pH modulates the activity and synergism of the airway surface liquid antimicrobials β-defensin-3 and LL-37

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The pulmonary airways are continuously exposed to bacteria. As a first line of defense against infection, the airway surface liquid (ASL) contains a complex mixture of antimicrobial factors that kill inhaled and aspirated bacteria. The composition of ASL is critical for antimicrobial effectiveness. For example, in cystic fibrosis an abnormally acidic ASL inhibits antimicrobial activity. Here, we tested the effect of pH on the activity of an ASL defensin, human β-defensin-3 (hBD-3), and the cathelicidin-related peptide, LL-37. We found that reducing pH from 8.0 to 6.8 reduced the ability of both peptides to kill Staphylococcus aureus. An acidic pH also attenuated LL-37 killing of Pseudomonas aeruginosa. In addition, we discovered synergism between hBD-3 and LL-37 in killing S. aureus. LL-37 and lysozyme were also synergistically important. Importantly, an acidic pH reduced the synergistic effects of combinations of ASL antibacterials. These results indicate that an acidic pH reduces the activity of individual ASL antimicrobials, impairs synergism between them, and thus may disrupt an important airway host defense mechanism.

Many organisms defend against infection by producing antimicrobials at the interface with the environment (1–4). In the mammalian respiratory system, the interface between the environment and the organism is a thin layer of airway surface liquid (ASL). To maintain sterile lungs and protect against bombardment with bacteria, mammalian lungs have evolved multiple protective mechanisms, one of which is antimicrobials in ASL that rapidly kill inhaled and aspirated bacteria (5–9).

ASL antimicrobial activity arises from a complex mixture of antimicrobials, proteins, and lipids that vary in size, structure, and abundance (10, 11). Many share cationic and hydrophobic features that render them amphipathic (2, 12–17). Two effectors of innate host defense are the defensins, which have β-sheet structures and three disulfide bonds, and the cathelicidins, which have linear α-helical structures (2, 3, 15, 18). Human β-defensin-3 (hBD-3) (19, 20) and the cathelicidin LL-37 (21–23) have broad antimicrobial spectrums, including activity against Staphylococcus aureus and Pseudomonas aeruginosa. At neutral pH, they are cationic and kill bacteria by disrupting the phospholipid membrane and dissipating the electrochemical gradient (4). Both hBD-3 and LL-37 retain activity under physiological ionic strength, and they both rapidly kill bacteria (19–23).

Several previous genetic studies highlighted the potential role of defensins as antimicrobials (24). Some suggested that polymorphisms in the defensin gene cluster modulate the phenotype of cystic fibrosis (CF) airway disease, including an association with chronic P. aeruginosa infection (25–27). In addition, a genetic variant in the promoter region of a defensin gene cluster was associated with reduced hBD-1 and hBD-3 transcript levels and a higher risk of persistent S. aureus nasal colonization in a cohort of healthy individuals (28). However, two reports did not identify an association, perhaps because the populations studied had different genetic backgrounds (29, 30). Polymorphisms in defensin genes are also associated with infections outside the lung (31–33). Also consistent with an important role in defense is the observation that airway insults and disease can increase defensin gene expression (34–36).

We recently obtained additional evidence for the contribution of ASL antimicrobials to host defense. Studying a porcine model of cystic fibrosis (CF) (37, 38), we found that loss of cystic fibrosis transmembrane conductance regulator (CFTR) anion channels reduced HCO₃⁻ secretion and decreased ASL pH (39, 40), results consistent with earlier findings in human airway epithelia (41–44). Importantly, the abnormally acidic pH partially inhibited bacterial killing by ASL (40). Those findings identified a host defense defect that may predispose CF airways to bacterial infection. We also reported that an acidic pH inhibited two ASL antimicrobials, lysozyme and lactoferrin (40). However, those experiments were performed with a low ionic strength that is optimal for antimicrobial activity. Thus, although lysozyme and lactoferrin may contribute to the inhibitory effect of an acidic ASL pH, other antimicrobial factors may make a greater contribution.

With this background, we tested the hypothesis that at a more physiological ionic strength, a reduced pH would inhibit the activity of hBD-3 and LL-37. A previous study showed the effect of a low ionic strength on synergism between antimicrobials (45). Therefore, here we also tested the hypothesis that hBD-3 and LL-37 exhibit synergism in killing bacteria and that a reduced pH would inhibit synergism.

**Results**

hBD-3 and LL-37 killing of S. aureus is Concentration-Dependent and Relatively Insensitive to Ionic Strength. We investigated antimicrobial activity using S. aureus because it is one of the first bacteria to

Significance

Although lungs are continuously bombarded by bacteria, pulmonary defense mechanisms normally keep them sterile. Those defenses include a complex mixture of antimicrobial peptides in the thin layer of liquid coating the airway surface. In cystic fibrosis, impaired bicarbonate secretion causes the airway surface liquid to become abnormally acidic. Here we found that an acidic pH impairs the ability of two key airway antimicrobial peptides, β-defensin-3 and LL-37, to kill bacteria. When these peptides were combined, they exhibited synergistic killing of Staphylococcus aureus, an organism that infects cystic fibrosis lungs. However, an acidic pH reduced their synergistic effect. Thus, an acidic pH impairs an important respiratory defense mechanism and may predispose the lungs of people with cystic fibrosis to bacterial infection.
tested the effect of pH on hBD-3 killing of S. aureus, we measured the reduction in luminescence with increasing hBD-3 concentrations at three different pH values. At pH 8.0, ~0.2 μg/mL hBD-3 reduced luminescence intensity by 50% (IC50) (Fig. 3B). At pH 7.4, the IC50 for hBD-3 was ~2.9 μg/mL. As pH was reduced to 6.8, the potency of hBD-3 fell further. The relative potency of hBD-3 against S. aureus at pH 6.8, 7.4, and 8.0 was ~1:5:7.5.

We also tested the hypothesis that a reduced pH would inhibit LL-37 killing of S. aureus. Fig. 3C shows that reducing the pH from 8.0 to 6.8 decreased the speed and extent of killing. However, in comparison with hBD-3, LL-37 activity was affected to a lesser extent as pH fell from 8.0 to 7.4 and 6.8 (Fig. 3C and D).

An Acidic pH Has Little Effect on hBD-3 Activity Against P. aeruginosa. Although reducing the pH decreased the anti-staphylococcal activity of both hBD-3 and LL-37, individual antimicrobials can have distinct abilities to kill various bacteria. Therefore, we tested the effect of these two antimicrobial peptides on P. aeruginosa, an important pathogen as CF airway disease progresses (47, 50). LL-37 was much more potent at killing P. aeruginosa than S. aureus (1 μg/mL in Fig. 4A vs. 100 μg/mL for S. aureus in Fig. 3C). As with S. aureus, an acidic pH attenuated LL-37 killing of P. aeruginosa (Fig. 4A). hBD-3 was less potent at killing P. aeruginosa than S. aureus (1 and 5 μg/mL in Fig. 4B and C vs. 1 μg/mL in Fig. 3D). However, in contrast to S. aureus, an acidic pH had minimal effects on hBD-3 anti-pseudomonal activity (Fig. 4B and C).

An Acidic pH Attenuates the Synergistic Activity of hBD-3, LL-37, and Lysozyme Against S. aureus. Although overlapping activities of multiple different ASL antimicrobials could defend the airways, synergism between antimicrobials could further enhance killing. We previously showed that a low ionic strength induced synergism between lysozyme, lactoferrin, and secretory leukocyte peptidase inhibitor in killing Escherichia coli (45). Therefore, we tested for synergistic interactions between antimicrobials at a higher ionic strength and examined the effect of pH.

We asked whether pH affected the speed of killing by hBD-3 and LL-37 when they were combined. Compared with hBD-3 and LL-37 used alone at pH 8.0, the combination of half the concentrations of hBD-3 and LL-37 reduced S. aureus luminescence more rapidly (Fig. 5A–C). At pH 7.4, the difference in speed of killing between the combination and the individual peptides was more marked, but at pH 6.8 the time-dependent advantage of the combination was minimal.
Our results suggest that hBD-3 and LL-37 have antibacterial activity as individual peptides and have an even greater effect through their synergistic interactions. Likewise, agents that show little antibacterial activity at relatively physiological ionic strength might contribute to host defense when combined with other antimicrobials. An example is lysozyme, which has a relatively high ASL concentration (250–500 µg/mL), but which our data and earlier work (8) show has minimal anti-S. aureus activity as ionic strength increases toward physiological values. Nevertheless, here we found that lysozyme had no synergistic interactions with hBD-3 and LL-37 against S. aureus even at pH 7.4. 

Thus, peptides and proteins that only kill bacteria at concentrations greater than those in ASL might still make important synergistic contributions to antibacterial defense. We also examined a triple combination of LL-37, hBD-3, and lysozyme. At pH 8.0, there was a strong synergistic interaction (Fig. 4C). However, as pH fell, the synergistic interactions were attenuated (Fig. 4B and C). We also tested this triple combination with higher concentrations of each factor and found that reducing pH attenuated synergism, suggesting that inhibition does not depend on the maximal concentrations of each factor (Fig. S1).

**Discussion**

Our results suggest that hBD-3 and LL-37 have antibacterial activity as individual peptides and have an even greater effect through their synergistic interactions. The data also indicate that a reduced pH inhibits their individual and synergistic actions and could therefore impair airway defense.

The concentrations of hBD-3 and LL-37 that were effective against S. aureus (~2 µg/mL for hBD-3 and ~100 µg/mL for LL-37) and P. aeruginosa (~5 µg/mL for hBD-3 and ~1 µg/mL for LL-37) are in the range of their expected ASL concentrations (0.5–5 µg/mL for hBD-3 and 25–100 µg/mL for LL-37) (10, 19, 53–56). However, our results suggest that synergistic interactions may be even more important than activity of individual agents. We also varied the ratio of two antimicrobial peptides/proteins, measured S. aureus luminescence, and plotted luminescence as a function of the fraction of each peptide in the mixture. A concave-down curve indicates synergism, a straight line indicates additivity, and a concave-up curve indicates antagonism (51, 52). Fig. 6A shows that LL-37 and hBD-3 had a synergistic effect on S. aureus killing at pH 8.0 and 7.4 that was eliminated at pH 6.8. We observed similar behavior with the combination of LL-37 and lysozyme (Fig. 6B). Although lysozyme and hBD-3 did not exhibit synergistic interactions, their combined effect was additive in killing S. aureus at pH 8.0 and 7.4. At pH 6.8, lysozyme and hBD-3 showed an antagonistic interaction (Fig. 6C).

We also examined a triple combination of LL-37, hBD-3, and lysozyme. At pH 8.0, there was a strong synergistic interaction (Fig. 7A). However, as pH fell, the synergistic interactions were attenuated (Fig. 7B and C). We also tested this triple combination with higher concentrations of each factor and found that reducing pH attenuated synergism, suggesting that inhibition does not depend on the maximal concentrations of each factor (Fig. S1).

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synergistic effects when combined with LL-37 or with LL-37 plus hBD-3 at high ionic strength.

The reported absolute values of ASL pH depend on several factors, including the method of assay; whether it is measured in vivo, ex vivo, in submucosal gland secretions, or in cultured epithelia; whether it is assayed in newborns or inflamed airways; and perhaps the host species (40, 42–44, 57, 58). There is also considerable within-study variation. The reported range of pH in non-CF is from ~6.8 to 7.8. However, and importantly, pH of CF ASL is consistently reported as lower than pH of non-CF ASL. Thus, the pH values that we tested are in the reported ranges for ASL.

The antimicrobial activity of cationic amphipathic peptides such as hBD-3 and LL-37 depends on electrostatic interactions between cationic domains of the peptide and the negatively charged head groups of phospholipids in the bacterial plasma membrane. The cationic peptides insert into and disrupt the bacterial plasma membrane (59, 60). Insight into how a more alkaline pH might increase activity comes from NMR experiments with synthetic, histidine-rich peptides and lipid bilayers (61, 62). Those studies indicate that the peptide assumes a position parallel to the lipid bilayer, weakening the membrane before insertion and then inserting and disrupting the membrane. At alkaline pH, the peptide carries a lower positive charge and thus inserts into the membrane more quickly. ASL cationic peptides may interact with the bacterial membrane in a similar fashion with corresponding effects of alkaline pH. Synergistic effects may occur when one peptide in a conformation parallel to the bacterial membrane facilitates insertion of other peptides (63). We speculate that such an effect might account for synergistic activity between hBD-3 and LL-37 as well as synergism for the combination of LL-37 and lysozyme; in addition to its enzymatic activity, lysozyme has nonenzymatic antibacterial activity dependent on its cationic and hydrophobic domains that disrupt bacterial plasma membranes (64–66). A possibility that we cannot exclude is that variations in pH might render S. aureus and P. aeruginosa more or less susceptible to antimicrobial factors. In addition, it is possible that pH might modulate the inhibitory effect of hBD-3 on S. aureus cell-wall biosynthesis. Previous studies found that the bactericidal activity of several defensins involves perturbation of cell-wall biosynthesis by binding to lipid II (67–71).

Our study has several advantages: we studied more than one ASL peptide/protein, the antimicrobials were used at concentrations similar to those found in ASL, we used pH values in the physiological range, the buffers had a high ionic strength, and S. aureus and P. aeruginosa have relevance to CF lung disease. Our study also has limitations. Bacterial inocula in vivo will usually be smaller than what we used, and thus we may have underestimated the potency of antimicrobials; those would have been missing from our preparations. ASL antimicrobials may bind to mucin or other factors that alter their antibacterial activity. ASL contains more peptide, protein, and lipid antimicrobials than we studied, and thus our preparation does not reproduce the complex mixture of individual factors and their synergism.

In cultured human and porcine airway epithelia, in vitro studies of airways, and in vivo studies in newborn piglets and neonatal humans, CF ASL is abnormally acidic (40, 42–44), consistent with the loss of CFTR-dependent HCO₃⁻ transport (39, 41). However, in older children and adults with CF, the effect of genotype in ASL pH is more variable (44, 58, 74). Whether inflammation, remodeling, infection, and/or age are factors that might circumvent the loss of CFTR anion channels and increase ASL pH in CF

![Fig. 6](image-url) Effect of pH on antimicrobial activity of combination of lysozyme, hBD-3, and LL-37. (A) S. aureus (Xen-29) luminescence (in RLU) with varying ratios of two antimicrobial peptides. The x-axis shows a ratio of antimicrobial peptides; from left to right there is an increasing ratio of one antimicrobial and a decreasing ratio of the other. (A) LL-37 and hBD-3 in combination (8 min incubation). (B) LL-37 and lysozyme in combination (30-min incubation). (C) Lysozyme and hBD-3 in combination (30-min incubation). Maximal combination (1.0) was 1 μg/mL for hBD-3, 100 μg/mL for LL-37, and 1 mg/mL for lysozyme. Data are mean ± SEM; some error bars are hidden by symbols. Results are from a single experiment in triplicate. Each experiment was repeated at least three times with similar results.

![Fig. 7](image-url) Ternary surface plot of the effect of pH antimicrobial activity on the combination of lysozyme, hBD-3, and LL-37. Data are S. aureus (Xen-29) luminescence (vertical axis, in RLU). The surface plot color and curvature correspond to changes in relative luminescence. Red and concave indicate less luminescence and low bacteria viability; blue and flat indicate more luminescence and high bacteria viability. Relative luminescence with each antimicrobial factor alone is indicated at top of each vertical axis in blue font. (Horizontal triangular axis indicates ratio of antimicrobial factors in triple combination). Maximal concentrations (1.0 on the axes) were 1 μg/mL for hBD-3, 100 μg/mL for LL-37, and 1 mg/mL for lysozyme. Data are shown at different times for each pH. (A) pH 8.0 after 2-min incubation. (B) pH 7.4 after 8-min incubation. (C) pH 6.8 after 30-min incubation. Results are an average of three experiments, each done in triplicate. Similar results were obtained with higher concentrations of antimicrobials (Fig. S1).
is unknown. However, we speculate that changes in ASL pH might contribute, in part, to variations in the species of bacteria that infect CF airways. For example, *S. aureus* is commonly cultured early and *P. aeruginosa* is cultured later in the course of CF airway disease in humans and pigs (46, 47, 75). Interestingly, a reduced pH impaired hBD-3 killing of *S. aureus*, but had minor effects on killing of *P. aeruginosa*. Although a reduced ASL pH might be important for initiating *S. aureus* infection at the onset of CF lung disease, the variety of ASL antimicrobials, their varied properties, and interactions between them leave much uncertainty.

In addition to CF, ASL pH may be abnormally reduced in asthma (76), chronic obstructive pulmonary disease (77), and acute respiratory distress syndrome (78). Acidic conditions can also be found at sites of infection outside airways, including in peritoneal fluid, pleural fluid, cerebral spinal fluid, and abscesses. In addition to inhibiting antimicrobial peptides and proteins, an acidic pH can reduce the efficacy of pharmaceutical antibiotics (79, 80). These observations suggest that an acidic pH might impair the effectiveness of antimicrobial defenses and antibiotics under several conditions.

Thus, perhaps raising pH might aid prevention and treatment of bacterial infection at multiple sites in addition to CF airways.

### Materials and Methods

**Materials and Bacteria.** ASL antimicrobial factors were included recombinant human lysozyme (Sigma-Aldrich), iron-unsaturated human milk lactoferrin (Sigma-Aldrich), recombinant human β-defensin-2 hBD-2 (Peprotech), recombinant human β-defensin-3 hBD-3 (Peprotech), and human LL-37 (Anaspec). Lysozyme and lactoferrin were dissolved in double-deionized water. Cationic peptides were dissolved in acidified water (0.01% acetic acid) that contained 0.1% BSA.

We used *S. aureus* Xen-29 (Caliper LifeSciences Bioware) and *P. aeruginosa* PA103. *S. aureus* Xen-29 was derived from *S. aureus* 2650, a pleural fluid isolate, which is also designated as NCTC8322. *S. aureus* Xen-29 possesses a stable copy of the modified *Photorhabdus luminescens luxABCDE* operon on a single genetic site on the bacterial chromosome. For maintenance of luminescence, the bacteria were grown in TSB in the presence of kanamycin (10 μg/ml). We also used *P. aeruginosa*, modified to express the lux operon on a pUCP19lux plasmid and grown in Tryptic Soy Broth in the presence of carbenicillin (40 μg/ml) to maintain selection.

Antimicrobial peptide activity was tested in a buffer composed of TSB [casein peptone 17 g/L, soya peptone 3 g/L, NaCl 5 g/L, K₃HPO₄ 2.5 g/L, glucose 2.5 g/L; 1% for *S. aureus* and 5% (v/voll) for *P. aeruginosa*] and supplemented with 10 mM potassium phosphate buffer with pH adjusted by varying the ratio of monobasic to dibasic phosphate. The ionic strength of this solution is calculated at 25 mM (8). Where indicated, 100 mM NaCl was added to the final buffer to achieve an ionic strength of 125 mM.

**Luminescence Antibacterial Assay.** Bacteria were grown overnight at 37 °C in medium described above, diluted 1:100, and grown to exponential phase. Bacteria were harvested by centrifugation and suspended in the 1% or 5% TSB buffer at the indicated pH and ionic strength. Bacteria (*S* × 10⁵ cfu) were incubated with antimicrobial factors in a 96-well plates (Optitray; Packard Instruments) in a total volume of 120 μL. Luminescence was measured with a luminometer (Spectra Max L, Molecular Devices) and reported as relative light units (RLU). A previous study determined that reductions in luminescence have an excellent correlation with a decrease in cfu (8). All experiments included control bacteria that did not receive antimicrobials but were incubated in buffer of identical ionic strength and pH. Data are shown as relative luminescence as a percentage of control (RLU percentage of control). Exposure of bacteria to the various pH values did not cause bacterial death (Fig. 52).

**Testing Combinations of Antimicrobials.** Experiments testing combinations of antimicrobials are often done using a checkerboard assay, and interactions are analyzed using isobolograms (81). In this study, we followed a more general approach (51, 52, 82) for several reasons. First, some factors (lysozyme) were completely inhibited at high ionic strength. Therefore, a full dose–response and IC₅₀ calculations to design a checkerboard experiment are not possible. Second, preliminary studies showed that these antimicrobial factors kill bacteria at different rates. Third, we wanted to study more than two factors in combination and the effect of pH on the combination (83).

### Statistical Analyses

For dose–response curves in Figs. 2B and 3B, we used a Hill equation. For time-kill curves in Fig. 3A, we used a one-phase decay equation. For ternary surface plots in Fig. 7, we used tmaplot (a MATLAB plugin by Carl Sandrock).

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