Effect of Apolactoferrin on Experimental Pneumococcal Otitis Media

Patricia A. Schachern, BS; Vladimir Tsuprun, PhD; Sebahatin Cureoglu, MD; Patricia A. Ferrieri, MD; David E. Briles, PhD; Michael M. Paparella, MD; Steven K. Juhn, MD

Objective: To find the effect of apolactoferrin administration on the middle and inner ears after experimentally induced pneumococcal otitis media.

Design: Histopathologic and morphometric analysis of the middle and inner ears.

Setting: University of Minnesota, Minneapolis.

Subjects: Ten chinchillas.

Interventions: The middle ear cavities of chinchillas were inoculated bilaterally with type 2 wild-type Streptococcus pneumoniae. Twenty-four hours later, the ears of 5 of the animals were injected with phosphate-buffered saline (PBS) and the other 5 with human apolactoferrin. The animals were killed 24 hours after the last injection. Bacterial counts were made of the middle ear effusions, and the cochleae were processed for histologic analysis. The thickness of the round window membranes and bacterial and inflammatory cell infiltration of the round window membranes, and scala tympani and damage of the hair cells and stria vascularis were compared for these 2 groups of animals.

Main Outcome Measures: Comparison of inflammatory and bacterial cells in the middle and inner ears, and damage to inner ear structures.

Results: Bacterial plate counts of middle ear effusions ($P = .005$) and the number of inflammatory cells in the round window membrane ($P = .047$) were significantly lower in the apolactoferrin group compared with the group treated with PBS.

Conclusion: Further investigation of apolactoferrin as a nonantibiotic approach for the treatment of otitis media and its complications is needed to confirm its safety and efficacy.


Author Affiliations:
Department of Otolaryngology (Ms Schachern and Drs Tsuprun, Cureoglu, Paparella, and Juhn) and Laboratory of Medicine and Pathology and Department of Pediatrics (Dr Ferrieri), University of Minnesota, Minneapolis; and Department of Microbiology, University of Alabama at Birmingham (Dr Briles).
chillas.10,11 Notably higher concentrations of lactoferrin-secreting cells have been found in tubotympanum of chin-
little or no development of bacterial resistance. Lactoferrin-
Wild-type D39, serotype 2 strain, was used in this study. The growth of bacteria and measurement of their con-
trolations were performed as previously described. 10 Briefly,
bacteria were grown in Todd-Hewitt broth (Bacto Todd-
Hewitt Broth, BD Diagnostics, Sparks, Maryland) containing
0.5% yeast extract (Bacto Yeast Extract, BD Diagnostics) and
plated on sheep blood agar plates. The bacterial strains were
stored in 10% glycerin at −80°C. After growing the colonies
were washed again in buffer, dehydrated in a graded series of
10% EDTA, washed in phosphate buffer, and postfixed in 1%
oscium tetroxide in phosphate buffer (pH 7.4) for 1 hour. They
then immersed in fixative for 2 hours, decalcified for 3 days in
10% EDTA, washed in phosphate buffer, and postfixed in 1%
oscium tetroxide in phosphate buffer (pH 7.4) for 1 hour. They
were washed again in buffer, dehydrated in a graded series of
ethanol followed by propylene oxide, and embedded in epoxy
resin. Samples were cut at a thickness of 1 µm and stained with
toluidine blue for light microscopic assessment.

METHODS

Wild-type S pneumoniae D39, serotype 2 strain, was used in this
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0.5% yeast extract (Bacto Yeast Extract, BD Diagnostics) and
plated on sheep blood agar plates. The bacterial strains were
stored in 10% glycerin at −80°C. After growing the colonies
were transferred into broth, incubated at 37°C until early log phase, and centrifuged at 2000 rpm for 15 min-
teats. Bacteria were suspended in 0.15M of phosphate-buffered
PBS, and optical densities were measured at 660 nm
before the desired concentration in PBS. Actual bacterial concentra-
tions were confirmed by plating multiple 10-fold dilutions onto
blood agar plates, inculating at 37°C in 5% to
10% carbon dioxide, and counting viable cells.

Animals were housed and fed under standard conditions at
our institutional animal care facility. Experiments were per-
formed on young chinchillas weighing 250 to 350 g, with normal
external auditory canals and tympanic membranes. The care
and use of animals were approved by the Institutional Animal
Care and Use Committee of the University of Minnesota, Min-
neapolis. All animals were anesthetized prior to intrabullar in-
oculations with a combination of ketamine, 100 mg/kg, and
acepromazine, 10 mg/kg. A total of 10 chinchillas were given
bilateral intrabullar inoculations with 0.5 mL of 300 colony-
forming units/mL of wild-type S pneumoniae per ear. Twenty-
four hours later, bullae of 5 of these animals were injected bi-
laterally with 0.5 mL of 1 mg/mL ALF (human ALF, L0520;
Sigma, St Louis, Missouri) in 0.1M PBS, and bullae from the other 5 animals were bilaterally injected with 0.5 mL of 0.1M
PBS.

Two days after bacterial inoculation, the animals were killed
by overdose of sodium pentobarbital, and their bullae were
removed. Bacterial counts in the middle ear effusions were esti-
mated using BD BBL Stacker plates (BD Diagnostics). The co-
chlea were perfused via the apex and oval window with 2%
glutaraldehyde in 0.2M phosphate buffer (pH 7.4). Samples were
then immersed in fixative for 2 hours, decalcified for 3 days in
10% EDTA, washed in phosphate buffer, and postfixed in 1%
osmium tetroxide in phosphate buffer (pH 7.4) for 1 hour. They
were washed again in buffer, dehydrated in a graded series of
ethanol followed by propylene oxide, and embedded in epoxy
resin. Samples were cut at a thickness of 1 µm and stained with
toluidine blue for light microscopic assessment.

Measurements of the thickness of the round window membrane
were made at the midpoint of the sample and midway
between the midpoint and the edge of the sample on each slide,
using a 10 × 10-U eyepiece grid calibrated in units of 0.16 µm.
Measurements were averaged. The areas of greatest inflamma-
tory cell infiltration in the round window membrane and scala
tympani were counted within the 10 × 10-U grid for each sample.
Measurements were not taken near the annulus, as this was not
present in every sample. Thickness and inflammatory cell in-
filtration were assessed at a magnification of ×600. Multiple
slides from each ear were averaged and the data presented per
car. To compare the types of inflammatory cells infiltrating the
round window membrane, the areas of greatest infiltration were
photographed at a magnification of ×600 and the images en-
larged for counting. The average number of polymorpho-
nuclear leukocytes (PMNs), lymphocytes, and macrophages were
compared for the 2 groups. Bacterial infiltration of the scala tym-
pani and neurons and damage of the organs of Corti and stria
vascularis were noted.

All results were expressed as mean ± SE. Differences of bac-
terial plate counts, thickness of the round window membrane,
and inflammatory cell counts in the round window mem-
brane and scala tympani between PBS- and ALF-treated ani-
mal groups were analyzed with paired samples t test using SPSS
software (SPSS Inc, Chicago, Illinois). Differences were con-
sidered to be significant if P < .05.

Table. Comparison of Disease in the ALF- and PBS-Treated Animal Groups

<table>
<thead>
<tr>
<th>Type of Treatment</th>
<th>PBS</th>
<th>ALF</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial plate count of MEEa</td>
<td>14 (3) [n = 7]</td>
<td>2 (1) [n = 7]</td>
<td>.005</td>
</tr>
<tr>
<td>RWM thickness, µm</td>
<td>29.6 (1.7) [n = 6]</td>
<td>22.4 (1.9) [n = 6]</td>
<td>.11</td>
</tr>
<tr>
<td>RWM inflammatory cells, all types/areaa</td>
<td>43.6 [n = 6]</td>
<td>15.6 [n = 6]</td>
<td>0.047</td>
</tr>
<tr>
<td>ST inflammatory cells, all types/area adjacent to RWMa</td>
<td>62 (38) [n = 6]</td>
<td>12 (6) [n = 6]</td>
<td>27</td>
</tr>
<tr>
<td>Bacteria in RWMb</td>
<td>5 of 5</td>
<td>1 of 6</td>
<td>NA</td>
</tr>
<tr>
<td>ST bacteria adjacent to RWMb</td>
<td>3 of 5</td>
<td>1 of 6</td>
<td>NA</td>
</tr>
<tr>
<td>Hair cell damageb</td>
<td>4 of 6</td>
<td>3 of 8</td>
<td>NA</td>
</tr>
<tr>
<td>Stria vascularis damageb</td>
<td>3 of 6</td>
<td>2 of 8</td>
<td>NA</td>
</tr>
<tr>
<td>Bacteria in area of neuronsb</td>
<td>4 of 6</td>
<td>2 of 8</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviations: ALF, apolactoferrin; MEE, middle ear effusion; n, number of ears used in statistical analysis; NA, not applicable; PBS, phosphate-buffered saline; RWM, round window membrane; ST, scala tympani.

aValues are given as mean ± SE.

bData are given as the number of ears involved. Nonparametric test was not performed because of the small number of animals; however, the percentage of ears with these pathologic changes have a trend to be higher in PBS-treated animals compared with those treated with ALF.
RESULTS

Two of the animals in the PBS-treated group were found dead on the final day of the experiment, presumably from the infection. One animal from the ALF-treated group died immediately after anesthesia administration, presumably from the anesthesia. At 48 hours after bacterial inoculation (24 hours after PBS or ALF injections) animals from both groups had middle ear effusions. The Table compares the findings of bacterial counts, round window membrane thickness, inflammation of the round window membrane and scala tympani, bacterial presence in the scala tympani and neurofilaments, and damage to the hair cells and stria vascularis. Bacterial plate counts of middle ear effusions and the number of inflammatory cells in the round window membrane were significantly lower in the ALF group compared with the group treated with PBS. Although histopathologic findings were not analyzed statistically, because of the limited number of animals, pathological changes did show a trend to be greater in the PBS-treated group. Figure 1A demonstrates the most severe findings of the round window membrane in the ALF-treated group, showing the round window membrane with only a slightly elevated thickness and a small number of inflammatory cells. Figure 1B demonstrates the most severe findings in the PBS-treated group, showing a large number of bacteria and inflammatory cells in the round window membrane and a substantially increased thickness of the round window membrane. Bacteria can be seen both in the round window membrane and the adjacent scala tympani. Figure 2A shows findings from this PBS-treated animal that are characteristic of the inner ear changes seen in both groups, including bacterial penetration of the scala tympani and neurofilaments, strial edema, hair cell damage, and bacterial cells (arrow) in this PBS-treated ear. B, Higher magnification shows bacteria (arrows) within the area of the neurofilaments. SM indicates scala media; SV, stria vascularis.

COMMENT

Antibiotic-resistant bacteria have become an increasing problem. Lynch and Zhanel\textsuperscript{14} recently reported that 15%
The innate immune system of the tubotympanum contains a number of naturally produced antimicrobial agents that kill various microorganisms. One component of this system is the iron-binding glycoprotein, lactoferrin, which is secreted into mucosal fluids. Pneumococcal carriage has been shown to be dependent on mucosal rather than systemic immunity, and lactoferrin has been shown to play an important role as a first line of host defense against infection and inflammation at the mucosal surface. The functions and mechanisms of lactoferrin are broad and include among others that it (1) has antimicrobial properties, (2) is a key component of the innate immune response at the mucosal barrier, (3) has an anti-inflammatory role, (4) has pleiotropic immunomodulatory activities affecting many cell types, and (5) has the ability to modulate cellular signaling pathways.

Lactoferrin protection is less effective against S pneumoniae than against other bacterial types because of the protective effect of one of its virulence factors, PspA, which is common to all serotypes of pneumococcus. PspA was shown to prevent activation of complement by reducing host complement-mediated clearance and phagocytosis. Binding ALF, the iron-free form of lactoferrin, was demonstrated to protect pneumococci against its bactericidal effects. The mechanism by which ALF kills pneumococci was suggested to be membrane destabilization. In vitro experiments have demonstrated that ALF can kill actively growing pneumococci at a concentration of 1 mg/mL. We used a much higher concentration of ALF in our experiments, in an effort to overwhelm the protective binding capacity of the PspA, thus making the pneumococci vulnerable to the ALF.

We found that middle ear administration of ALF (the commercially available iron-free form of lactoferrin), after experimentally induced pneumococcal otitis media, reduced the number of bacteria in the middle and inner ears and damage of the round window membrane and inner ear compared with the PBS-treated controls. The safety of human lactoferrin peptide 1-11 was tested in a sequential, randomized, double-blind, placebo-controlled study using ascending single and multiple intravenous doses in healthy volunteers, and open-label, single intravenous doses in autologous human stem cell transplant recipients. It was shown to be well tolerated in both populations, with few possibly related side effects. Further studies, perhaps using topical application of exogenous ALF (or its peptides) alone or in combination with other antimicrobial and/or anti-inflammatory agents via tympanostomy tubes for the treatment of acute otitis media, are warranted.

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Correspondence: Vladimir Tsuprun, PhD, Department of Otolaryngology, University of Minnesota Medical School, LRB, 2001 Sixth St SE, Minneapolis, MN 55455 (tsupr001@umn.edu).

Author Contributions: All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Schachern, Tsuprun, Cureoglu, Ferrieri, Briles, Paparella, and Juhn. Acquisition of data: Schachern, Tsuprun, Cureoglu, and Ferrieri. Analysis and interpretation of data: Schachern, Tsuprun, Cureoglu, Ferrieri, Briles, and Juhn. Drafting of the manuscript: Schachern, Tsuprun, Cureoglu, and Briles. Critical revision of the manuscript for important intellectual content: Schachern, Tsuprun, Cureoglu, Ferrieri, Briles, Paparella, and Juhn. Statistical analysis: Tsuprun, Cureoglu, and Briles. Administrative, technical, and material support: Schachern, Tsuprun, Cureoglu, Ferrieri, and Juhn. Study supervision: Ferrieri, Briles, Paparella, and Juhn.

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REFERENCES

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Figure 3. Histogram shows a significant difference between the phosphate-buffered saline (PBS)- and apolactoferrin (ALF)-treated groups of animals. A, Bacterial plate counts of middle ear effusion (P=.005). B, Inflammatory cell infiltration of the round window membrane (RWM) (P=.047). Error bars indicate standard errors.


