduces the growth of bacteria, suggesting activation of endogenous antibacterial factors (6, 7)**.** Loss of antibacterial peptide function represents one of several changes that underlie the molecular basis for chronic lung infection associated with CF (14). The ability of *Pseudomonas aeruginosa* to produce high levels of alginate, to grow as a biofilm in CF lung, and to express LPS molecules lacking O antigen-side chains, with lipid A alterations that increase bacterial resistance to antibacterial peptides (15) also contribute to the complex phenomena of CF lung inflammation.

Anionic polymers of DNA and F-actin present in CF sputa in addition to cationic antibacterial peptides may also bind and mask neutrophil protease and chemokines such as IL-8. Reduction of CF sputum viscosity by rhDNase I or gelsolin *in vitro* was demonstrated to increase the proportion of free IL-8 and the IL-8-dependent neutrophil chemotactic activity of sputum supernatants (16), but in an earlier study the increase in CF sputum protease activity after rhDNase I treatment was found to be a transient event

and the trend over the 6 months was a reduction in both neutrophil elastase and IL-8 concentration (17).

Previous experiments showing that different cationic antibacterial molecules present in CF sputum form bundled aggregates with DNA and F-actin (6) suggest that depolymerization of those bundles may release antibacterial agents trapped in their interior. In this study, for the first time, we demonstrate that factors that depolymerize DNA and F-actin can liberate LL37 peptide from bundled structures in CF sputum and increase bactericidal activity. Additionally we found that the ability of LPS present in CF sputum (18) to inactivate the antibacterial function of the LL37 peptide may explain why the function of antibacterial components present in CF sputum was only partially restored after treatment with rhDNase I, gelsolin or poly-ASP (6, 7). The finding that the ability of LPS to inactivate LL37 activity may be prevented by lipopolysaccharide-binding protein (LBP)-derived peptides indicates that a combination of factors that dissolve DNA/F-actin aggregates with LPS-binding agents may be more effective than either treatment alone to prevent or reverse the chronic infection taking place in CF airways.

Materials and methods:

Materials. Tryptic Soy Broth and Pseudomonas Isolation Agar were acquired from DIFCO (Kansas City, MO). Poly-aspartic acid, LPS (*Pseudomonas aeruginosa;* serotype 10; LPS from PA), and human placental DNA were purchased from Sigma (St Louis, MO). Dornase alfa (recombinant human deoxyribonuclease I – rhDNase I; Pulmozyme) was from Genentech Inc. (South San Francisco, CA), LL37 and rhodamine B-labeled

LL37 peptides were from Bachem (King of Prussia, PA). Human lipopolysaccharide binding protein (LBP) peptide, amino acids 86-99, which binds LPS in solution and inhibits LPS-induced cell responses as well as LPS interactions with protein (19, 20) , was purchased from HyCult Biotechnology (Canton, MA). LBPW91A mutant peptide in which tryptophan 91 was substituted by alanine, causing lack of LPS binding (20) was obtained from GenScript (Piscataway, NJ).

Bundles of DNA and F-actin. G-actin was prepared from rabbit skeletal muscles as previously described (21) and polymerized to obtain F-actin. Heterologous bundles of these polymers were formed after mixing DNA (2 mg/ml) with F-actin (0.2 mg/ml) and rhodamine B-labeled LL37 peptide (10 μM)) after a 10 minute incubation at RT.

Sputum samples. CF sputum samples were collected by spontaneous expectoration from patients attending the University of Pennsylvania Health System Adult Cystic Fibrosis Center at Presbyterian Hospital (IRB 803255). The samples were diluted in buffer containing 140 mM NaCl, 10 mM TRIS, 0.2 mM $CaCl_2$ (pH 7.4) or PBS, vortexed and treated for 1 hour with rhDNase I (5 - 30 μg/ml), gelsolin (0.5 - 2 μM), poly-Asp (50 μM) or their combination. They were then centrifuged (10 min, 15500 x g) to pellet the remnant cells, bacteria and bundle structures. Samples used for experiments presented in Figures 2 and 4 were obtained from independent collections.

Optical microscopy. Bundles of DNA and F-actin and samples of CF sputum were viewed using a Leica microscope (Bannockbum, IL) using a 40x objective. Images were acquired using a Cool SNAP(HQ) camera (Trenton, NJ). F-actin was labeled with rhodamine (TRITC) phalloidin (Sigma, St Louis, MO) and DNA was labeled with YOYO-1 (Molecular Probes, Boulder CO).

Immunohistochemical studies. Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded CF sputum sections and rat lung (data not shown; this condition was used as a positive control for antibody reactivity) using a rabbit anti-hCAP-18/LL37 antibody that cross-reacts specifically with human and rat cathelicidin (H-075-06, used at 1:100 dilution; Phoenix Pharamceuticals Inc, Belmont CA). Paraffin-embedded materials were sectioned at 5 μm thickness and floated on distilled water at 45^{o}C. Sections were mounted on slides and placed in the oven at 57^{o}C overnight. The sections were deparaffinized according to standard procedures and quenched with 0.9% hydrogen peroxide in methanol for 30 minutes. The sections were incubated with primary antibody at 37^{o}C for 60 minutes, washed with 1% PBSA (1% BSA in PBS), and subjected to binding with secondary antibody (biotinylated goat anti-Rabbit IgG, 1:400 dilution). Amplification was performed with a Vectastain ABC kit and a horse-radish peroxidase (HRP) detection system was used to colocalize peroxidase activity with a 3,3'-diainobenzidine (DAB) substrate. The sections were counterstained with hematoxylin.

Immunoblot analysis. Samples of CF sputum were diluted 1:1 with 0.9% NaCl, mixed by vortexing with rhDNase I, poly-ASP, gelsolin or a combination of rhDNase I + gelsolin or rhDNase I + poly-ASP, and centrifuged. A whole dilution of CF sputum, or supernatant was added to the gel sample buffer, boiled for 10 min and subjected to electrophoresis on a 10% SDS-PAGE or 16.5% Tris-Tricine SDS-PAGE peptide analysis gel from Bio-Rad (Philadelphia, PA) for LBP protein and LL37 peptide analyses, respectively. After electrophoresis, proteins were transferred to nitrocellulose membranes (Immobilon-NC, Millipor) that were blocked by incubation in 5% (w/v) non-fat dry milk

dissolved in TTBS (150 mM NaCl, 50 mM TRIS, 0.05% Tween 20, pH 7.4). After transfer to the membrane, proteins were probed for 1 h with a polyclonal anti-LBP or monoclonal anti-LL37/CAP18 antibody (clone 1-1C12, 1:250 dilution, HyCult Biotechnology Canton, MA) in TTBS. HRP-conjugated secondary antibodies were used at a 1:20000 dilution in TTBS. Immunoblots were developed with Kodak BioMax MR film using an HRP-targeted chemiluminescent substrate. The relative amount of LL37 peptide and LBP protein in each lane was determined by gel densitometry, followed by image analysis with ImageQuant software.

LPS extraction and detection. LPS extraction from CF sputum and *Pseudomonas aeruginosa* culture (positive control) was performed using an LPS extraction kit (17144) from Intron Biotechnology (Boca Scientific, Boca Raton FL) according to the manufacturer's instructions. Qualitative and quantitative analyses were made using a QCL-1000 Chromogenic end-point LAL assay (50-647U) from Cambrex (Walkersville, MD).

Binding of LL37 and LBP peptides to LPS. To evaluate if the LBP peptide can compete with LL37 to bind LPS we performed a binding assay based on previously describe techniques (20). Flat-bottomed multi-wells polystyrene plates were coated with LPS (2 nmol/well; LPS from PA) by incubation in 0.2% trichloroacetic acid at 37^{o}C for 15 h. Plates were then washed with PBS/0.1% Tween 20, and the rhodamine B–labeled LL37 peptide (2.55 nmol/well) was added, alone or with LPS (2 nmol), LBP peptide (2.5 nmol) or LBPW91A peptide (2.5 nmol), After 1H of incubation, plates were washed and

the binding of rhodamine B-LL37 peptide was detected fluorimetrically (I_{ex} = 544 / I_{em}= 590) using a multiple plate reader (Fluoroskan Ascent FL, Labsystems Inc, MA).

Antimicrobial activity. The bactericidal activity of LL37 peptide was measured as previously described (22). Kanamycin-resistant *Pseudomonas aeruginosa* (PAO1) were grown to mid-log phase at 37^{o}C, resuspended in PBS (140 mM NaCl, 7.5 mM Na_2HPO_4, pH 7.4) and brought to 10^8 CFU/ml (assuming that an optical density at 620 nm of 0.35 corresponds to 10^8 CFU/ml). They were then diluted 10 times in 100 μl of PBS containing LL37, LBP peptide, LL37 + LPS, or the combination of LPS + LBP peptide + LL37. Similarly, tubes containing a dilution of CF sputum samples (1:20) were treated with rhDNase I or rhDNase I + LBP peptide (5 μM). After one hour of incubation at 37^{o}C, the suspensions were placed on ice and diluted 10- to 1000- fold in PBS and 10 μl aliquots were spotted on Pseudomonas Isolation Agar plates for overnight culture at 37^{o}C. The number of colonies in the duplicate samples at each dilution was counted the following morning, and the number of colony forming units/ml (CFU/ml) of the individual mixture was determined from the dilution factor.

Results

Visualization of DNA and F-actin in CF sputum. Individual or mixed bundles of DNA and F-actin form when these anionic polyelectrolytes are mixed with cationic antimicrobial peptides (6) or histones (7). In this study, we observed that bundles formed after mixing DNA and F-actin with rhodamine B-labeled LL37 peptide (Figure 1). Addition of fluorescent phalloidin or YOYO-1 to control samples of F-actin and DNA

alone results in uniform, low-intensity labeling without any detectable structures, when phase contrast observation was performed (upper row on panel A, Figure 1). When rhodamine-labeled LL37 was added to F-actin and DNA, bundles formed that were positive for fluorescence of both the filaments and LL37, indicating an ability of the LL37 peptide to induce the formation of these structures (middle and bottom row of Figure 1A). As shown in Figure 1B, similar bundles containing DNA and F-actin are found in the complex environment of whole CF sputum.

Immunohistochemical probing of CF sputum sections with anti-LL37/hCAP18 antibody. Microscopic images of CF sputum after immunohistochemical evaluation with anti-LL37/hCAP-18 antibody are shown in figure 1C. The presence of DAB-positive, elongated structures (indicated with arrows) positively labeled with hematoxylin, which labels DNA (upper panel), indicates the presence of the LL37 peptide and/or its parent protein, in bundles of CF sputum. The high intensity of the DAB signal suggests that LL37 and hCAP18 are present in CF sputum at high concentration.

Immunoblotting analysis of LL37 peptide in CF sputum. Panel A of figure 2 shows results from immunoblotting analysis of six different CF sputum samples with an anti-LL37/hCAP18 antibody. The two upper rows compare representative immunoblots of samples that were obtained from CF patients who were (S1) or were not (S2) receiving Pulmozyme treatment before CF sputum collection. The analysis revealed that LL37 peptide is mostly bound to the contents of CF pellets (compare lane 1 representing the total amount of LL37 peptide in the whole volume of CF sputum, versus lane 2

representing the amount of LL37 peptide in the supernatant of a corresponding volume). Densitometry analysis of the blots of 6 patient sputum samples showed that the LL37 peptide present in the CF sputum supernatant fraction accounts for between 5.4 to 35% of its total concentration. Besides detectable heterogeneity among the analyzed samples regarding LL37 concentration, we found that treatment of CF sputum with rhDNase I (lane 3), gelsolin (lane 4), poly-ASP (lane 5) or a combination of rhDNase I with gelsolin (lane 6) or with poly-ASP (lane 7) increases the amount of LL37 in the supernatant fraction to various extents (9-73%). In all samples, the combination of rhDNase I with gelsolin or poly-ASP was found to be more effective in increasing the LL37 peptide concentration in the supernatant fraction than any individual treatment. This observation is in agreement with the previously reported ability of gelsolin (13) and poly-ASP (7) to increase rhDNase I activity in addition to their effects on CF sputum rheological properties. The release of LL37 was specific since the concentration of soluble LBP, which does not bind actin or DNA did not follow the pattern of changes in LL37 concentration (Figure 2B). The release of antibacterial peptides from DNA/F-actin bundles was found to have a significant but incomplete effect on CF sputum bacterial load (6) and motivated a search for other CF components that may interfere with bactericidal activity.

Bacterial endotoxin and LBP protein are present in CF sputum. Samples of CF sputum obtained from five patients testing positive for chronic infection with *Pseudomonas aeruginosa* (four currently receiving Pulmozyme) were analyzed for the presence of LPS and LBP. Both total volume and supernatant (obtained after centrifugation) of CF sputum

(1:10 dilution in endotoxin free H_20) were subjected to LPS extraction and quantitative analysis with an LAL assay or electrophoresis followed by immunoblotting analysis with an anti-LBP antibody. This study revealed that the average concentration of LPS in the total volume of CF sputum was 40.9 ± 19.1 EU/ml (~ 0.02 μM), and ~ 50% of this amount was identified in the sputum supernatant fraction (19.0 ± 4.9 EU/ml). Because of the unknown and probably low efficiency of LPS extraction from CF sputum, and unknown dilution of CF sputum caused by expectoration, these values must be interpreted qualitatively, as documenting relative amounts of LPS rather than absolute concentrations, which may be significantly higher.

A centrifugation force applied to the CF sputum supernatant fraction (10 min at 13000 RPM) normally causes sedimentation of intact bacteria. Therefore, LPS in the supernatant represents an LPS fraction already released from the bacterial wall. As shown in panel B of figure 2, beside LL37 peptide, an acute-phase reactant lipopolysaccharide binding protein (LBP), which also binds bacterial LPS, is present in CF sputum mostly in its supernatant fraction. This finding suggests that in the supernatant of CF sputum, LPS may link with the LL37 peptide**,** antibodies to LPS (23) and LPB protein, one of the critical molecules regulating the airway response to LPS (24, 25). Even if the average, free concentration of LPS in CF sputum is low, its distribution in the whole volume may differ, rising specifically in regions of bacterial growth, which are the principle targets for antibacterial peptides. The presence of LBP in CF sputum suggests that LPS in CF airways may be presented to TLRs (26) and stimulate the local production of inflammatory cytokines, especially by type II pneumocytes (27).

Inactivation of LL37 by LPS is reversed by LBP. Evaluation of LL37 peptide interaction with LPS-coated plates in the presence of LPS, LBP and LBPW91A revealed that binding of rhodamine B-LL37 peptide (2.55 nmol) to LPS (2 nmol) coated plates decreased in the presence of LPS (2 nmol) and LPB peptide (2.5 nmol) by 52 ± 8% and 39 ± 12% respectively, whereas a control LBPW91A peptide (2.5 nmol) had no effect on LL37 binding to the LPS-coated surface.

One consequence of the interaction of LPS with LL37 is the inhibition of its antibacterial activity. LPS alone had no effect on *Pseudomonas aeruginosa* (PAO1) bacteria when evaluated using a bacteria killing assay (Figure 3). LL37 at a concentration ~1 μM kills almost all bacteria (Figure 3A), but the addition of LPS (0.5 - 5 μM) restored bacterial growth to the original level. These data confirm the functional interaction of LL37 with LPS. LPB peptide prevented LPS from inhibiting the antibacterial function of LL37. When the LL37 peptide (1 μM) was treated with LPS (0.5 μM) in the presence of LBP peptide (1 μM), its antibacterial activity was restored (Figure 3B). At the concentration used in this assay, LBP peptide alone had no significant effect on PAO1 bacteria growth. The specificity of the LBP peptide's ability to prevent inactivation of LL37 by LPS was confirmed by the lack of effect of an LPBW91A mutant peptide that lacks LPS binding.

Bacterial load of CF sputa after treatment with a combination of Pulmozyme and LBP peptide. LBP peptide alone appear to have no significant effect on CF sputum bacterial outgrowth, but CFU from 4 of 6 analyzed samples was reduced after rhDNase I treatment (Figure 4). Combination of rhDNase I with 5 μM LBP peptide had more distinct effect

(10-35 % decrease of CFU in sputum samples). This result suggests that antibacterial peptides released from CF sputum bundles are partially inhibited by LPS, and LPS effect can be prevented by LPS neutralizing molecules such as LBP peptide.

Discussion

Airway inflammation is recognized as a major factor in the pathogenesis of cystic fibrosis lung disease. While there appears to be no immune deficiency in patients with CF, the defect in cystic fibrosis transmembrane conductance regulation increases the susceptibility of the lungs to endobronchial infections by bacteria (28-30). One of the mechanisms proposed to explain this susceptibility to pulmonary infection points to possibly reduced antibacterial properties in the layer of fluid that lines the airways (1, 3). Previous attempts to increase the bactericidal activity of cystic fibrosis ASF were focused on airway epithelial cell transfection with hCAP18 to increase local production of LL37 (9), engineered exogenous antibacterial sequences that display high activity against *Pseudomonas aeruginosa* (31), peptide mimetic molecules (32) or salt-insensitive antimicrobial peptides (33). Limiting the inflammatory response in the CF lung may also be effective in slowing the course of the disease. The mechanisms by which the inflammatory response occurs in the CF lung have also been considered as new therapeutic targets (34). The lack of antibacterial activity of cystic fibrosis ASF coexisting with a normal or higher (8) concentration of antibacterial peptides indicates local inactivation of antimicrobial factors in CF sputum. Both DNA (10) and F-actin (11) present in CF sputum are anionic polyelectrolytes with negative surface charges sufficiently high that positively charged antibacterial peptides, which are otherwise

soluble, may be sequestered at the surface of the polymers and lead to their aggregation into bundles. Interactions of DNA and F-actin with antibacterial peptides contribute to the altered viscoelastic properties of sputum and result in inactivation of various antimicrobial factors (6, 7). Strategies to recover endogenous antibacterial function of peptides present in CF sputum may have advantages over expression or introduction of exogenous antimicrobial factors as therapeutic approaches to treat CF lung infection. Modulation of the CF environment to overcome its ability to inhibit bactericidal activity will be necessary also for effective use of exogenous antibacterial peptides as well as for optimal function of antibacterial factors overexpressed after gene therapy.

Based on previous observations that agents which dissolve CF sputa bundles had limited effect on CF bacterial load (7) we attempted to identify other factors that inactivate bactericidal activity in CF airways and the mechanisms through which they act. We found that factors that depolymerize CF bundles of DNA and F-actin simultaneously release cationic antibacterial molecules. This was shown by an increase in LL37 peptide concentration in the supernatant fraction of CF sputum (Figure 2). Based on the ability of LPS to inactivate LL37's antibacterial function (35), we propose that LPS released from the bacterial wall, which is observed in CF sputum, also contributes to the loss of bactericidal activity of CF ASF. The ability of LPS to inactivate LL37 activity can be prevented by an LBP-derived peptide, known for its ability to modulate LPS cellular effects. This finding suggests that a combination of factors that dissolve DNA/F-actin aggregates with LPS binding agents may represent a potential for the treatment of chronic infection in CF airways.

The inhibition of LL37 peptide by LPS reported here, and prevention of this effect by the LPB peptide is likely to be only one aspect of the LBP peptide modulatory effect on LL37-LPS interactions. CAP18/LL37-LPS complexes may also activate cell receptors, as has been shown for the soluble form of the LPS CD14 receptor that can either inhibit or enhance cellular response to LPS (36). LBP can also either enhance or inhibit the cellular response to LPS, depending in part on the presence of other LPS-binding molecules to which LBP may facilitate transfer (37). The finding that LL37 may competes with LBP for binding LPS suggests that at inflammatory sites in the respiratory system (38) and elsewhere, the delivery of LPS to cellular receptors, lipoprotein, or other targets, mediated by LBP would be perturbed when CAP18/LL37 levels change. The dual role of LBP and CD14 in innate immunity has been recently established (39), and the potential that CAP18/LL37 may also participate in the interactions of these proteins with LPS (40) deserves future investigation.

Acknowledgments

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Figures:

Figure 1. Presence of DNA and F-actin in mixed bundles formed after the addition of rhodamine B-labeled LL37 peptide (A) and in CF sputum (B) was visualized by Alexa-Fluor phalloidin labeling for F-actin (upper and middle row) and YOYO-1 labeling for DNA (upper and bottom row), respectively. The morphology of corresponding samples is shown by phase contrast microscopy (left column). Data shown are representative of three experiments. Immunohistochemical staining of CF sputum with anti-LL37/hCAP-18 antibody (C). Data shown are representative of two experiments.

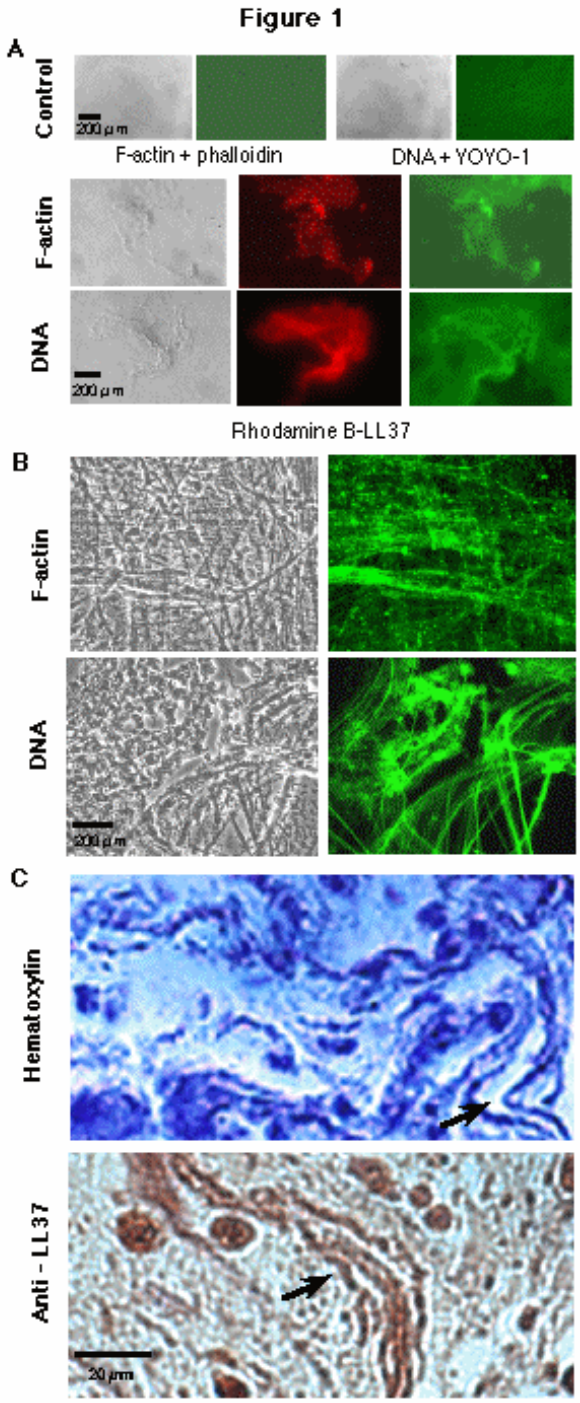


Figure 2. Presence of LL37 (A) and LBP (B) in CF sputum detected using

immunoblotting analysis with monoclonal antibodies to human LL37/CAP-18 and human LBP protein, respectively. Upper row represents analysis of the individual samples (S1- and S2 obtained respectively, from patient treated or nor with rhDNase I before sample collection). Lanes: 1- total amount of LL37 (A) or LBP (B) in the samples of CF sputa, lanes 2-7 represent the amount of LL37 (A) or LBP (B) detected in equal volumes of supernatant without treatment, after the addition of rhDNase I (30 μg/ml), gelsolin (2μM), poly-ASP (50 μM), rhDNase I (30 μg/ml) + gelsolin (2μM) or rhDNase I (30 μg/ml) + poly-ASP (50 μM), respectively. Quantitative immunoblot analysis represents the concentration of LL37 or LBP in samples as % of total concentration in the whole volume of individual CF sputa. Each data point represents the mean of 6 and 5 different samples for LL37 and LBP respectively. Differences between mean data in this experiment were evaluated by Student's *t*-test, with $p<0.05$ being taken as the level of significance ($^{a}p<0.05$ vs. the respective value in supernatant; statistically significant).

Figure 2

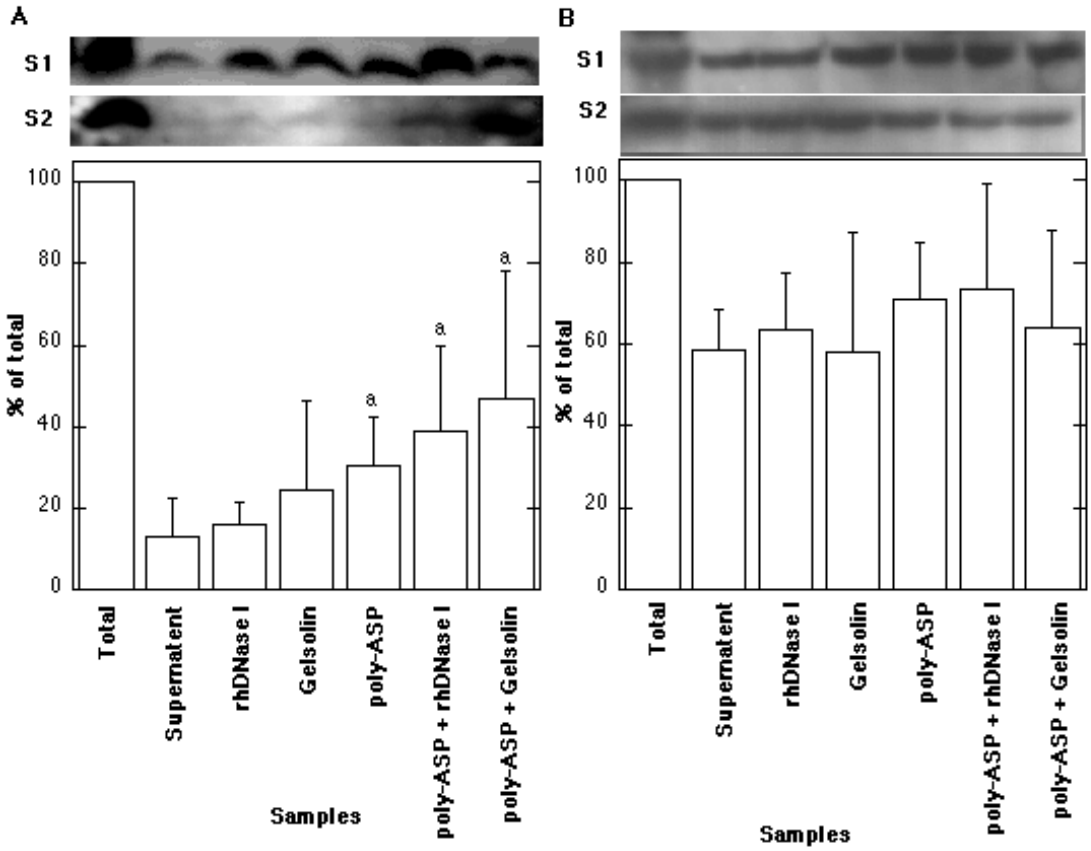


Figure 3. Viability of *Pseudomonas aeruginosa* after a 1 hour incubation with varying

concentrations of LL37 peptide (circles) or a combination of LL37 (1 μM) + LPS (0.1-5 μM) (triangles) The effect of peptides was calculated by taking the number of CFU of no treatment sample (growth control) as a 100%. (A). The ability of LBP peptide to prevent LPS-mediated inhibition of LL37 bactericidal activity (B). In this experiment, viability of *Pseudomonas aeruginosa* was evaluated in the presence of LL37 peptide, LPB peptide and LBW91A peptide (1 μM of each) or their combination in the presence of LPS from PA (0.5 μM) as indicated in sample descriptions. Each data point represents the mean of 4 experiments. Differences between mean data in this experiment were evaluated by Student's *t*-test, with $p<0.05$ being taken as the level of significance (^{d} $p<0.001$ vs. the respective value; statistically significant).

Figure 3

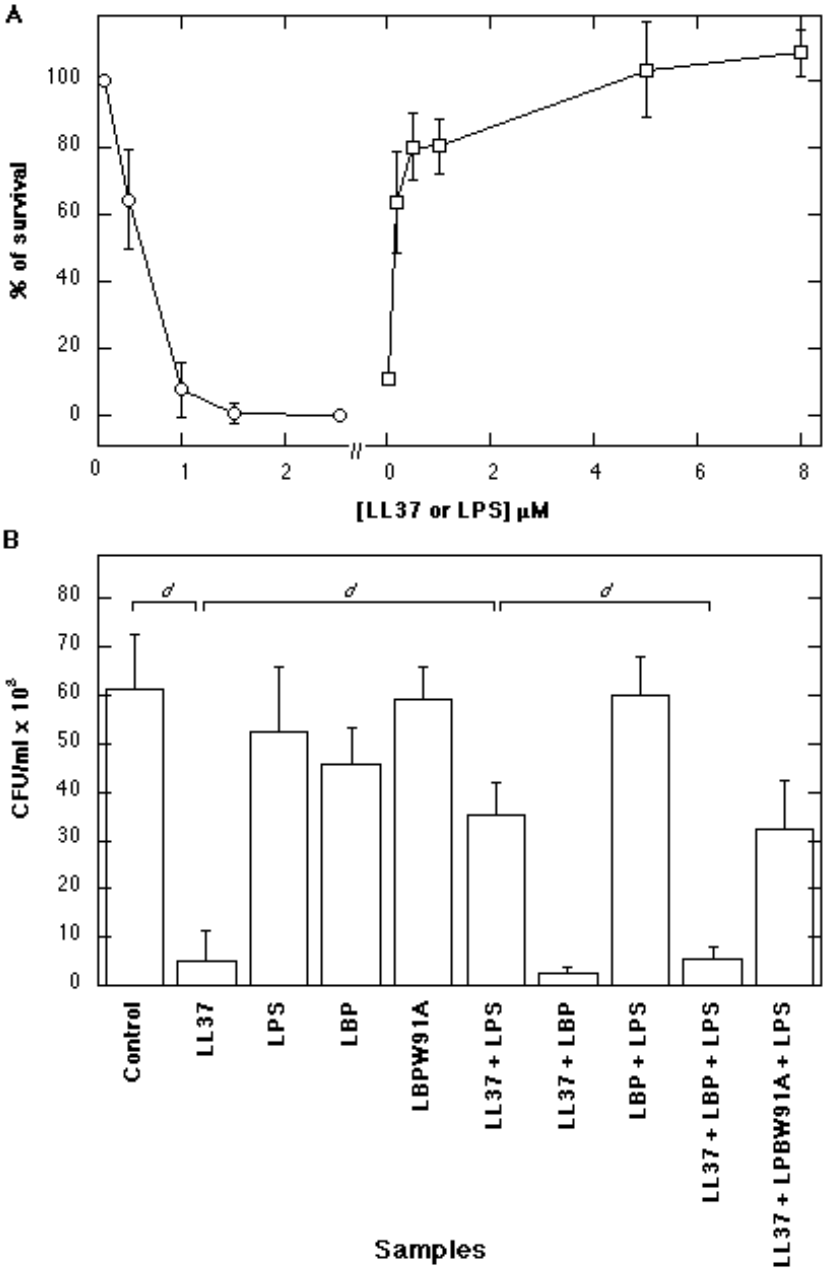
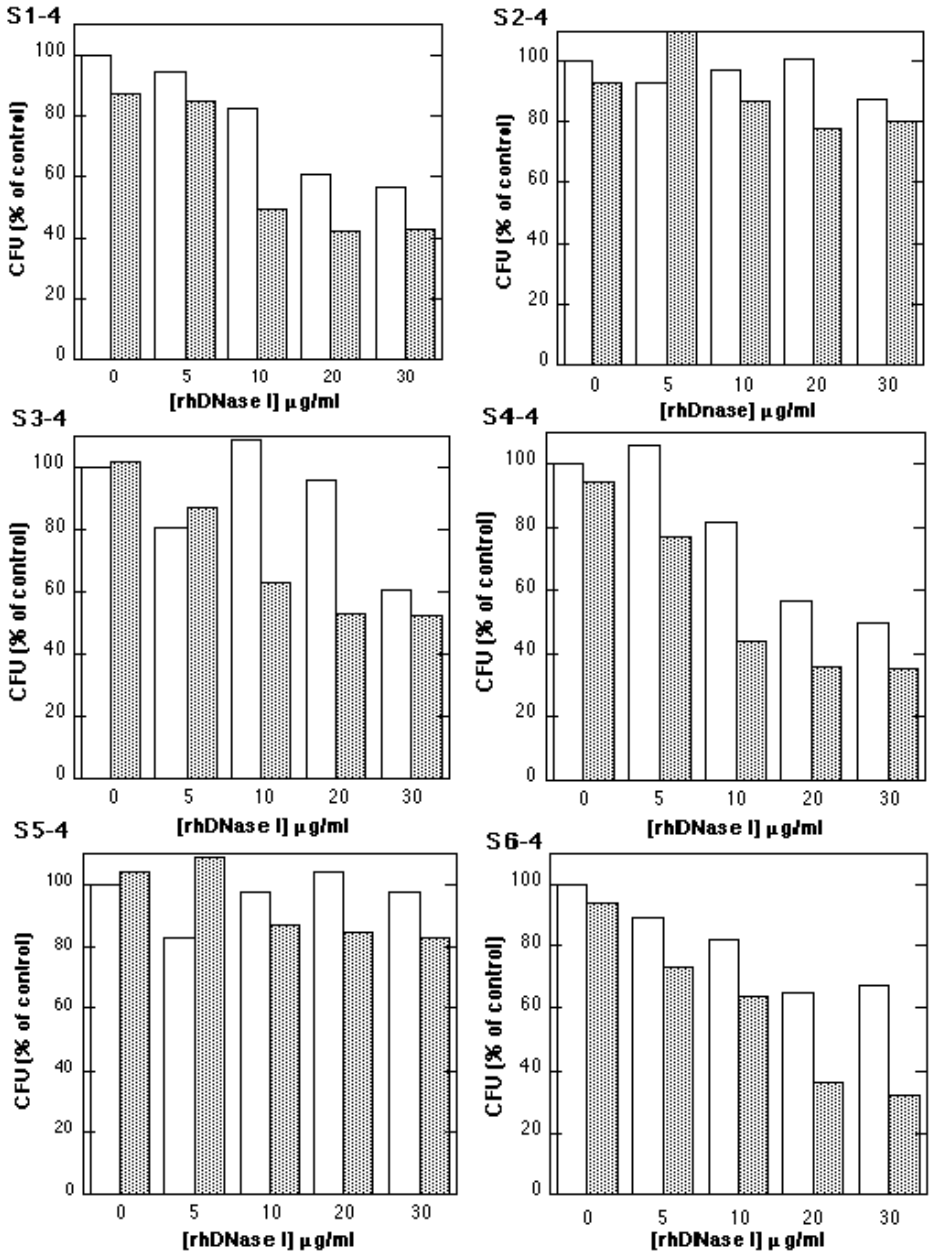


Figure 4. Bacterial load of CF sputa (six different samples, S1-4 - S6-4), after treatment

with rhDNase I (empty column) or a combination of rhDNase I with 5 μM LBP peptide (dark column). Data were normalized by taking the number of CFU of untreated samples (growth control) as 100%.

Figure 4



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