Synergistic and additive killing by antimicrobial factors found in human airway surface liquid

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Received 21 February 2000; accepted in final form 11 May 2000

Singh, Pradeep K., Brian F. Tack, Paul B. McCray, Jr., and Michael J. Welsh. Synergistic and additive killing by antimicrobial factors found in human airway surface liquid. Am J Physiol Lung Cell Mol Physiol 279: L799–L805, 2000.—Airway surface liquid contains multiple factors thought to provide a first line of defense against bacteria deposited in the airways. Although the antimicrobial action of individual factors has been studied, less is known about how they work in combination. We examined the combined action of six antimicrobial peptides found in airway surface liquid. The paired combinations of lysozyme-lactoferrin, lysozyme-secretory leukocyte protease inhibitor (SLPI), and lactoferrin-SLPI were synergistic. The triple combination of lysozyme, lactoferrin, and SLPI showed even greater synergy. Other combinations involving the human β-defensins, LL-37, and tobramycin (often administered to cystic fibrosis patients by inhalation) were additive. Because the airway surface liquid salt concentration may be elevated in cystic fibrosis patients, we examined the effect of salt on the synergistic combinations. As the ionic strength increased, synergistic interactions were lost. Our data suggest that the antibacterial potency of airway surface liquid may be significantly increased by synergistic and additive interactions between antimicrobial factors. These results also suggest that increased salt concentrations that may exist in cystic fibrosis could inhibit airway defenses by diminishing these synergistic interactions.

cystic fibrosis; innate immunity; lysozyme; β-defensins; lactoferrin

THE HUMAN AIRWAYS ARE PROTECTED against infection by both the adaptive and innate immune systems. Important components of innate defense are antimicrobial factors that provide a first line of defense against bacteria deposited in the airways. This liquid contains multiple antimicrobial factors, including lysozyme, lactoferrin, and secretory leukocyte protease inhibitor (SLPI), that are secreted by submucosal glands and surface epithelia as well as several small antimicrobial peptides known as β-defensins and cathelicidins (3, 4, 25, 29). Lysozyme and lactoferrin are the most abundant antimicrobial fac-

tors in the airway, present in the sputum at ~1 mg/ml (5, 10). SLPI has been measured in nasal secretions at 10–80 μg/ml (6, 18). Estimates of human β-defensin (HBD)-2 concentration in ASL vary from 0.01 to 10 μg/ml (6, 25), whereas LL-37 and HBD-1 (4, 6, 25) have been detected, but their concentrations are not established.

The antimicrobial shield provided by these factors has several important features. Antimicrobial factors are continually present in ASL and are thus able to kill bacteria as soon as they are deposited. Furthermore, these factors kill bacteria without inflammation and have activity against a broad range of microorganisms. Although controversy remains, some studies suggest that the antimicrobial activity of these factors is impaired by high salt concentrations that may exist in cystic fibrosis (CF) ASL (8, 26). This disruption of innate defense may contribute to the susceptibility of cystic fibrosis patients to bacteria that do not normally infect the lung and the persistent inflammation that eventually results in respiratory failure.

It is interesting that ASL has evolved to contain multiple antimicrobial factors. There are several potential reasons for this. One is that multiple factors might provide redundancy in maintaining the sterility of the lower respiratory tract, a requirement for survival. Multiple factors could also have evolved to increase the spectrum of antimicrobial activity. An additional possibility, which we addressed in this study, is that the antimicrobial potency of ASL is increased by the presence of multiple factors. This could occur if some factors interact additively, that is, combined activity equals the sum of individual activities; or synergistically, that is, combined activity is greater than the sum of individual activities. Increased potency produced by such interactions could make the ASL a more effective defense against bacteria and prevent the emergence of resistant organisms.

There are examples of other systems in which the activity of native antimicrobial factors is enhanced in combination. Lactoferrin and secretory IgA in human
breast milk kill enteric bacteria not affected by either agent alone (23). Two peptides found in rabbit neutrophil granules, bacterial permeability increasing factor and p15s, also act synergistically in killing extracellular pathogens (33). In addition, previous studies (3, 4) have suggested that lactoferrin and lysozyme may enhance the activity of HBD-2 and the cathelicidin LL-37.

The purpose of our studies was twofold. The first goal was to examine how combining ASL antimicrobial factors affects their potency. Although previous reports (3, 4, 6, 8, 18, 25, 29) have described the antimicrobial properties of several ASL factors, less is known about how they work in combination. We examined this question using two different methods developed to measure interactions between antibiotics: the checkerboard method and the time-kill method. The second goal was to examine the effect of increased NaCl concentrations on the combined antimicrobial activity of ASL factors. This issue relates to the pathogenesis of CF lung disease because several studies (2, 8, 13, 32, 34) have suggested that ASL salt concentrations are elevated in CF. However, other studies (12, 16, 20) have suggested that salt concentrations in normal and CF ASL may not differ. We examined six antimicrobial factors found in the ASL: lysozyme, lactoferrin, SLPI, HBD-1 and HBD-2, and the cathelicidin LL-37. Interestingly, these factors are present in combination not only in ASL but also in other mucosal secretions including tears, saliva, urine, and breast milk (11, 14, 19, 24, 30; McCray P, personal communication). Thus examining their combined action may increase our understanding of innate defenses at several mucosal surfaces that are routinely exposed to bacteria.

**METHODS**

**Antimicrobial agents, bacterial strains, and culture conditions.** Recombinant human lysozyme and lactoferrin purified from human milk were obtained from Sigma (St. Louis, MO). Recombinant human SLPI was obtained from R&D Systems (Minneapolis, MN). All were reconstituted in sterile H₂O. Recombinant HBD-1 and HBD-2 peptides were made with the use of an insect cell-baculovirus expression system as described previously (25, 30). The cathelicidin LL-37 was synthesized by the solid-phase method, with a model 433a peptide synthesizer (Applied Biosystems, Foster City, CA) and was purified by reverse-phase HPLC (Waters Delta Prep, Milford, MA). Peptide concentration was determined by quantitative amino acid analysis on a Beckman 6300 amino acid analyzer (Palo Alto, CA).

**Luminescence antibacterial assay.** Determining the nature of an antimicrobial interaction requires assessing the antibacterial effect of many different combinations of agents. Because some of the recombinant peptides were available in very small quantities, we required an assay of antimicrobial activity that was quantitative and utilized a sensitive indicator organism. For these reasons, we utilized a luminescence assay of bacterial viability that has been described previously (25). The bacteria used were *Escherichia coli* DH5α (GIBCO BRL, Life Technologies, Grand Island, NY) engineered to express the luminescence plasmid pCGLS1 (7). *E. coli* were grown in Luria-Bertani medium at 30°C with shaking. For maintenance of pCGLS1, ampicillin (100 μg/ml) was included in the growth medium.

Bacteria were grown to late log phase at 30°C, centrifuged, and resuspended in 10 mM KH₂PO₄ buffer, pH 7.2, with 1% Luria-Bertani medium (ionic strength, 25 mM). In some studies, the ionic strength was increased by the addition of NaCl as indicated. Bacteria (5 × 10⁶ colony-forming units (cfu)) were incubated with the antimicrobial agents in 96-well plates (Optiplate, Packard Instruments, Meriden, CT) in a total volume of 150 μl. After incubation at 30°C for 4 h, luminescence was measured with a luminometer (Anthos Labtech Wals, Salzburg, Austria or Optiplate, Packard Instruments) and was reported as relative light units. The concentration of antimicrobial factor that decreased luminescence to 50% of control values (EC₅₀) was used for calculations in the checkerboard assay (see Checkerboard assay of combined antimicrobial activity). In a previous study, Travis et al. (29) determined that reductions in luminescence have an excellent correlation with a decrease in colony-forming units.

**Checkerboard assay of combined antimicrobial activity.** In the checkerboard test (17), serial dilutions of two antimicrobial agents were mixed together in a microtiter plate so that each row (and column) contained a fixed amount of one agent and increasing amounts of the second agent. The concentrations of antimicrobials ranged from approximately twice the EC₅₀ to seven serial twofold dilutions below this amount. Each plate also contained a row and column in which a serial dilution of each agent was present alone. Bacteria (5 × 10⁶ cfu) were then added to each well, and bacterial luminescence was measured 4 h later. A dose-response curve was generated for each row and column, and the amounts of the two agents required in combination to produce the EC₅₀ was calculated.

For each combination experiment (composed of a row or column in the matrix), the fractional inhibitory concentration (FIC) of each agent was calculated. The FIC of a factor is the concentration that kills when used in combination with another agent divided by the concentration that has the same effect when used alone (9, 17). The FIC index for the combination of A and B is the sum of their individual FIC values. Each checkerboard test generates many different combinations, and, by convention, the FIC values of the most effective combination are used in calculating the FIC index. The FIC index defines the nature of the interaction. Antimicrobial factors with additive interactions have a FIC index of ~1; an FIC index < 1 defines synergistic interactions; combinations with an FIC index > 1 are antagonistic (9, 17). Furthermore, the more synergistic a combination, the lower the FIC index; the more antagonistic, the higher the FIC index.

The results of the checkerboard assay can be represented graphically by plotting the FIC values on a graph known as an isobologram (9, 17). On the x- and y-intercepts are plotted the EC₅₀ values of the two agents when used alone. The intervening points are formed by pairs of FIC values from different combination experiments generated by the checkerboard matrix. If the two agents have additive antimicrobial activity, the line connecting the x- and y-intercepts and the intervening points (the isobol) will be straight. If the two agents have synergistic antimicrobial activity, the FIC values of each agent will be lower, and the isobol will be concave. For factors that are antagonistic in combination, the isobol will be convex. Furthermore, the degree of concavity (or convexity) reflects the degree of synergy (or antagonism). An important advantage of the checkerboard assay is that one can determine the effect of different conditions on an antibiotic interaction by examining how the shape of the isobol (or the FIC index) changes. This is true because for any given set of conditions, the isobologram and the FIC index are always
normalized to the activity of the antimicrobials when used alone. Thus even when changes in conditions change the individual EC_{50} of an agent, the degree of synergy or antagonism between two agents can still be determined by comparison with the additive situation.

**Time-kill assay of combined antimicrobial activity.** The time-kill method (17) evaluates combined antimicrobial action by measuring the effect of a subinhibitory concentration of one agent on the killing ability of another over time. We combined lysozyme (at concentrations approximating the EC_{50}) with subinhibitory concentrations of other ASL factors (lactoferrin, SLPI, HBD-1, HBD-2, and LL-37). The concentration of lysozyme in ASL far exceeds that of most of the other peptides (4, 6, 25, 27, 29). Therefore, testing the combined effect of two agents in this manner may better reflect the situation in vivo. A synergistic interaction is considered to be present if the killing ability of lysozyme is increased by a subinhibitory concentration of the other factor. An antagonistic interaction is present if the antimicrobial effect of lysozyme is inhibited by the second agent. Because one agent is used at subinhibitory concentrations, this assay cannot distinguish additive interactions (combined activity equals the sum of individual activities) from indifferent interactions (combined activity equals that of the most active agent used alone).

To assess the activity of ASL factors in combination with lysozyme via the time-kill method, four time-kill curves, generated in parallel, were required. Bacteria were incubated with 1) lysozyme alone (at concentrations approximating the EC_{50}), 2) the second agent at subinhibitory concentrations (defined as one-half the concentration that produced no decrease in luminescence), 3) a combination of the two agents, and 4) buffer alone to determine the control level of luminescence. Luminescence was measured at 30 min and approximately every hour thereafter for 5 h. The data are plotted as a percentage of the control luminescence value.

**RESULTS**

**Evaluation of pairs of antimicrobials with the checkerboard assay.** The antimicrobial activities of human lysozyme, lactoferrin, SLPI, recombinant HBD-1 and HBD-2, and synthetic LL-37 were determined with the use of the luminescence assay. All agents killed bacteria in a dose-dependent manner; Table 1 shows the potencies expressed as the EC_{50}. EC_{50} for factors tested at higher salt concentrations are also shown.

Because synergy and antagonism are defined by their deviation from the additive situation, we first validated the checkerboard assay by testing two solutions containing the same agent. By definition, this interaction is additive. On the isobologram, additive interactions generate a straight line; synergistic interactions, a concave line; and antagonistic interactions, a convex line. Figure 1 shows that lysozyme combined with itself produced a straight line, indicating additivity. In similar tests, lactoferrin, SLPI, and tobramycin combined with themselves all showed additive interactions (data not shown).

Next, we evaluated pairs of ASL factors. Combinations of lysozyme with HBD-1, HBD-2, and LL-37 produced additive interactions (Fig. 1). In contrast, the combined action of lysozyme and lactoferrin, lysozyme and SLPI, and lactoferrin and SLPI were synergistic as reflected by the concavity of the isobol (Fig. 2). Interestingly, synergism was observed over the entire range of concentrations tested, although the highest degree of synergy was obtained when equally effective concentrations of the two agents were used together. This is evidenced when the nadir of the isobol occurs at approximately equal FICs for the two agents (at the midpoint of the isobol).

The combined effect of these factors can be represented mathematically via the FIC index (Table 2). As indicated by the isobolograms, the combinations of lysozyme-lactoferrin, lysozyme-SLPI, and lactoferrin-SLPI interacted synergistically (FIC index <1). The other pairs tested acted in an additive manner (FIC index ~1). None of the combinations tested showed antagonism.

**Evaluation of pair interactions with the use of time-kill assays.** We also evaluated antimicrobial combinations with the time-kill assay, which assesses different
aspects of combined activity than the checkerboard test. The checkerboard method evaluates interactions between factors over an equally effective range of concentrations, whereas the time-kill method assesses the combined effect of a subinhibitory concentration of one agent with an effective dose of another. This may better reflect the situation in ASL because lysozyme is present at a significantly higher concentration than SLPI, the β-defensins, and LL-37 (4, 6, 25, 27, 29). Furthermore, the time-kill test gives a dynamic picture of the combined effect. This allows one to determine if a combination kills bacteria at a different rate than agents used alone.

Using this method, we found that subinhibitory concentrations of lactoferrin, SLPI, and LL-37 were synergistic with lysozyme; a greater proportion of bacteria were killed and the rate of killing increased (Fig. 3). In contrast, HBD-1 or HBD-2 combined with lysozyme showed either additivity or indifference, results that cannot be distinguished by this test. The results of the checkerboard and time-kill assays agreed in all cases except for the combination of lysozyme and LL-37; the checkerboard method suggested an additive interaction, whereas the time-kill method suggested synergy. This difference may be accounted for by the different ratio of the factors used in the two tests.

### Table 2. FIC indexes of ASL antimicrobial factor combinations

<table>
<thead>
<tr>
<th>Combination</th>
<th>FIC Index</th>
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<tbody>
<tr>
<td>Lysozyme and lactoferrin</td>
<td>0.23 ± 0.02</td>
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<tr>
<td>Lysozyme and SLPI</td>
<td>0.31 ± 0.16</td>
</tr>
<tr>
<td>Lysozyme and HBD-1</td>
<td>1.06 ± 0.15</td>
</tr>
<tr>
<td>Lysozyme and HBD-2</td>
<td>1.07 ± 0.07</td>
</tr>
<tr>
<td>Lysozyme and LL-37</td>
<td>0.99 ± 0.03</td>
</tr>
<tr>
<td>Lactoferrin and SLPI</td>
<td>0.45 ± 0.08</td>
</tr>
<tr>
<td>HBD-1 and HBD-2</td>
<td>1.14 ± 0.09</td>
</tr>
<tr>
<td>HBD-2 and LL-37</td>
<td>1.16 ± 0.15</td>
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Values are means ± SD; n = 3 experiments. FIC, fractional inhibitory concentration.

Fig. 2. Isobolograms showing synergistic interactions between ASL antimicrobial factors tested in pairs. SLPI, secretory leukocyte protease inhibitor. See Fig. 1 legend for details.

Fig. 3. Antimicrobial action of ASL factor combinations as measured by the time-kill method. Each graph shows the effect of combining a subinhibitory concentration of a different factor with lysozyme at concentrations approximating the EC_{50}. L.U., relative light units. ▲, Antimicrobial activity of lysozyme alone; □, other indicated factor alone; ●, combined effect of lysozyme and the other indicated factor. Lysozyme in combination with lactoferrin, LL-37, and SLPI showed synergy, whereas lysozyme in combination with HBD-1 and HBD-2 showed either additive or indifferent interactions. Data are results of 1 experiment done in triplicate; each experiment was done at least twice with similar results.
Interaction between lysozyme and tobramycin. Because tobramycin is frequently administered to people with CF by the inhaled or intravenous route, we evaluated its combined action with lysozyme. The checkerboard assay showed an additive interaction (FIC index 1.04), and the time-kill assay indicated either an additive or indifferent interaction (Fig. 4).

Evaluation of three factors in combination. Because lysozyme, lactoferrin, and SLPI showed synergism when tested in pairs, we explored the possibility that they might have even greater synergy in triple combination. To assess this, we modified the checkerboard assay as described by Yu et al. (31). To reduce the number of dose-response curves needed, we took advantage of the fact that the greatest interaction occurred when similarly effective concentrations of agents were combined, i.e., combinations that produced points near the midpoint of the isobologram. The FIC index of the triple combination was 0.086, more than 2.5 times lower than the FIC index of the factors in pairwise combinations. Thus the EC$_{50}$ for killing could be achieved with a small fraction of the concentrations required individually or in pairs. For example, for the most efficacious combination tested, the dose of lysozyme could be reduced by $\frac{1}{15}$, lactoferrin by $\frac{1}{50}$, and SLPI by $\frac{1}{500}$ compared with the amounts of these individual factors required to achieve the same effect.

Effect of ionic strength on antimicrobial interactions. Increased ionic strength inhibits the antimicrobial activity of several factors (3, 25, 29). However, it is not known whether an increased ionic strength affects synergistic interactions. To test this, we performed checkerboard assays on the lysozyme-lactoferrin, lysozyme-SLPI, and lactoferrin-SLPI combinations at varying levels of NaCl. Although an increase in ionic strength increases the EC$_{50}$ for each individual agent, the checkerboard test corrects for this so that the shape of the isobol and the FIC index still define the combined effect. Figure 5 shows the effect of increasing the NaCl concentration on the synergistic interaction between lysozyme and lactoferrin. As the ionic strength increased, synergy was lost, and at an ionic strength of 65 mM, the combined effect of lysozyme and lactoferrin was, at best, additive. Table 3 shows the effect of ionic strength on the FIC index. The synergistic interactions involving SLPI were also salt sensitive. Because the individual antimicrobial effect of SLPI was very sensitive to ionic strength, we could not test its combined activity at ionic strengths $>45$ mM. These data suggest that where synergistic interactions occur, they are inhibited by increasing ionic strength.

DISCUSSION

The ASL makes complex and multifaceted contributions to the defense of the lung against inhaled and aspirated bacteria. It physically protects the airway epithelium, it promotes ciliary clearance, and mucus in ASL binds bacteria (28). ASL contains surfactant proteins that enhance phagocytosis of bacteria, immunoglobulins that bind bacteria, and lactoferrin that deprives bacteria of iron (21, 22). ASL also contains many substances that directly kill bacteria (6, 25, 29). In this

![Fig. 4. Combined action of lysozyme and tobramycin as assessed by the checkerboard (top) and time-kill (bottom) methods. $\text{L}$, Lysozyme alone; $\text{H}$, tobramycin alone; $\text{F}$, lysozyme and tobramycin combined. Experiments were repeated twice with similar results.](http://ajplung.physiology.org/)

![Fig. 5. Isobolograms showing the effect of ionic strength on the synergistic interaction between lysozyme and lactoferrin. Ionic strength was adjusted to indicated values by addition of NaCl. Results are from 1 experiment done in triplicate.](http://ajplung.physiology.org/)
study, we found that, when tested in combination, the antimicrobial activity of six ASL proteins increased, some combinations were additive, and others were synergistic. The triple combination of lysozyme, lactoferrin, and SLPI was very synergistic. None of the antimicrobial combinations tested interacted in an antagonistic manner.

The data also show that increasing the NaCl concentration inhibited synergistic interactions. This loss of synergism was independent of the inhibitory effects of salt on the individual factors. If our studies reflect the situation on the airway surface in vivo, the increased ASL NaCl concentration that may exist in CF (2, 8, 13, 32, 34) would impose a double hit on the antimicrobial activity of the ASL. Not only would salt inhibit the individual action of the factors, it would also attenuate their synergistic interaction. This double effect could amplify the impact that a relatively modest increase in salt levels has on ASL antimicrobial activity. [Note, however, that some studies (12, 16, 20) suggest that ASL NaCl concentration is not increased in CF.]

The methods we used to examine combined activity have several advantages. We examined activity over a broad range of concentrations. We used two complementary techniques to assess combined action and obtained similar results with both. In addition, the luminescence assay provides a reliable indicator of bactericidal activity, which may be the most physiologically relevant end point in terms of maintaining a sterile lower respiratory tract.

Our study has two main limitations. First, because of the large number of assays required to measure combined activity and the scarcity of the recombinant peptides, these studies were performed with a sensitive indicator organism, E. coli DH5α. Our studies establish the concept that ASL antimicrobial factors may have increased activity in combination. Additional studies with the CF pathogens Pseudomonas aeruginosa and Staphylococcus aureus, as well as with other organisms that do not commonly infect the lung, would also be of interest. Second, the study was done in vitro under specific assay conditions. There is uncertainty about several variables in vivo including the concentrations of ASL antimicrobials, the exact ASL salt concentration, and the relevant bacterial inoculum. Furthermore, there may be heterogeneity of all these conditions along the airway surface in vivo. Despite uncertainty about conditions at the site of action, in vitro testing can predict the action of antibiotic combinations in vivo. Clinical studies (1, 15) have shown that patients with serious infections who are treated with antibiotic combinations that are synergistic in vitro have better clinical outcomes than patients treated with combinations that are not.

The antimicrobial interactions that we observed could enhance the defense function of ASL in several ways. First, the overall potency of ASL could be significantly increased by additive and synergistic interactions between factors. If such interactions occur in vivo, a larger number or perhaps more resistant organisms could be killed. Empirical support for this idea comes from a recent study of nasal secretions by Cole et al. (6). They found that the antimicrobial activity of boiled human nasal secretions could not be reproduced by adding back the most abundant antibacterial substances, lysozyme and lactoferrin. This result suggests that the antimicrobial potency of nasal secretions may depend on interactions between multiple factors. Second, the combined actions that we observed suggest that a given antimicrobial effect may be achieved with lower concentrations of factors. Third, our studies that used the time-kill assay suggest that the rate of killing by ASL antimicrobial factors is increased in combination. This may be advantageous to the host because it might reduce the time available for bacteria to develop a more virulent phenotype, for example, that associated with biofilm formation. Finally, although not directly addressed by our study, the enhanced activity of combinations of antimicrobials might prevent emergence of strains resistant to endogenous antimicrobials.

The multiplicity of antimicrobial factors in ASL is remarkable. Because survival depends on maintaining sterile lower airways, the redundancy may have evolved as part of a fail-safe system that also provided an antimicrobial spectrum wider than that obtained with a single or a few factors. Our data suggest an additional advantage afforded by the evolution of numerous factors: multiple factors offer the opportunity for synergistic and additive interactions in killing bacteria.

We thank Dr. Hong Peng Jia for assistance with production of recombinant peptides, Dr. Barbarb-Ann D. Conway for the luminescence assay of bacterial killing, and Drs. E. P. Greenberg, Ronald N. Jones, Sue Travis, and Joseph Zabner for helpful discussions.

This work was supported by National Heart, Lung, and Blood Institute (NHLBI) Grant HL-61234. P. Singh was supported by a Cystic Fibrosis Foundation Leroy Mathews Award and NHLBI Grant K08-HL-04173-01. M. J. Welsh is an Investigator of the Howard Hughes Medical Institute.

Table 3. Effect of ionic strength on the FIC index of synergistic combinations

<table>
<thead>
<tr>
<th>Ionic Strength</th>
<th>25 mM</th>
<th>45 mM</th>
<th>55 mM</th>
<th>65 mM</th>
</tr>
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<tbody>
<tr>
<td>Lysozyme-lactoferrin</td>
<td>0.23(0.18–0.29)</td>
<td>0.41(0.36–0.46)</td>
<td>0.64(0.59–0.70)</td>
<td>1.17*(0.99–1.36)</td>
</tr>
<tr>
<td>Lysozyme-SLPI</td>
<td>0.31(0.24–0.38)</td>
<td>0.63*(0.58–0.69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactoferrin-SLPI</td>
<td>0.46(0.31–0.59)</td>
<td>0.76*(0.70–0.82)</td>
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</table>

Values are means; nos. in parentheses, range. We compared values at 25 mM ionic strength with the highest ionic strength tested. *P < 0.05.
REFERENCES


