A Role for Pneumolysin but Not Neuraminidase in the Hearing Loss and Cochlear Damage Induced by Experimental Pneumococcal Meningitis in Guinea Pigs

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We investigated the roles of pneumolysin and neuraminidase in the pathogenesis of deafness and cochlear damage during experimental pneumococcal meningitis. Anesthetized guinea pigs were inoculated intracranially with 7.5 log_{10} CFU of either (i) wild-type Streptococcus pneumoniae D39 (n = 8), (ii) PLN-A, a defined isogenic derivative of D39 deficient in pneumolysin (n = 5), or (iii) ∆NA1, a new derivative of D39 deficient in neuraminidase constructed by insertion-duplication mutagenesis of the nanA gene (n = 5). To quantify hearing loss, the auditory nerve compound action potential evoked by a tone pulse was recorded from the round window membrane of the cochlea every 3 h for 12 h. The organ of Corti was intravitally fixed for subsequent examination by high-resolution scanning and transmission electron microscopy. All animals sustained similar meningial inflammatory responses. PLN-A induced significantly less hearing loss than D39 over the frequency range of 3 to 10 kHz. Levels of mean hearing loss at 10 kHz 12 h postinoculation were as follows: D39, 50 dB; ∆NA1, 52 dB (P = 0.76 versus D39), and PLN-A, 12 dB (P < 0.0001 versus D39). The mean rates of hearing loss at 10 kHz were 4.4 dB/h for D39, 4.3 dB/h for ∆NA1, and just 1.0 dB/h for PLN-A (P < 0.0001 versus D39). Suppurative labyrinthitis was universal. PLN-A induced the accumulation of less protein in the cerebrospinal fluid (P = 0.04 versus D39). Infection with D39 and ∆NA1 induced significant damage to the reticular lamina, the sensory hair cells, and supporting cells of the organ of Corti. By contrast, after infection with PLN-A, the organ of Corti appeared virtually intact. Pneumolysin seems to be the principal cause of cochlear damage in this model of meningogenic deafness. No clear pathogenic role was demonstrated for neuraminidase.

Acute bacterial meningitis can result in deafness in spite of aggressive antibiotic treatment and supportive care. Infection with Streptococcus pneumoniae causes a sensorineural hearing loss in between 21 and 50% of patients (14, 17, 37) and for unknown reasons seems to be the most harmful of the common meningeval pathogens. In previous studies, we demonstrated that pneumolysin is a potent ototoxic agent (2, 13). We now describe a new model of experimental meningitis where we used isogenic mutants of S. pneumoniae unable to express pneumolysin (PLY) or neuraminidase to investigate the contribution of these factors to hearing loss.

Deafness after meningitis is almost certainly caused by cochlear damage. Suppurative labyrinthitis has been observed in postmortem studies of meningitis (15, 19, 31) and in experimental meningitis induced by systemic or intracranial inoculation (6, 9, 20, 38). Organisms, inflammatory cells, and soluble inflammatory mediators can spread from the inflamed subarachnoid space to the perilymphatic space of the inner ear via the cochlear aqueduct, which is usually patent (33). Specific ultrastructural lesions of the organ of Corti (the sensory organ of hearing) have been found in experimental Escherichia coli and Haemophilus influenzae type B meningitis in rabbits (27) and in E. coli and pneumococcal meningitis in guinea pigs (39). In a recent study of childhood bacterial meningitis, otoacoustic emissions (a measure of cochlear function) were impaired within 6 h of admission in all those who were to sustain long-term deafness, suggesting that the cochlea sustains early damage (35).

Meningeal infection triggers the release of many harmful inflammatory mediators (32). Bacterial products themselves could also be toxic for the cochlea. Two factors of particular interest in pneumococcal infection are pneumolysin (PLY), a cholesterol-binding hemolysin, and neuraminidase, an enzyme which cleaves N-acetylneuraminic acid from mucin, glycoproteins, and gangliosides.

PLY has many putative roles in the pathogenesis of pneumococcal disease and is expressed by all pathogenic strains of S. pneumoniae (30). In particular, we have shown that microperfusion of purified recombinant PLY into the scala tympani of the cochlea of the guinea pig causes severe electrophysiological and ultrastructural damage to the organ of Corti (13). Intracisternal injection of purified PLY provokes a meningeval inflammatory response in the rabbit, but there is little difference in the inflammatory response induced by intracisternal challenge with viable S. pneumoniae D39 or by its PLY-deficient isogenic derivative, PLN-A (18). Therefore, it is not certain if PLY has any pathogenic role in the subarachnoid space.

Evidence for the role of neuraminidase in the pathogenesis of meningitis is also conflicting. Neuraminidase is expressed by all clinical isolates of S. pneumoniae (22, 28). Although intracerebral inoculation of a crude neuraminidase preparation in mice causes neurological symptoms and death (23), intrathecal...
inoculation of dogs with a similar preparation causes no symptoms even though it reduces the sialic acid content of subcellular cortical organelles (29). In patients with pneumococcal meningitis, the development of coma and a poor outcome is proportional to the concentration of free N-acetylneuraminic acid in the cerebrospinal fluid (CSF) (28).

In an attempt to resolve the contribution of these pneumococcal products to meningogenic hearing loss, we inoculated groups of guinea pigs intracranially with wild-type S. pneumoniae strain D39 or with isogenic derivatives deficient in either neuraminidase (PLN-A) (5) or neuraminidase (ΔNAI) (described below). Previous studies have assessed hearing loss by monitoring brain stem auditory evoked responses (6–8, 21, 27). To improve precision, we assessed hearing loss by recording the transient auditory nerve cochlear action potential (CM) and cochlear microphonics potential (CM) directly from the round window membrane of the cochlea (12). Ultrastructural damage to the organ of Corti was investigated with high-resolution scanning and transmission electron microscopy (SEM and TEM), respectively (26).

This work was presented in abstract form at the 7th International Congress for Infectious Diseases, Hong Kong, 10 to 13 June 1996 [39a].

MATERIALS AND METHODS

Construction of ΔNAI and PLN-A. S. pneumoniae serotype 2 strain D39 (NCTC 7466) is encapsulated and fully virulent in a mouse model of pneumococcal bacteremia (5). Construction of its PLY-deficient isogenic derivative PLN-A has been described previously (5). The recent isolation of the neuraminidase gene nanA (10) enabled us to construct an isogenic derivative of strain D39 deficient in neuraminidase (ΔNAI) by insertion duplication mutagenesis following direct transformation of D39 with plasmid DNA. A 631-bp fragment was isolated from the nanA structural gene by digestion with XbaI and HincIII. The fragment was purified by agarose gel electrophoresis, recovered using a Band Prep kit (Pharmacia), and cloned into the vector pGEM+ host 5 (25). This vector replicates in S. pneumoniae at 28°C but not at 37°C. The construct pNAI was transformed into D39 by the method of Yothers et al. (40) and plated out for growth at 28°C on blood agar supplemented with erythromycin (1 μg/ml).

A colony was selected and grown overnight at 28°C in brain heart infusion broth (BHI) supplemented with erythromycin (5 μg/ml) before being plated on selective blood agar plates for growth at 37°C. Colonies were picked from 37°C plates and incubated in erythromycin selection at 37°C overnight. Cells were collected and assayed for neuraminidase activity. Those colonies giving negative enzyme activity were subjected to DNA isolation and Southern blotting to confirm the nature of the construct (data not shown).

Phenotypy.

Mannan A carried by the pneumococcal capsules from the CSF of animals inoculated with PLN-A or ΔNAI were tested for erythromycin resistance by susceptibility techniques. CSF leukocytes were counted in an improved Neubauer chamber. The remainder of the CSF was centrifuged (13,000 × g, 5 min, 20°C), and the supernatant was stored at −20°C for up to 4 months prior to protein assay. The concentration of protein in the CSF was measured with a standard Bio-Rad microassay.

Measurement of hearing loss.

Pneumococcal meningitis was initiated by intracranial inoculation of 106 CFU of strain D39 or with isogenic derivatives deficient in neuraminidase (ΔNAI) by insertion duplication mutagenesis following direct transformation of D39 with plasmid DNA. A 631-bp fragment was isolated from the nanA structural gene by digestion with XbaI and HincIII. The fragment was purified by agarose gel electrophoresis, recovered using a Band Prep kit (Pharmacia), and cloned into the vector pGEM+ host 5 (25). This vector replicates in S. pneumoniae at 28°C but not at 37°C. The construct pNAI was transformed into D39 by the method of Yothers et al. (40) and plated out for growth at 28°C on blood agar supplemented with erythromycin (1 μg/ml).

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A standardized pneumococcal inoculum was prepared by the method of Adewar et al. (1). Erythromycin (5 μg/ml) was added to all liquid media when PLN-A or ΔNAI was prepared to maintain selection pressure. Outbred MF1 mice were inoculated by intraperitoneal injection of 106 CFU of stock D39 (or its derivative) in 0.2 ml of phosphate-buffered saline (PBS; Gibco). Twenty-four hours later, the moribund or dead animals were perfused via the round window with glutaraldehyde (2.5% [wt/vol] glutaraldehyde oxalic acid; POole, United Kingdom). All animals underwent tracheotomy to protect the airway from nasal secretions. The rectal temperature was maintained at 38°C by a thermococcal heating pad. Fluid replacement was given as 0.9% (wt/vol) saline by intraperitoneal injection at a rate of 66 ml/kg/24 h. The inoculum (0.1 ml, 3 × 107 CFU) was gently instilled into the subarachnoid space through a burr hole 1 mm in diameter drilled in the outer table of the left parietal skull bone. The defect was plugged with a smear of silicone sealant.

Twelve hours after inoculation, CSF was taken from the cisterna magna, blood was sampled from the tail veins, and the animals were killed by cervical dislocation. Viable bacterial counts were performed by standard dilutional techniques. CSF leukocytes were counted in an improved Neubauer chamber. The remainder of the CSF was centrifuged (13,000 × g, 5 min, 20°C), and the supernatant was stored at −20°C for up to 4 months prior to protein assay. The concentration of protein in the CSF was measured with a standard Bio-Rad microassay.

Auditory stimuli consisted of sinusoidal pure tone bursts of 1- ms duration and <0.1-ms rise and fall, phase-locked to 0°, and delivered at a rate of 10 Hz via a piezoelectric ceramic transducer (Motorola) coupled to the ear bar with 4 cm of plastic tubing. Maximum achievable sound level was 102 dB sound pressure level at 10 kHz and 112 dB sound pressure level at 3 kHz.

Auditory assessments were performed every 3 h for 12 h in both ears. To record the CAP, stimuli were presented at 1-kHz intervals from 3 to 10 kHz. A fine insulated silver electrode was placed on the round window membrane to record electrical activity. Potentials were amplified (×20,000; NL100 & NL104, Neurolog, Welwyn Garden City, United Kingdom), bandpass filtered (100 to 6 kHz, 40 dB/oct) and notch filtered (50 Hz, 100 dB/oct) with a computer averaged by using a MacLab 2e (AD Instruments, Hastings, Australia) and a PowerMac 6100/60 (Apple, Stockley Park, United Kingdom). The intensity of the stimulus was adjusted with an attenuator (Fieldpoint 2010) so that the mean peak-to-peak potential of the N1-P1 wave was 2 V at the electrode. The stimulus intensity required to evoke this 100-μV criterion level was defined as the CAP threshold.

For brevity, a rise in the CAP threshold and “hearing loss” are considered synonymous.

The peak potential at the time of the first peak was also recorded. Tone bursts (3 kHz, 100 ms) were delivered at a rate of 2 Hz in 10-dB steps from 82 to 112 dB SPL. The maximum achievable peak-to-peak CM amplitude 5 ms poststimulus was used for analysis.

Electron microscopy. The methods for fixation and the preparation of guinea pig cochlea for high-resolution SEM have been described in detail previously (26). Immediately after death, both temporal bones were removed. The cochleae were perfused via the round window with glutaraldehyde (2.5% [wt/vol] in 0.05 M phosphate buffer [pH 7.2]) and then immersed in fixative for at least a week. The modiolus was dissected out, dehydrated in acetone, and critical point dried in liquid CO2. Specimens were attached with Araldite (apex upright) onto copper SEM holders, sputter coated with platinum, and examined in a JEOL 120CXII TEM fitted with a lanthanum hexaboride filament and a TESMACAN scanning attachment. Images were obtained with a secondary electron detector and accelerating voltage of 40 kV. To examine the undersurface of the basilar membrane (bordering scala tympani), specimens were carefully prised off the SEM holder, inverted, and remounted so that the basilar surface of the first cochlear turn was upright. The specimen was then recoated with platinum. Morphological damage was assessed by high-resolution SEM (×30, A). In each group, seven (D39) or six (ΔNAI and PLN-A) cochleas were examined by SEM. The cochleae were coated with gold, and photographs were taken immediately after demise. Ultrathin sections were cut at 90 nm with a diamond knife, mounted on Formvar/
TABLE 1. Electrocochleographic findings 12 h after intracranial inoculation of $3 \times 10^7 S. pneumoniae$

<table>
<thead>
<tr>
<th>Strain (n)</th>
<th>Hearing loss (measured by CAP recording)*</th>
<th>CM potential (as proportion of baseline) at 3 kHz</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 kHz</td>
<td>3 kHz</td>
</tr>
<tr>
<td>D39 (15)</td>
<td>50 ± 0</td>
<td>42 ± 9</td>
</tr>
<tr>
<td>PLN-A (10)</td>
<td>12 ± 6</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>∆NA1 (10)</td>
<td>52 ± 13</td>
<td>45 ± 13</td>
</tr>
</tbody>
</table>

* Numbers refer to number of cochleas assessed. Values are means ± 95% CI unless indicated otherwise.

Statistical analysis. Animals were assigned to one of three groups and inoculated with D39 (n = 8), PLN-A (n = 5), or ∆NA1 (n = 5). The major quantitative endpoint was the difference between CAP loss induced by wild-type D39 and that induced by PLN-A or ∆NA1 at a stimulus frequency of 10 kHz 12 h postinoculation. Subsidiary endpoints included the difference in the rate of hearing loss between the three groups (at 10 kHz) and the difference in loss of CM potential (evoked at 3 kHz).

For comparison between groups, the null hypothesis was that no difference was expected in any parameter measured. Data were tested for normality with the Shapiro-Wilk test. For hearing loss data, independence between left and right ears within each group was confirmed with Spearman’s rank correlation coefficient so the data were pooled. Quantitative bacterial counts and CSF leukocyte counts were log transformed. Differences between groups were compared by Student’s t test, if the variances were similar and the data were normal, or by the Mann-Whitney U test, if variances were unequal (even if the data were normal) or the data were nonnormal. Confidence limits at 95% significance (or as close as possible for the Mann-Whitney U test) for the difference between the means or medians were calculated by using Arcus 3.01 statistical software. Rates of hearing loss were compared by using grouped linear regression and analysis of variance (ANOVA).

RESULTS

Electrophysiological findings. Animals inoculated with PLY-deficient PLN-A sustained significantly less hearing loss than those inoculated with wild-type D39 or neuraminidase-deficient ∆NA1, as judged by CAP recording 12 h postinoculation (p.i.) (Table 1). At a stimulus frequency of 10 kHz, the group inoculated with D39 sustained a mean hearing loss of 50 dB (95% confidence interval [CI], 41 to 59 dB), those inoculated with ∆NA1 lost 52 dB (95% CI, 39 to 65 dB; $P = 0.76$ versus D39 by Student’s t test), while those inoculated with PLN-A lost just 12 dB (95% CI, 6 to 18 dB; $P < 0.0001$ versus D39 by the Mann-Whitney U test). The difference was apparent across the frequency range tested (3 to 10 kHz) with peak hearing loss occurring at a stimulus frequency of either 6 or 7 kHz in all groups. The mean rates of hearing loss at 10 kHz were 4.4 dB/h for D39, 4.3 dB/h for ∆NA1, and just 1.0 dB/h for PLN-A ($P < 0.0001$ for D39 versus PLN-A by ANOVA) (Fig. 1).

In normal ears and ears with a moderate hearing loss (<30 dB), successive compound action potentials were uniform, with a small initial P0 wave and identical N1-P1 waves at precisely the same latency. In ears which had lost substantial hearing (>30 dB), the shape of the CAP altered as a greater stimulus was needed to regain the reference level (not shown). A prominent P0 wave became apparent, and successive CAP waveforms were dissimilar in height and latency. As hearing loss progressed, the CAP became refractory to every third or fourth stimulus and finally could no longer be elicited.

CM elicited at 3 kHz declined steadily in almost all experiments from 3 h p.i. onward. However, CM was less affected in animals inoculated with PLN-A than those inoculated with D39 or ∆NA1 (Table 1). There was a strong overall correlation between final CM (as a proportion of baseline) and hearing loss (as measured by CAP recording at 10 kHz) (Spearman’s rank correlation coefficient $r = -0.7$; 95% CI for $r = -0.8$ to $-0.5$; $P < 0.0001$).

Induction of meningitis. The mean quantities of $S. pneumoniae$ inoculated were similar in all three groups (Table 2). Inoculation of $3 \times 10^7$ CFU $S. pneumoniae$ provoked a rapid (<3 h) leukocytosis in the CSF (data not shown). All animals were bacteremic at 12 h p.i. CSF leukocytosis was not significantly affected by the lack of either PLY or neuraminidase, although this was not a primary endpoint. The concentration of protein in the CSF at 12 h p.i. was significantly lower in the group inoculated with PLN-A. There were no significant differences in the concentration of viable pneumococci in the CSF or the blood at 12 h p.i. Thus, when inoculated intracranially, the inability of $S. pneumoniae$ to express either PLY or neuraminidase did not appear to affect bacterial replication within the CSF or bloodstream or alter the magnitude of the meningial inflammatory response. The amount of protein within the CSF was lower in animals infected with PLN-A.

Phenotypic analysis. The PLN-A inoculum had no detectable hemolytic activity. The neuraminidase activity of the ∆NA1 inoculum was 0.55 mU/mg of cell protein by MUAN cleavage fluorescence assay (the lower limit of detection). Organisms recovered from the CSF and the blood of animals.
inoculated intracranially with PLN-A or ΔNA1 were fully re-
sistant to erythromycin. Organisms recovered from the CSF
and blood of animals inoculated with PLN-A had no detect-
able hemolytic activity. Organisms recovered from the CSF
of two animals inoculated intracranially with ΔNA1 expressed no
neuraminidase activity (<0.5 mU/mg of cell protein) after
growth in either selective or nonselective medium.

**Ultrastructural findings.** Representative scanning and trans-
mision electron micrographs are shown in Fig. 2 and Fig. 3,
respectively. There was clear evidence of ultrastructural dam-
age to the organ of Corti in animals inoculated with D39 or
ΔNA1. This was most pronounced in the basal turn and af-
fected three key elements of the organ of Corti: the sensory
hair cells and their stereocilial hair bundles, the supporting
cells, and the nerve endings associated with the hair cells. In
marked contrast, animals inoculated with PLY-deficient PLN-
A exhibited visible damage only to the nerve endings. Irrespec-
tive of the genotype of the inoculum, all animals sustained a
labyrinthitis, and by SEM, organisms attached to the scala
tympanic surface of the basal membrane could be demon-
strated. Indeed, on dissection, the perilymphatic space was
frequently filled with a plug of inflammatory debris. The basilar
membrane was intact in all TEM sections examined.

(i) **Damage to sensory hair cells and stereocilial hair bun-
dles.** In animals inoculated with D39 or ΔNA1, damage to
outer hair cells (OHCs) was extensive, especially if hearing loss
exceeded 40 dB (Fig. 2a and b). Damaged OHCs were swollen
and vacuolated with breaks in both the apical and basal plasma
membranes (Fig. 3a). The site of the basal body (the remnant
of the kinocilium) appeared to be a particular weak point, with
cell contents sometimes extruding out through a breach in the
cell membrane at this point. Mitochondria were vacuolated.
Intercellular junctions were disrupted, and at times the cattic-
ular plate seemed to be lifted clear of the bordering phalangeal
cell processes (Fig. 2b). Hair bundle stereocilia were fused
together (Fig. 2b), and the plasma membranes were poorly
defined. Inner hair cells (IHCs) were in general much less
affected. The stereocilia of a few IHC sensory hair bundles
were fused together or disrupted (Fig. 2c), but often IHC hair
bundles were relatively undamaged (Fig. 2d). In animals inco-
nulated with PLN-A, the organ of Corti appeared virtually nor-
mal by SEM (Fig. 2e and f). With TEM, although some OHCs
apparently lacked turgor, no breaks in the sensory cells plasma
membrane were found (Fig. 3b) and intercellular junctions
remained intact.

(ii) **Damage to supporting cells.** Shallow craters developed
in the apical surface of inner sulcus cells in almost all animals
inoculated with D39 (Fig. 2c) and ΔNA1 (Fig. 2d). Cratering of
supporting cells was often associated with localized damage to
hair bundles of IHCs (Fig. 2c). Phalangeal cells were swollen
and contained vacuolated mitochondria (Fig. 3a). In animals
inoculated with PLN-A, cratering of supporting cells was seen
just once; most supporting cells were intact.

(iii) **Damage to the neural elements.** All three inocula in-
duced pathological damage in nerve endings as judged by TEM
(Fig. 3b). Mitochondria within nerve endings were vacuolated
and highly abnormal, and there was disruption of nerve ending
plasma membranes. With PLN-A infection, the changes were
less severe but still apparent (Fig. 3b).

**DISCUSSION**

The primary purpose of this study was to compare hearing
loss and ultrastructural cochlear damage induced by a wild-
type pneumococcal strain (D39) and by isogenic derivatives
deficient in PLY (PLN-A) or neuraminidase (ΔNA1) in a mod-
el of experimental meningitis. The ultrastructural damage that
we observed in pneumococcal meningitis strongly resembles
that due to microperfusion of purified PLY into the cochlea:
damage to hair bundle stereocilia, cratering of the apical sur-
face of hair cells and supporting cells, and extrusion of hair
cells from their adjacent supporting cells (13). When we in-
duced meningitis with PLN-A, there was almost no ultrastruc-
tural cochlear damage and significantly less hearing loss at all
frequencies tested. The reticular lamina and associated tight
junctions, hair bundles, and the apical surfaces of sensory cells
and supporting cells all remained intact, although hair cell
nerve endings sustained some minor damage. It is striking that
the very ultrastructural changes induced by cochlear perfusion
with PLY are those that are absent after meningitis due to
PLN-A. Thus, at least in this experimental model, the majority
of cochlear damage and hearing loss sustained can be ascribed
to PLY.

Studies with the isogenic mutant PLN-A have yielded much
information about the pathogenic role of PLY in pneumococ-
cal infection. PLN-A fails to replicate as well as D39 in the
mouse alveolus (11) and causes significantly less ultrastructural
damage to isolated human respiratory mucosa (34). Moreover,
PLN-A cannot sustain continued exponential growth in mouse
bacteremia (3). Thus, PLY plays a number of important roles
in establishing pneumococcal infection. In our study, PLN-A
was still able to induce a significant meningeal inflammatory
response. This finding is consistent with the study of Friedland
et al., who found no difference in the concentration of leuko-
cytes or tumor necrosis factor alpha in the CSF when rabbits
were inoculated intracisternally with either D39 or PLN-A
(18). Thus, PLY seems not to augment the meningeal inflam-
atory response. In contrast to the study of Friedland et al.
(18), PLN-A induced less protein accumulation in the CSF of
infected animals. This finding suggests that PLY may play a
role in mediating changes in permeability of the blood-brain
barrier. We have suggested a similar role for PLY in mediating
changes in permeability of the alveolar cell-capillary barrier of
the lung during pulmonary infection (35a).

All prior studies of hearing loss in experimental meningitis
have relied on monitoring brain stem auditory-evoked re-

**TABLE 2. Viable bacterial counts and CSF inflammatory response in guinea pigs 12 h after intracranial
inoculation of 3 × 10^7 S. pneumoniae**

<table>
<thead>
<tr>
<th>Strain (n)</th>
<th>Viable counts (log_{10} CFU/ml)</th>
<th>CSF parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum</td>
<td></td>
<td>Leukocytes (log_{10}CFU/ml)</td>
</tr>
<tr>
<td></td>
<td>CSF</td>
<td>Blood</td>
</tr>
<tr>
<td>D39 (8)</td>
<td>7.5 ± 0.2</td>
<td>8.7 ± 0.5 (n = 6)</td>
</tr>
<tr>
<td>PLN-A (5)</td>
<td>7.5 ± 0.3</td>
<td>8.1 ± 0.4</td>
</tr>
<tr>
<td>ΔNA1 (5)</td>
<td>7.5 ± 0.0</td>
<td>9.0 ± 0.3 (n = 4)</td>
</tr>
</tbody>
</table>

* Unless stated otherwise, values are means ± 95% CI.

** Significantly different (P < 0.05) from value for D39 group by Mann-Whitney U test.
FIG. 2. Scanning electron micrographs of the apical surface of the basal turn of the organ of Corti in guinea pigs 12 h after intracranial inoculation of $3 \times 10^7$ CFU *S. pneumoniae*. (a) D39. There is extensive damage to many OHCs, with ballooning of the apical surface and destruction of the stereocilial hair bundles (arrow). Row 1 cells are particularly affected. (b) D39. The cuticular plate of this row 1 OHC has become torn from the surrounding phalangeal cells (arrow). The hair bundle is almost completely effaced. (c) D39. Extensive craters in the apical surface of border cells and inner sulcus supporting cells (asterisk) can be seen. In this area, stereocilial hair bundles of the IHCs are irregular. (d) D39A1. Cratering in an inner sulcus cell (asterisk) and disruption of OHC hair bundles (arrow) can be seen. (e) PLN-A. In marked contrast, the organ of Corti appears here to be completely intact, with no damage to the OHC hair bundles and no craters in the supporting cells. (f) PLN-A. Regular OHC hair bundles with intact intercellular junctions are apparent.
FIG. 3. Transmission electron micrographs of the basal turn of the organ of Corti 12 h p.i. prepared in the same way as for Fig. 2. (a) D39. The row 2 OHC on the left is swollen with defects in its plasma membrane and pathological stereocilia (arrow). Mitochondria in both hair cells and neighboring supporting cells are abnormal and vacuolated. The contents of an inner phalangeal cell are extruding through its apical surface (asterisk). (b) PLN-A. The reticular lamina and associated tight junctions are clearly intact across the entire width of all three OHCs. However, nerve endings at the base of the row 2 OHC are abnormal (arrow), containing some vacuolated mitochondria.
sponses (6–8, 21, 27). This has a measurement error of about ±10 dB. Rabbits inoculated with 5 × 10^5 CFU of S. pneumoniae by intracranial injection sustain a progressive hearing loss as assessed by click- and tone-evoked brain stem response audiometry (6). This correlates with the development of a meningeal inflammatory response (8) and can be reduced by antibiotic treatment (7). In this model, hearing loss affects high frequency (10 kHz) before low frequency (3 kHz) and is lost at a rate of 3 dB/h, similar to the 4 dB/h observed in our experiments. Our robust and reproducible electrophysiological technique allowed us to measure hearing loss at multiple frequencies to a precision of around 1 dB and record changes in the CM potential. This is to our knowledge the first description of electrophysiological recording in experimental bacterial meningitis and shows unequivocally that hearing loss occurs at the level of the cochlea. The more complete audiogram data obtained in the guinea pig suggest that the most audiologically sensitive area of the cochlea is the most susceptible to the lesions induced by pneumococcal meningitis. This observation may be explained by longitudinal gradations of hair cell structure and metabolism and endolymphatic potential (36) in the cochlea, rather than the base-to-apex gradient of inflammation proposed by Bhatt et al. (8). Short-term experimental meningitis induced by H. influenzae or E. coli in rabbits induces ultrastructural lesions similar to those reported here, with patchy damage of the hair cells, supporting cells, and nerve endings of the organ of Corti once hearing loss exceeds 20 dB (27).

In this experimental model, there were no distinguishable differences between wild-type S. pneumoniae D39 and its neuraminidase-deficient derivative ΔnanA in terms of meningeval inflammation, growth in the CSF, bacteremia, or cochlear damage. The data that we present are among the first available on the behavior of a neuraminidase-deficient pneumococcal mutant in vivo. Defining the exact role of neuraminidase enzyme activity in pathogenesis is complicated by the presence of a second neuraminidase gene, termed nanB, close to nanA on the pneumococcal chromosome (4). The work described here shows that nanA has no role in meningeal deafness in this model. Berry et al. state that nanA-deficient mutants have residual enzyme activity due to the production of nanB (4). We could detect no neuraminidase activity from nanA mutants within the sensitivity limits of our assay. The contribution of nanB to pathogenicity awaits the construction of appropriate bacterial mutants. The existing evidence that neuraminidase has a role in the pathophysiology of pneumococcal meningitis is inconclusive and circumstantial (22, 23, 28, 29). This study does not support any significant role for neuraminidase in the pathogenesis of meningitis once intracranial pneumococcal infection is established.

We turn now to the possible mechanisms underlying the cochlear toxicity of PLY and hearing loss in meningitis. Nitric oxide seems to be one important pathological mediator. Perfusion of the cochlea with the NO synthase inhibitor N^2-methyl-l-arginine almost completely protects it from the devastating effects of PLY (2). The pattern of cochlear damage induced by PLY perfusion or pneumococcal meningitis can also be reproduced by cochlear perfusion with the nitric oxide donor sodium nitroprusside (2). Whatever the mechanism, definite breakdown of the reticular lamina is observed only with S. pneumoniae that express PLY. Breakdown of the reticular lamina would allow mixing of cochlear fluids and hence raise the perilymphatic potassium ion concentration. This in turn would dissipate the endocochlear potential which is essential for mechanoelectrical transduction and hair cell function. The pronounced decline in CM as the CAP fell confirms impairment of OHC function. Apart from its effects on transduction, an elevated perilymphatic potassium ion concentration would in turn trigger an increase in the rate of firing of single nerve units followed by depolarization block (24). The accumulation of extracellular glutamate generated by spontaneous excitation could also cause wider pathological effects. Loss of uniformity of the CAP during wild-type meningitis suggests some impairment of afferent neural transmission. The presence of a large P0 wave (thought to reflect summed excitatory postsynaptic potentials) (16) implies that hair cell depolarization and synaptic release were in part maintained even when there was a substantial hearing deficit. Damage to nerve endings was a consistent finding even in the absence of PLY, although much of this damage was localized to nerve endings bordering OHCs and would not be expected to affect the CAP. Measurement of the endocochlear potential, cochlear fluid sampling, and single-fiber recording during experimental meningitis would shed further light on these mechanisms.

In conclusion, our data show that hearing loss in experimental pneumococcal meningitis is due to the action of the bacterial toxin PLY, which induces specific and devastating cochlear damage. Meningeal inflammation induced by PLY-deficient S. pneumoniae does not in itself result in significant cochlear damage or hearing loss. Neuraminidase-deficient S. pneumoniae appear to behave as wild-type organisms during intracranial infection. A deeper understanding of the events that lead to meningeal deafness may lead to successful adjuvant therapies that might ameliorate or even prevent this feared complication.

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REFERENCES


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