GENETICS AND CELL BIOLOGY

Alcohol Use Disorders Affect Antimicrobial Proteins and Anti-pneumococcal Activity in Epithelial Lining Fluid Obtained via Bronchoalveolar Lavage

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Abstract — Aims: Our overall objective was to examine whether characteristics of epithelial lining fluid (ELF) from subjects with alcohol use disorders (AUDs) obtained via bronchoalveolar lavage (BAL) contribute to their predisposition to pneumococcal pneumonia. We sought to compare the anti-pneumococcal activity of acellular human BAL from subjects with AUDs to matched controls. Further, differences in BAL lysozyme activity and lactoferrin concentrations between these two groups were examined to determine the effect of AUDs on these antimicrobial proteins. Methods: BAL was performed in subjects with AUDs and matched controls. Acellular BAL was used at varying concentrations in an in vitro killing assay of Streptococcus pneumoniae, type 2, and the percent kill of organisms per microgram per milliliter total BAL protein was ascertained. Lysozyme activity and lactoferrin concentrations were measured in BAL from subjects and controls at measured concentrations of BAL protein. Results: AUD subjects (n = 15) and controls (n = 10) were enrolled in these investigations who were balanced in terms of smoking history. Using a mixed effect model, across the range of BAL protein concentrations, killing of pneumococcus tended to be less potent with BAL fluid from AUD subjects. Additionally, lysozyme activity and lactoferrin concentrations were significantly lower in the AUD group. Conclusions: The predisposition for pneumococcal pneumonia among those with AUDs may be in part mediated through effects of alcohol on substances within ELF that include antimicrobial proteins. Clarifying the composition and activity of ELF antimicrobial proteins in the setting of AUDs via investigations with human BAL fluid can help establish their contribution to the susceptibility for pulmonary infections in these individuals.

INTRODUCTION AND BACKGROUND

Severe community-acquired pneumonia (CAP) is the most common infectious disease leading to death in developed countries, and ranks as the eighth leading cause for overall death in the USA (Niederman, 2009; van der Poll and Opal, 2009). Despite medical progress in antibiotic development and better understanding of its pathogenesis, Streptococcus pneumoniae has remained the most commonly isolated pathogen in CAP for the past century (Watson et al., 1993). Moreover, although vaccines against the most common serotypes of S. pneumoniae are widely available and recommended for use among the elderly and immunocompromised individuals, there are sparse data supporting their efficacy in mitigating the morbidity and mortality associated with S. pneumoniae infections (Fine et al., 1996; Huss et al., 2009).

Alcohol use disorders (AUDs), including alcohol abuse and alcohol dependence, are independently associated with the development of pneumococcal pneumonia (DeRoux et al., 2006; Fernandez-Sola et al., 1995). This increased morbidity in those with AUDs may be related to an increased incidence of bacteria in the setting of pneumococcal pneumonia with attendant development of sepsis (Gentile et al., 2003), as well as the propensity to develop acute lung injury (ALI), a syndrome associated with the development of non-cardiogenic pulmonary edema and respiratory failure (Moss et al., 1996, 1999).

Alterations in pulmonary innate immunity among those with AUDs probably contribute to the underlying predisposition for these individuals to develop pneumococcal pneumonia. Several innate immune factors affected by alcohol that may predispose to pneumococcal pneumonia have been identified, primarily in animal models (Davis et al., 1991). These include a reduction in anti-pneumococcal surfactants (Lazic et al., 2007; Rubins et al., 1996; Sozo et al., 2009), defects in mucociliary clearance of pathogens (Sisson, 1995; Sisson et al., 1999; Wyatt et al., 2003), dysfunction of alveolar macrophages via impaired phagocytic and antibacterial ability (Bagasra et al., 1988; Wallaert et al., 1991), and decreased inflammatory mediator production by these cells (Antony et al., 1993; D’Souza et al., 1996; Standiford and Danforth, 1997; Omidvari et al., 1998). However, the effect of AUDs on antimicrobial proteins ubiquitous throughout the respiratory tract has remained relatively unexplored.

The alveolar epithelium is covered with a thin film of surface liquid, often referred to as epithelial lining fluid (ELF), that represents ~1% of the volume recovered in a bronchoalveolar lavage (BAL) procedure (Rennard et al., 1986). The ELF contains a variety of antimicrobial proteins, including lysozyme, lactoferrin, secretory leukoprotease...
inhibitor (SLP)-1, surfactants, among others (reviewed in Travis et al., 2001). When microorganisms invade the lung, submucosal gland epithelial cells and myeloid lineage cells respond by modulating the manufacture and secretion of antimicrobial proteins such as these (Rogan et al., 2006). Lysozyme, a 14 kDa enzyme, is quantitatively the most abundant antimicrobial peptide in ELF, and is bactericidal in vitro against Gram-positive pathogens including some Streptococci (Thompson et al., 1990). Lactoferrin is an iron-binding glycoprotein with MW 80 kDa that is the second most abundant antimicrobial protein in the respiratory tract (Thompson et al., 1990). One of its major antimicrobial properties is chelating iron, a necessary growth factor for both Gram-positive and -negative pathogens (Alkawash et al., 1999; Singh et al., 2000). Lysozyme may also synergize with lactoferrin to be bactericidal against some Gram-negative pathogens (Cole et al., 2002; Singh et al., 2000). The effect of AUDs on the composition of antimicrobial proteins in human subjects is unclear at present. However, qualitative and quantitative differences in the antimicrobial properties of ELF from individuals with AUDs could contribute to the predisposition for pneumococcal disease in this population.

We performed BAL on subjects with AUDs and healthy controls as a means to obtain ELF to assess the effects of AUDs on its antimicrobial protein composition. We hypothesized that BAL fluid from otherwise healthy subjects with AUDs would have a less potent microbicidal effect against S. pneumoniae in an in vitro assay compared with BAL fluid from controls. We further hypothesized that lysozyme activity and lactoferrin quantity would be decreased in BAL fluid from subjects with AUDs.

METHODS

Subject screening, recruitment and enrollment

Subjects with AUDs were recruited at the Denver Comprehensive Addictions Rehabilitation and Evaluation Services (Denver CARES) center, an inpatient facility affiliated with Denver Health and Hospital System in Denver, CO, USA. Control subjects without AUDs were recruited from the Denver VA Medical Center’s smoking cessation clinic, and approved flyers posted on the University of Colorado Denver’s medical campus. The institutional review boards at all participating sites approved this study and all subjects provided written informed consent prior to their participation in this protocol (Colorado Multiple Institutional Review Board Protocol Number 06-1088).

Subjects with alcohol abuse were eligible to participate if they met all of the following criteria at study entry: (1) an Alcohol Use Disorders Identification Test (AUDIT) score of ≥8 for men, or ≥5 for women (2) alcohol use within 7 days prior to enrollment, and (3) age of ≥21. The AUDIT questionnaire is a standardized survey to detect current and previous alcohol abuse that has been validated in a variety of clinical settings (Reinert and Allen, 2002).

In an effort to minimize the effects of co-morbidities, AUD subjects and controls were ineligible to participate in the study if they met any of the following criteria: (1) prior medical history of liver disease (documented history of cirrhosis, total bilirubin ≥2.0 mg/dL or albumin <3.0), (2) prior medical history of heart disease (documentation of ejection fraction <50%, myocardial infarction or severe valvular dysfunction), (4) prior medical history of renal disease (end-stage renal disease requiring dialysis or a serum creatinine ≥2 mg/dL), (5) prior medical history of lung disease defined as an abnormal chest radiograph or spirometry (FVC or FEV1 <75%), (6) concurrent illicit drug use defined as a positive toxicology screen, (7) prior history of diabetes mellitus, (8) prior history of HIV infection, (9) failure of the patient to provide informed consent, (10) pregnancy, (11) abnormal nutritional risk index (Detsky et al., 1984, 1987). Potential subjects aged >55 years were also excluded to minimize the presence of concomitant but asymptomatic co-morbidities.

The control subject group was selected to balance with the AUD subject group in terms of current tobacco use, age and gender. Stated more specifically, we sought to enroll equal percentages of smokers and men in each group, and also to ensure that the two groups were similar in age. Therefore, the major difference between the two groups, by design, was the diagnosis of AUD. Balancing of the two groups helped to ensure that any differences observed would be related to alcohol use history instead of these other confounders. Further, to meet eligibility as a control, control subjects must have had AUDIT scores of <3, and not have consumed alcohol within the prior month.

Bronchoscopy with BAL

Bronchoscopy procedures were performed in the inpatient Clinical and Translational Research Centers (CTRCs) at the University of Colorado Hospital. All procedures were performed utilizing telemetry monitoring and standard conscious sedation protocols as previously described (Hunninghake et al., 1979). The bronchoscope was wedged into a subsegment of either the right middle lobe or the lingula. Three 50-mL aliquots of sterile, room temperature 0.9% NaCl were sequentially instilled (150 mL total) and recovered with gentle aspiration. The second and third aliquots were combined and used in subsequent experiments as representative of the distal airspaces, whereas the first aliquot was discarded. BAL contents were then transported to the laboratory in sterile 50-mL conical tubes.

Laboratory processing

BAL fluid was immediately centrifuged (500g, 5 min) after collection to separate cellular and acellular components. Acellular fluid (containing ELF) was utilized in all subsequent assays.

Pneumococcal killing assay

The ability of acellular BAL fluid to kill S. pneumoniae was determined using an in vitro assay described below.

Preparation of live pneumococcal suspension. Streptococcus pneumoniae type 2 (ATCC, Manassas, VA) bacterial isolates were grown on blood agar plates (Remel, Lenexa, KS) overnight at 37°C with 5% CO2. A suspension was made from colonies swabbed from the blood agar plate and placed in trypticase soy broth (Remel). The inoculated
cultures were incubated at 37°C with 5% CO₂ for 2–5 h to achieve a log phase growth. The log phase bacteria were centrifuged at 1500g for 10 min to pellet the bacteria. The pellet was washed once with sterile phosphate-buffered saline and centrifuged again at 1500g for 10 min. The supernatant was decanted and the pelleted bacteria were resuspended in Hank’s Balanced Salt Solution (HBSS; Mediatech, Manassas, VA) with 15% glycerol, aliquotted and stored at −80°C. The colony forming units (CFUs) per milliliter of the bacteria was determined by 1:10 serial dilutions in sterile H₂O that was streaked onto blood agar plates using 100 µL of dilution and using the sterile technique. The plates were grown overnight at 37°C with 5% CO₂. The following day, for every dilution, the number of colonies was counted and the following equation used: number of colonies × dilution factor × volume plated. The average of all of the dilutions was then multiplied by the total volume of the culture to give the final CFU per milliliter.

Preparation of acellular BAL fluid. We observed in preliminary experiments that acellular BAL fluid had negligible bacterial activity in the in vitro pneumococcal killing assay (described below). Presuming that this was related to the dilution of ELF antibacterial proteins by saline used in the BAL procedure, subjects’ BAL fluid was vacuum concentrated and then resuspended with sterile water in serial dilutions to create aliquots with known protein concentrations. In this way, the efficacy of a known amount of BAL protein in killing *S. pneumoniae* could be assessed in a systematic fashion between subjects. For each subject and control, 9 mL of acellular BAL fluid was vacuum concentrated via a Thermo Scientific SpeedVac (room temperature × 4 h) immediately after collection. This volume was chosen as it provided adequate amounts of protein after vacuum concentration for these experiments, while additional non-concentrated BAL fluid could be used for later experiments. The pellet remaining (containing antimicrobial proteins) was then reconstituted in 500 µL of sterile molecular grade H₂O (Mediatech). The total protein concentration was then measured by using the Coomassie (Bradford) Protein Assay Kit (Pierce, Rockford, IL). *Lysozyme activity* from each sample was measured using the EnzChek Lysozyme Assay Kit (Molecular Probes, Eugene, OR) using a 1:1 dilution of sample was measured using the EnzChek Lysozyme Assay Kit (Molecular Probes, Eugene, OR) using a 1:1 dilution of sample to 1× reaction buffer. Plates were measured using standard fluorescein filters corrected for background fluorescence. Lysozyme activity was determined from the standard curve. For a subset of AUD subjects and controls, lysozyme activity was also assessed in samples that had previously been vacuum concentrated to determine the effect of this procedure on lysozyme’s activity. *Lactoferrin* was quantified in acellular BAL by ELISA using the BIOXYTECH Lactof EIA (OXISResearch, Foster City, CA). BAL fluid was used undiluted unless measured concentrations were >100 ng/mL, in which case it was diluted 1:1 with 0.9% NaCl and re-measured. Final concentrations of lactoferrin were adjusted to the total protein present in 100 µL of acellular BAL fluid.

Pneumococcal killing assay. Dilutions of acellular BAL fluid with known protein concentrations in a final volume of 30 µL were pipetted into sterile 96-well cell culture plates (BD Biosciences, San Jose, CA). Immediately thereafter, 20 µL of *S. pneumoniae* type 2 (ATCC) at a concentration of 7 × 10⁵/mL in HBSS; Mediatech, 40 µL of HBSS and 10 µL of sterile H₂O were added. Separate wells were also filled with dilutions of commercially available heat inactivated bovine serum albumin (BSA; Thermo Scientific, Rockford, IL), identical amounts of *S. pneumoniae* solution and sterile water. Dilutions of the BSA approximated the protein concentrations in acellular BAL fluid. Experiments were performed with BSA, a protein without significant bactericidal properties, to help confirm the validity of our assay in determining whether BAL fluid was truly bactericidal. We expected that killing effects by BSA would be negligible in our assay, and that superior killing would be observed with BAL fluid. Three wells per 96-well plate containing only *S. pneumoniae*, HBSS and sterile H₂O were used as the standard reference for bacterial growth. All experimental and control conditions were performed in triplicate. After wells were filled accordingly, the entire plate was placed on a plate shaker set at 200 rpm in a 5% CO₂ incubator for 2 h at 37°C. Afterwards, the contents of each well were diluted 1:6 with sterile H₂O and 100 µL of that dilution was streaked using sterile technique onto blood agar plates (Remel, Lenexa, KS). The plates were incubated overnight in a 5% CO₂ incubator at 37°C. The following day, the number of bacterial colonies (CFUs) was counted from each blood agar plate in a blinded manner (Fasching et al., 2007; Standish and Weiser, 2009). To calculate the percent kill of *S. pneumoniae* by the subjects’ fluids (or albumin), the following equation was used: \[ \frac{\{\text{CFUs with bacteria only} - \{\text{CFUs with bacteria + BAL fluid}\}\} \times 100}{\text{CFUs with bacteria only}} \times 100 \]

Antimicrobial protein measurements

To measure antimicrobial proteins within acellular BAL fluid, samples from subjects and controls were first adjusted to achieve a protein concentration of 25 µg/mL after measuring total protein using the Coomassie (Bradford) Protein Assay Kit (Pierce, Rockford, IL). *Lysozyme activity* from each sample was measured using the EnzChek Lysozyme Assay Kit (Molecular Probes, Eugene, OR) using a 1:1 dilution of sample to 1× reaction buffer. Plates were measured using standard fluorescein filters corrected for background fluorescence. Lysozyme activity was determined from the standard curve. For a subset of AUD subjects and controls, lysozyme activity was also assessed in samples that had previously been vacuum concentrated to determine the effect of this procedure on lysozyme’s activity. *Lactoferrin* was quantified in acellular BAL by ELISA using the BIOXYTECH Lactof EIA (OXISResearch, Foster City, CA). BAL fluid was used undiluted unless measured concentrations were >100 ng/mL, in which case it was diluted 1:1 with 0.9% NaCl and re-measured. Final concentrations of lactoferrin were adjusted to the total protein present in 100 µL of acellular BAL fluid.

Statistics

To assess the relationship between acellular BAL fluid protein and percent kill, a mixed effect model (Laird and Ware, 1982) was used after first log-transforming protein concentrations. The mixed effect model is an extension of linear regression that accounts for correlation between repeated observations from each subject. It was necessary to use this model in these investigations to account for the repeated measures (i.e. the different dilutions of reconstituted BAL fluid from each subject) in the data set. Given the number of subjects in the analysis, only a random effect (i.e. a random intercept but no random slope) was included for each subject. Doing so allowed the mean level of percent kill to differ slightly for each subject, and is how the correlation between the observations on each subject was modeled. This model was fit for all participants (both AUD and control
groups taken together), and separately for AUD and control groups.

The Wilcoxon rank sum test was used to determine whether there were statistically significant differences in BAL fluid lactoferrin concentrations and lysozyme activity between AUD and control subjects. This test was also used to assess the relationship between percent kill and smoking history, as well as percent kill and gender. Univariate analyses were also conducted to determine whether correlations existed between lactoferrin concentration/lysozyme activity and age, smoking and gender. A paired t-test was utilized to compare lysozyme activity between acellular BAL fluid before and after vacuum concentration procedures to determine the effect of concentration on this enzyme’s activity. A P-value of <0.05 was considered statistically significant.

RESULTS

Fifteen subjects with AUDs were enrolled as well as 10 smoking-matched controls (Table 1, P = 1.0 for tobacco use). The median FEV₁ and FVC spirometric values were within normal for both groups (median >80% for both values). There were no significant differences in the age or percentage of women in the subjects and controls. The NRI for subjects and controls characterized them as ‘well-nourished’, and did not differ significantly between the groups. The average volume of BAL fluid recovered from a 150-mL saline instillation did not differ between subjects with AUDs and controls (57% [48–63%] versus 45% [31–58%], P = 0.23).

AUDIT scores for the subjects with AUDs far exceeded the diagnostic limit for alcohol abuse and dependence (median 31 with 25–75% CI of 16–35). The median AUDIT score in controls was zero. In general, subjects with AUDs drank nearly every day of the week with consumption of a median of 8 standard drinks per day (25–75% CI, 6–11).

Acellular BAL fluid from subjects with AUDs and controls was examined for its efficacy in killing S. pneumoniae. Data from one AUD subject and one control were not able to be analyzed due to suboptimal bacterial growth in the control wells. Two control subject samples (both smokers) were not tested as the yield of BAL post-bronchoscopy was insufficient to test. Therefore, the final analysis consisted of data from 14 subjects with AUDs and 7 controls who remained balanced on the basis of smoking (P = 0.52), age (P = 0.80) and gender (P = 0.57). Subjects and controls had on average a range of three different protein concentrations of their BAL fluid tested for its ability to kill S. pneumoniae. The total protein for all reconstituted samples and serial dilutions contained a median protein concentration of 2200 µg/mL (25–75%, 880–16,000 µg/mL). Total protein concentrations did not differ significantly between AUD subjects and controls (P = 0.14). In univariate analysis, no relationship was observed between percent kill and smoking history (P = 0.82), although only 3 individuals were non-smokers out of the 21 examined. Further, there was no relationship between percent kill and age (P = 0.64), or gender (P = 0.56).

To further explore the effect of BAL fluid proteins on killing of S. pneumoniae in the context of AUDs, a mixed effects model was utilized (Table 2). Parameter estimates were calculated for the intercept and log of the protein concentration (in µg/mL). Confidence intervals and P-values were calculated for the parameter estimate of log(protein concentration) for the entire cohort, the group of AUD subjects and the group of control subjects. A significant association between percent kill and total protein concentration was observed (P < 0.0001) for the entire cohort of subjects, with a one unit increase in log protein concentration associated with a 16.3 (95% CI 10.9–21.6) unit increase in percent kill (Fig. 1). The association between percent kill and BAL total protein remained when assessing the association in AUD subjects only (P < 0.0002), and in control subjects only (P < 0.0001, Fig. 2). Although not statistically different, for a given concentration of BAL fluid protein, killing of S. pneumoniae tended to be less potent in subjects with AUDs. Stated more specifically, for AUD subjects, a one unit increase in log protein concentration was associated with a 14.6 (95% CI 7.6–21.5) unit increase in percent kill, whereas for control subjects, a one unit increase in log protein concentration was associated with a 20.0 (95% CI 11.5–28.4) unit increase in percent kill (Table 2).

Lysozyme activity and lactoferrin concentration within acellular BAL fluid were then measured from AUD subjects (n = 15) and a subset of controls (n = 6) with sufficient fluid remaining for testing after killing assay experiments were completed. Demographics of this subset are highlighted in Table 3. Of note, the median age of the control subjects in this subset was younger than that of AUD subjects; however, these subjects and controls did not significantly differ in terms of tobacco use. For a given quantity of protein (25 µg), the lysozyme activity within lavage from subjects with AUDs was ~6-fold lower when compared with controls (P < 0.01, Fig. 3). Concentrations of the antibacterial protein lactoferrin were also measured and standardized to the amount of lavage protein. Paralleling what was observed for lysozyme among subjects with alcohol abuse, lactoferrin concentrations were also significantly lower among those with AUDs (P < 0.02, Fig. 4). For the group as a whole,
Fig. 1. Relationship of percent kill of live *S. pneumoniae* in culture to acellular bronchoalveolar lavage fluid (BAL) total protein for all subjects (alcohol use disorders and controls). The ability of BAL fluid total protein from subjects and controls was examined for its ability to kill *S. pneumoniae* over a range of dilutions. A significant association between percent kill and total protein concentration was observed ($P < 0.001$). The line is the estimate of the mean percent kill as a function of log (protein concentration) for all subjects combined. A one unit increase in log protein concentration associated with a 16.3 (95% CI 10.9–21.6) unit increase in percent kill. $n = 66$, total observations, average three observations per subject or control.

Fig. 2. Relationship of percent kill of live *S. pneumoniae* in culture to acellular bronchoalveolar lavage fluid (BAL) total protein from subjects with AUDs (open circles) and control subjects (solid triangles). The estimate of the mean percent kill as a function of log (protein concentration) for all subjects only (dotted line) and for control subjects only (dashed line) is also illustrated. The slope is significant for the AUD subject group ($P < 0.0002$) and for the control subject group ($P < 0.0001$). For a given concentration of BAL fluid protein, killing of *S. pneumoniae* tended to be less potent in subjects with AUDs. AUD subjects, $n = 14$; control subjects, $n = 7$.

Table 3. Demographics of subjects with AUDs and controls utilized in *lysozyme and lactoferrin* experiments

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Subjects with AUDs ($n = 15$)</th>
<th>Control subjects ($n = 6$)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median</td>
<td>45 [39–47]</td>
<td>29 [18–37]</td>
<td>0.009</td>
</tr>
<tr>
<td>Gender, no. of women</td>
<td>2/15 (13%)</td>
<td>3/6 (50%)</td>
<td>0.11</td>
</tr>
<tr>
<td>Smoking</td>
<td>12/15 (80%)</td>
<td>5/6 (83%)</td>
<td>0.68</td>
</tr>
</tbody>
</table>

there was no association observed between either lysozyme activity or lactoferrin concentration and smoking ($P = 0.89$ and 0.96, respectively), or gender ($P = 0.48$ and 0.82). However, a significant negative association between lysozyme activity and age ($P < 0.007$) and lactoferrin concentration ($P < 0.0001$) was determined to be present. Given the younger age of our control group for these experiments, we conducted a subsequent analysis of lysozyme activity and lactoferrin concentrations that excluded subjects over the median age for the entire group (43 years) to account for age as a potential confounder (10 subjects with AUDs and 1 control). Therefore, this latter analysis was limited to five subjects with AUDs and five controls aged <43 years. In this subset of younger subjects, lactoferrin concentrations remained significantly less in those with AUDs (0.53 [0.47–0.91] versus 2.80 [1.35–3.57] ng/µg, $P < 0.01$). Additionally, a trend for decreased lysozyme activity among those with AUDs persisted (26.3 versus 74.2 U/µg, $P = 0.30$).

In additional analyses with samples from both subjects with AUDs ($n = 6$) and controls ($n = 6$), we determined that vacuum concentration of BAL fluid was associated with a decrease in lysozyme activity by 18% ($P = 0.006$). The magnitude of decrease was similar in both AUD subjects (21%) and controls (17%).

**DISCUSSION**

Our experiments are the first, to our knowledge, to describe an antimicrobial effect of human ELF obtained via BAL against *S. pneumoniae*, the most common pathogen in CAP, particularly among those with AUDs. Further, this study identified novel causes for the increased susceptibility to pneumococcal pneumonia in individuals with AUDs. Differences in antimicrobial protein composition, and activity of lysozyme and lactoferrin, probably contribute to the alterations in innate observed in those with AUDs. Importantly, the present study identifies potentially addressable factors that may decrease the incidence of pneumococcal pneumonia among those with AUDs. A decrease in the incidence of pneumonia among these individuals is an important public health issue, as an estimated 50% of patients with pneumonia have an AUD (Goss et al., 2003). Moreover, these patients have more severe symptoms, longer and more expensive hospitalizations and higher mortality (Fernandez-Sola et al., 1995; Perlino and Rimland, 1985; Saitz et al., 1997). As a result, there are more AUD-associated deaths from pneumonia when compared with the number of deaths due to alcohol-related liver disease.
Effect of AUDs on BAL Composition and Activity

Lysozyme activity was significantly decreased in subjects with alcohol use disorders compared with smoking-matched controls. Data presented as medians (middle line within box) with 25–75% confidence intervals (bottom and top of box, respectively). Lysozyme activity was significantly decreased in subjects with alcohol use disorders.

![Fig. 3. Lysozyme activity in bronchoalveolar lavage fluid per 25 µg total protein in subjects with alcohol use disorders compared with smoking-matched controls. Data presented as medians (middle line within box) with 25–75% confidence intervals (bottom and top of box, respectively). Lysozyme activity was significantly decreased in subjects with alcohol use disorders.](https://academic.oup.com/alcalc/article-abstract/45/5/414/184787)

Lactoferrin concentration in bronchoalveolar lavage fluid corrected for total protein in subjects with alcohol use disorders compared with smoking-matched controls. Data presented as medians (middle line within box) with 25–75% confidence intervals (bottom and top of box, respectively). Lactoferrin concentrations were significantly decreased in subjects with alcohol use disorders.

![Fig. 4. Lactoferrin concentration in bronchoalveolar lavage fluid corrected for total protein in subjects with alcohol use disorders compared with smoking-matched controls. Data presented as medians (middle line within box) with 25–75% confidence intervals (bottom and top of box, respectively). Lactoferrin concentrations were significantly decreased in subjects with alcohol use disorders.](https://academic.oup.com/alcalc/article-abstract/45/5/414/184787)

Lysozyme and lactoferrin are manufactured by serous epithelial cells or resident alveolar macrophages (Yeh et al., 2007). Additionally, breakdown products of ethanol could affect antimicrobial proteins post-translationally. For example, metabolism of alcohol produces acetaldehyde that in vitro has been shown to decrease the activity of lysozyme by 62% (Brecher et al., 1995). It is unclear which of these alterations in anti-pneumococcal defense elicited by alcohol is most important in increasing the predisposition for pneumococcal disease among those with AUDs, but each has a potential role.

Our group focused on examining differences in lysozyme and lactoferrin in acellular BAL fluid as these are quantitatively the most abundant antimicrobial proteins in lung (Travis et al., 2001) and therefore potentially have the most potent effect on eliminating the pneumococcus in the airways. Lysozyme functions in antimicrobial defense through its ability to cleave N-acetylMuramic acid, the cell wall material that helps bacteria maintain their shape. It is found ubiquitously throughout the airway, and has been associated with better killing and improved survival in infection with group B Streptococci and mucoid Pseudomonas aeruginosa in animal models (Akinbi et al., 2000). Lactoferrin has not only antibacterial but anti-inflammatory properties. The synergism that has been reported between various antimicrobial proteins may explain the presence of multiple antimicrobial proteins in ELF, including those present in only minute quantities. More recently, antimicrobial proteins and peptides have gained additional recognition as immune regulators, possessing activity in the neutralization of LPS, chemotactic activity, wound healing and activity in adaptive immunity (Diamond et al., 2009).

It is certainly possible that alcohol consumption may have affected not only lysozyme and lactoferrin, but also additional proteins important in the immune response against pneumococci that ultimately contributed to our killing assay observations. In order to specifically identify antimicrobial proteins whose concentration or activity are most prominently affected by AUDs, investigations with BAL fluid utilizing more sophisticated techniques (i.e. proteomics, crystallography) would be helpful (Baker and Baker, 2004; Merkel et al., 2005) to clarify the type and quantity of antimicrobials present in BAL fluid. One important family of proteins to assess in future investigations consists of the surfactant proteins. In ovine models, alcohol exposure of pregnant ewes in late gestation has been determined to result in decreased expression of mRNA for surfactant proteins A, B and D (Lazic et al., 2007; Sozo et al., 2009). Although the effect of AUDs on human surfactant proteins is not known at present, decreased expression of these proteins could be an additional contributor to the susceptibility for pulmonary infections, and might also be a relevant factor in these individuals’ susceptibility to ALI (Moss et al., 1996). Future investigations can also help establish if modifications resulting from alcohol or its metabolites are operative that might affect protein function. For example, our lactoferrin assay did not allow us to differentiate between apolactoferrin (iron-desaturated) and hololactoferrin (iron-saturated) forms of this protein in BAL fluid, although the presence of iron binding has been reported to result in conformational and functional changes to this protein (Baker and Baker, 2004; Norrby, 2004). By clarifying the distribution and function of these pulmonary antimicrobial proteins in the context of AUDs, mechanisms underlying the predisposition to pulmonary infections (and, potentially, their severity) in this population may be established.

While AUDs are related to a myriad of effects on the pulmonary host response, concomitant tobacco might also be...
expected to influence this response. For our current investigations, we examined the effects of AUDs in cohort of predominantly smokers, believing this to be a clinically relevant approach in that the majority of individuals with AUDs also smoke. Tobacco smoke exposure has been associated with an increased risk for pulmonary infections, including invasive pneumococcal disease (Nuorti et al., 2000). Many alterations in host immunity elicited by tobacco smoke have been reported, including depressed mucociliary clearance (Foster et al., 1985), increased bacterial adhesion (Raman et al., 1983), decreased pulmonary surfactant (Honda et al., 1996) and impaired function of host innate immune cells (e.g. alveolar macrophages) (Hodge et al., 2007). As discussed previously, several of these same immune mediators are similarly altered by AUDs, suggesting the possibility of an additive or even synergistic effect between AUDs and smoking. However, the previously reported effects of tobacco smoking on lysozyme and lactoferrin concentrations in lung contrast with our current observations in those with AUDs. For example, alveolar macrophages from smokers have been reported to secrete significantly more lysozyme than alveolar macrophages from non-smokers (Hinman et al., 1980). Moreover, concentrations of airway fluid lysozyme and lactoferrin among asymptomatic smokers were determined previously to be ~2-fold higher than in non-smokers (Thompson et al., 1990), and more recent data utilizing proteomic techniques have confirmed this 2-fold increase in lysozyme (Merkel et al., 2005). Our investigations accounted for the potential effect of smoking on lysozyme activity and lactoferrin concentrations in acellular BAL fluid by sampling a similar percentage of smokers in the AUD and control groups; therefore, differences in these outcome variables independent of smoking history are more likely to be detected. We ultimately enrolled very few non-smokers with AUDs due to the high prevalence of tobacco use in our population. As such, we cannot make assumptions about the effect of AUDs in the absence of smoking on our outcome variables.

Although this work provides impetus for additional investigations of antimicrobial proteins in those with AUDs, it is not without certain limitations. Although we attempted to perform all described assays on samples from every subject and control, we were limited by the amount of BAL fluid obtained from each individual, and that a modest number of individuals were enrolled. However, subjects with AUDs and controls were balanced in terms of smoking history and had no co-morbidities. This served to diminish between-subject variability and strengthens the validity of our comparisons. The killing assay that we utilized to measure the antimicrobial activity of ELF fluid proteins does not precisely replicate the in vivo environment. For example, antimicrobial effects of innate immune cells, such as alveolar macrophages, were not measured with this assay, and protein concentrations utilized in these experiments could differ from those in native ELF. However, use of this assay enabled us to experiment specifically with antimicrobial proteins from human subjects rather than a commercially available product. Although some lysozyme activity was found to be lost during vacuum concentration, we were able to perceive an effect on pneumococcal killing proportionate to the amount of protein present, and the effect was similar between AUD subjects and controls. The assay could be modified to explore the specific interactions between antimicrobial proteins and cells within collected BAL to further address these issues. We acknowledge that we examined the antimicrobial effects of one strain of *S. pneumoniae*, and our results might differ with other strains or pathogens. We elected to utilize a strain of a pathogen that had been utilized successfully in killing assay experiments in our laboratory, and is an important pulmonary pathogen in humans, particularly those with AUDs. Modifying this assay with the use of other strains of *S. pneumoniae* or other pathogens important in CAPs in those with AUDs should be examined in the future.

Pulmonary antimicrobial proteins are an important component of innate immunity. The effect of AUDs on these proteins has not been examined; however, AUDs are known to deleteriously affect other innate immune functions, so it seems plausible that antimicrobial proteins could be affected as well. Acellular BAL fluid from subjects with AUDs, who were otherwise healthy save for their excessive use of alcohol, tended to have a less potent anti-bacterial effect on the *S. pneumoniae* than did acellular BAL from healthy individuals, suggesting that antimicrobial proteins may be affected by the consumption of alcohol. These effects may be mediated by differences in the antimicrobial proteins lysozyme and lactoferrin, as well as the activity of other proteins and peptides. Clarifying the composition and activity of ELF proteins in AUDs can help establish their contribution to the prevention of pulmonary infections in this setting, and may provide avenues to develop novel prophylactic therapies against the development of pneumococcal pneumonia for individuals with AUDs.

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