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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) ΔNA1, 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 meningeal 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; ΔNA1, 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 ΔNA1, 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 ΔNA1 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 meningeal 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 meningeal 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

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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 pneumolysin (PLN-A) (5) or neuraminidase (ΔNA1) (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 both the auditory nerve compound action potential (CAP) 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 Conference for Infectious Diseases, Hong Kong, 10 to 13 June 1996 [39a].)

MATERIALS AND METHODS

Construction of ΔNA1 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 (ΔNA1) 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 *Xba*I and *Hin*dIII. The fragment was purified by agarose gel electrophoresis, recovered by using a Band Prep kit (Pharmacia), and cloned into the vector pG+host 5 (25). This vector replicates in *S. pneumoniae* at 28°C but not at 37°C. The construct pNA1 was transformed into D39 by the method of Yother 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 BHI with 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).

Phenotypic analysis. Organisms recovered from the CSF of animals inoculated with PLN-A or ΔNA1 were tested for erythromycin resistance by susceptibility disk assay. In selected cases, single colonies were inoculated into 10 ml of BHI with or without erythromycin (5 μg/ml), incubated overnight at 37°C, and then pelleted. Cell lysates were prepared by ultrasonication. Total cell protein was determined by the Bio-Rad (Munich, Germany) microassay.

PLY activity was detected by a standard hemolytic assay. Twofold serial dilutions of the cell lysates were mixed with an equal volume of 2% sheep erythrocytes and incubated for 30 min at 37°C.

Neuraminidase activity was assayed fluorometrically by adding 20 μl of cell lysate to 200 μl of 150 μM 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUAN; Sigma) in a 100 mM citrate-phosphate buffer at pH 6.5 and incubating the mixture for 16 min at 37°C. The reaction was stopped by adding 2 ml of 0.5 M Na_2CO_3. Fluorescence was excited at a wavelength of 366 nm with an emission wavelength of 446 nm. A unit of enzyme activity was defined as the amount releasing 1 μmol of 4-methylumbelliferone from MUAN in 1 min at 37°C. The lower limit of detection of the assay was 0.5 mU/mg of cell protein while the wild-type D39 expressed about 6.4 mU/mg of cell protein. Assays were performed in duplicate on two separate occasions.

Preparation of inocula. A standardized pneumococcal inoculum was prepared by the method of Alwmark et al. (1). Erythromycin (5 μg/ml) was added to all liquid media when PLN-A or ΔNA1 was prepared to maintain selection pressure. Outbred MF1 mice were inoculated by intraperitoneal injection of 10^5 CFU of stock D39 (or its derivative) in 0.2 ml of phosphate-buffered saline (PBS; Gibco). Twenty-four hours later, blood obtained by cardiac puncture was inoculated onto Columbia agar plates. After overnight culture, four to five colonies of the mouse-passaged pneumococci were transferred into 10 ml of BHI and cultivated overnight. Bacteria were harvested by centrifugation (2,600 × *g*, 15 min, 20°C) and resuspended in fresh BHI to remove any autolysin present after overnight culture. Serum broth was prepared by adding as cryoprotectant 17% (vol/vol) heat-inactivated fetal calf serum (Gibco) to BHI and was filter sterilized (0.2-μm pore-size filter; Whatman). Fresh serum broth (10 ml) was inoculated with 400 μl of pellet suspension (final optical density at 500 nm of 0.7) and incubated for 5 h. Aliquots of 1 ml were rapidly frozen and stored at −70°C. Viable counts were performed in triplicate on one of the aliquots 24 h later. Prior to inoculation, an aliquot was defrosted at 20°C, centrifuged in a benchtop microcentrifuge (12,000 × *g*, 1 min, 20°C), washed with ice-cold sterile PBS, recentrifuged and then resuspended in fresh ice-cold PBS to an estimated concentration of 3×10^8 CFU/ml. Dilutions of the inoculum for viable counting were plated shortly after inoculation in all experiments.

Experimental meningitis. All experiments were conducted in accordance with UK Home Office regulations governing care of animals undergoing scientific procedures and were performed in an electrically screened and soundproofed laboratory. Pigmented guinea pigs of either sex weighing between 500 and 700 g were terminally anaesthetized by intraperitoneal injection of urethane (5.5 ml of 25% [wt/vol] ethyl carbamate in distilled water per kg of body weight; Sigma, Poole, United Kingdom). All animals underwent tracheotomy to protect the airway from nasal secretions. The rectal temperature was maintained at 38°C by a thermostatically controlled 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; 3×10^7 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 obtained by intracardiac puncture, and the animal was killed by brain stem destruction. 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.

Measurement of hearing loss. After tracheotomy, the temporalis and occipital muscles were reflected and the outer cartilaginous auditory meati were cut close to the skull, with careful attention to hemostasis. The animal was secured in a stereotactic frame, and two hollow brass ear bars were inserted into the meati. The posterior ridges of both auditory bullae were opened with bone forceps to expose the cochlea and the round window membrane. The middle ear cavity was kept dry by mopping with fine tissue wicks.

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; NL115), and notch filtered at 50 Hz. Sixteen sweeps of 5-ms duration were 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 (Hatfield 2105) so that the mean peak-to-peak potential of the N1-P1 wave was 2 V, equivalent to 100 μV 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 CM potential at the round window 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 processing of the 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 this fixative for at least a week. The modiolus was dissected out, dehydrated in acetone, and critical point dried in liquid CO_2. 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 TEMSCAN 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 basal surface of the first cochlear turn was upright. The specimen was then recoated with platinum. Morphological damage was assessed by high-resolution SEM (≈30 Å). In each group, seven (D39) or six (ΔNA1 and PLN-A) cochleas were examined by SEM.

For TEM, the modiolus was fixed in 2.5% (vol/vol) glutaraldehyde overnight and then postfixed in 1% osmium tetroxide for 15 min. A wedge of the organ of Corti was cut from the basal turn. This was dehydrated in graded ethanol dilutions, transferred through propylene oxide, and vacuum embedded in an Epon-substitute resin overnight at 80°C. After all bone was trimmed away, ultrathin sections were cut at 90 nm with a diamond knife, mounted on Formvar-

TABLE 1. Electrocochleographic findings 12 h after intracranial inoculation of 3×10^7 *S. pneumoniae*

Strain (*n*)	Hearing loss (measured by CAP recording)[a]				CM potential (as proportion of baseline) at 3 kHz	
	10 kHz		3 kHz			
	dB loss	Difference[b]	dB loss	Difference[b]	Median (range)	Difference[b]
D39 (15)	50 ± 9		42 ± 9		0.18 (0–0.9)	
PLN-A (10)	12 ± 6	25 to 51[c]	18 ± 4	12 to 34[c]	0.65 (0.3–1.0)	0.26 to 0.65[d]
ΔNA1 (10)	52 ± 13	−16 to 12	45 ± 13	−17 to 10	0.12 (0–0.39)	−0.24 to 0.11

[a] Numbers refer to number of cochleas assessed. Values are means ± 95% CI unless indicated otherwise.
[b] 95% CI for the differences between the means or medians compared to D39 group.
[c] $P < 0.0001$ by Mann-Whitney *U* test.
[d] $P = 0.0003$ by Mann-Whitney *U* test.

coated grids, and stained with uranyl acetate and lead citrate in a CKB Ultrostainer. Grids were examined in a JEOL 120CXII TEM at an accelerating voltage of 60 or 80 kV. In each group, one (D39) or two (ΔNA1 and PLN-A) cochleas were examined by TEM.

Statistical analysis. Animals were assigned to one of three groups and inoculated with D39 ($n = 8$), PLN-A ($n = 5$), or ΔNA1 ($n = 5$). The major quantitative endpoint was the difference between CAP loss induced by wild-type D39 and that induced by PLN-A or ΔNA1 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 ΔNA1, 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 ΔNA1 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 ΔNA1, 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 P_0 wave and identical N_1-P_1 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 P_0 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 ΔNA1 (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 ρ, −0.7; 95% CI for ρ −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 3×10^7 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 meningeal 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 ΔNA1 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

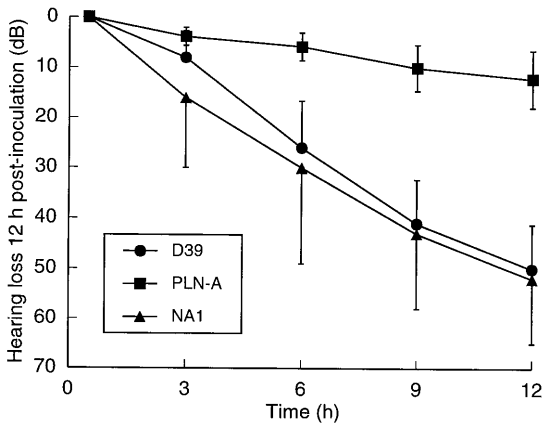

FIG. 1. Rate of hearing loss at 10 kHz after intracranial inoculation of guinea pigs with 3×10^7 CFU of *S. pneumoniae* (as judged by round window CAP recording). Bars represent 95% CI. PLN-A causes a significantly slower hearing loss than D39 ($P < 0.0001$ by ANOVA).

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

Strain (*n*)	Viable counts ($\log_{10}$ CFU/ml)			CSF parameters	
	Inoculum	CSF	Blood	Leukocytes ($\log_{10}$/ml)	Protein (g/liter) (median [range])
D39 (8)	7.5 ± 0.2	8.7 ± 0.5 (*n* = 6)	6.6 ± 0.5 (*n* = 5)	4.0 ± 0.5 (*n* = 6)	2.1 (0.4–4.0) (*n* = 6)
PLN-A (5)	7.5 ± 0.3	8.1 ± 0.4	6.0 ± 2.9 (*n* = 4)	3.7 ± 0.3	0.7 (0.4–0.8)[b]
ΔNA1 (5)	7.5 ± 0.0	9.0 ± 0.3 (*n* = 4)	6.1 ± 1.1 (*n* = 3)	4.3 ± 0.3 (*n* = 4)	2.4 (1.1–3.9) (*n* = 4)

[a] Unless stated otherwise, values are means ± 95% CI.
[b] Significantly different ($P = 0.04$) from value for D39 group by Mann-Whitney *U* test.

inoculated intracranially with PLN-A or ΔNA1 were fully resistant to erythromycin. Organisms recovered from the CSF and blood of animals inoculated with PLN-A had no detectable 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 transmission electron micrographs are shown in Fig. 2 and Fig. 3, respectively. There was clear evidence of ultrastructural damage to the organ of Corti in animals inoculated with D39 or ΔNA1. This was most pronounced in the basal turn and affected 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. Irrespective of the genotype of the inoculum, all animals sustained a labyrinthitis, and by SEM, organisms attached to the scala tympanic surface of the basilar membrane could be demonstrated. 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 bundles. 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 cuticular 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 inoculated with PLN-A, the organ of Corti appeared virtually normal 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 induced 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 model 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 surface of hair cells and supporting cells, and extrusion of hair cells from their adjacent supporting cells (13). When we induced meningitis with PLN-A, there was almost no ultrastructural 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 pneumococcal 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 leukocytes 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 inflammatory 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-

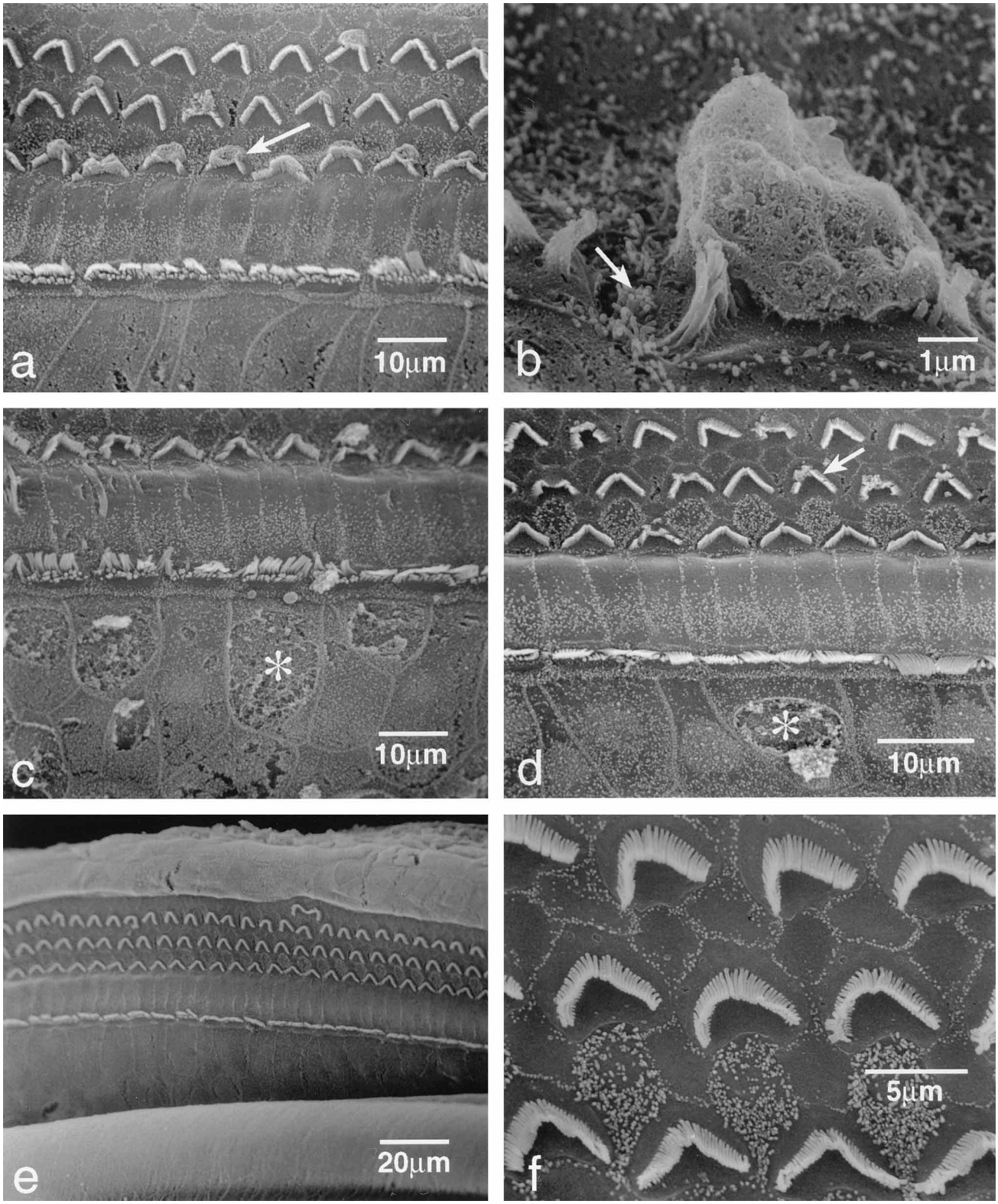

FIG. 2. Scanning electron micrographs of the apical surface of the basal turn of the organ of Corti in guinea pigs 12 h after intracranial inoculation of 3×10^7 CFU *S. pneumoniae*. (a) D39. There is extensive damage to many OHCs, with ballooning of the apical surface and destruction of the stereocilial 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) ΔNA1. 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.

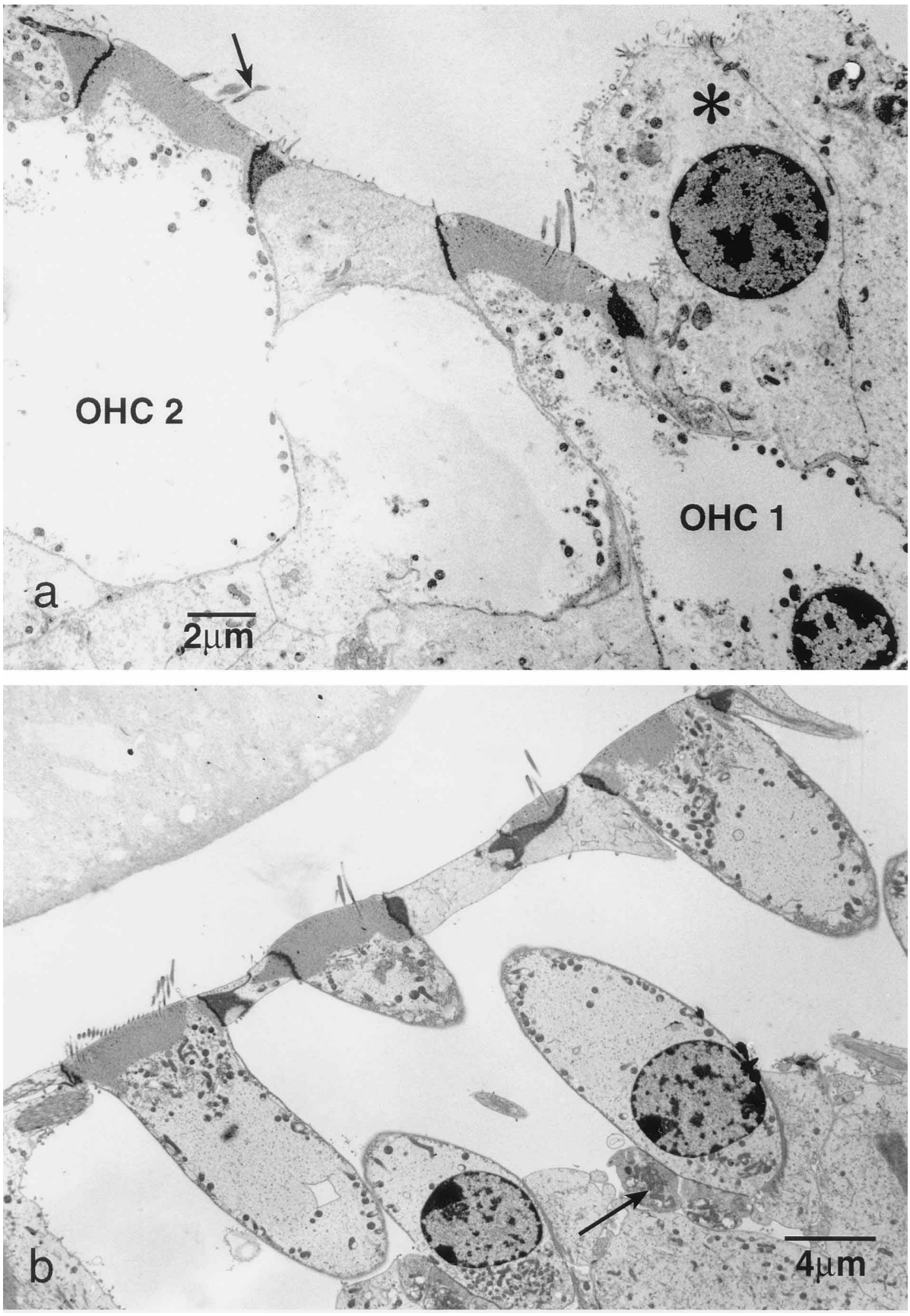

FIG. 3. Transmission electron micrographs of the basal turn of the organ of Corti 12 h p.i. prepared in the same way as for Fig. 2. (a) D39. The row 2 OHC on the left is swollen with defects in its plasma membrane and pathological stereocilia (arrow). Mitochondria in both hair cells and neighboring supporting 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 intracisternal 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 electrocochleographic 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 electrocochleographic 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 ΔNA1 in terms of meningeal 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 meningogenic 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^G-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 P_0 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 meningogenic deafness may lead to successful adjuvant therapies that might ameliorate or even prevent this feared complication.

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