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An in vivo tracer study of noise-induced damage to the reticular lamina[1]

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Abstract

An in vivo tracer was used to determine if the reticular lamina and/or the cell membranes abutting the endolymphatic space are temporarily disrupted after intense noise exposure (4-kHz OBN, 108-dB SPL, 1.75 h). Using a double-barreled micropipette, the endolymphatic potential (EP) was recorded and artificial endolymph containing 10% carbon particles was injected into the endolymphatic space either 0 days or 28 days post-exposure. The cochleae were fixed 30–45 min post-injection, then dehydrated, embedded in plastic and dissected as flat preparations. Damage in the organ of Corti (OC) was quantified, the location of carbon was determined, and some OC segments were then sectioned radially. EP averaged 72 ± 5 mV in five controls. These cochleae had carbon tracer in the endolymphatic space only. Four of five noise-exposed chinchillas examined 3–4 h post-exposure had a low EP (30 ± 6 mV). The cochleae from these 0-day animals had several focal lesions in which nearly all outer hair cells had just degenerated. At these lesions, carbon was attached to cell membranes and debris between the reticular lamina and basilar membrane. By transmission electron microscopy, discontinuities were found in the apical membranes of sensory and supporting cells. Carbon particles were found in the cytoplasm of these cells. Four of five animals examined at 28 days had an average EP of 70 ± 11 mV. The cochleae from these animals had multiple lesions in the basal turn, all of which were healed by phalangeal scars or squamous epithelial cells. In these cochleae, no carbon was found within the OC. Acute disruption of the reticular lamina and the apical membranes of sensory and supporting cells from noise appears to be a major mechanism to account for degeneration in the cochlea that spreads or continues for days to weeks post-exposure.

Key words: Noise; Tracer; Endolymphatic potential; Holes in reticular lamina; Membrane discontinuities; Chinchilla

1. Introduction

The apical membranes of hair cells and phalangeal processes of supporting cells form the reticular lamina of the organ of Corti (OC) (e.g. Engström et al., 1966; Bohne, 1976a). The reticular lamina serves as a boundary between two fluids with very different ionic compositions (e.g. Jahnke, 1975; Salt and Konishi, 1986; Ikeda and Morizono, 1990). Endolymph has a high potassium and a low sodium concentration and bathes the apical surface of the OC, including the hair-cell stereocilia. The fluid within the OC (sometimes called Cortilymph; Engström, 1960) has a low potassium concentration (Johnstone et al., 1989), fills the tunnel and spaces of Nuel in the OC and bathes the basolateral surfaces of the hair cells. Within the OC, the plasma membranes of hair cells and supporting cells are joined at their apices by tight junctions (Jahnke, 1975; Gulley and Reese, 1976). The cell membranes and tight junctions at the surface of the reticular lamina prevent the intermixing of endolymph and the fluid in the OC.

When damaged hair cells degenerate, they are replaced in the reticular lamina by phalangeal scars. De-

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Abbreviations: dB, decibel; EP, endolymphatic potential; ES, endolymphatic space; IHC, inner hair cell; MNF, myelinated nerve fibers; OBN, octave-wide band of noise; OC, organ of Corti; OHC, outer hair cell; PTS, permanent threshold shift; SPL, sound pressure level; StV, stria vascularis; TEM, transmission electron microscopy

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pending on which hair cell is degenerating, the phalangeal processes from two to three Deiters' cells, one to two outer pillar cells, or two inner phalangeal cells enlarge to form the scar (e.g. Engström et al., 1966; Bohne, 1976a). If the cochlea sustains so much damage that all sensory and supporting cells in a particular area degenerate, a region of total loss of the OC or an 'OC wipeout' will develop. Because all supporting cells degenerate in OC wipeouts, phalangeal scars cannot form. Rather, a squamous epithelial scar replaces the missing OC on the basilar membrane and seals off the open ends of the tunnel space.

The question of whether or not holes develop in the boundary between endolymph and the OC fluid spaces as hair cells are degenerating has been debated for more than 30 years. There are published data that either refute this notion (e.g. Lim and Melnick, 1971; Hunter-Duvar et al., 1982; Forge, 1985; McDowell et al., 1989; Raphael and Altschuler, 1991) or support it (e.g. Bohne, 1971; Lim, 1976; Bohne and Rabbitt, 1983; Fredelius et al., 1988). None of these previously published studies is definitive because a morphological examination of fixed tissue cannot assess the in vivo integrity of the reticular lamina.

It is important to determine if there is a temporary breakdown of the reticular lamina during and/or shortly after exposure to noise. A breakdown of the reticular lamina would result in the intermixing of cochlear fluids and may partly account for continuing loss of sensory and supporting cells long after the noise has terminated (i.e. secondary degeneration (Bohne and Harding, 2000)). If this hypothesis is true, it may be possible to develop treatment strategies to limit secondary degeneration and minimize the extent of noise-induced hearing loss.

The present study was designed to test the hypothesis that reticular lamina holes develop and endolymph leaks into the OC after a severe noise exposure. At two time intervals post-exposure (i.e. 0 and 28 days), the endolymphatic potential (EP) was recorded and tracer particles were injected into the endolymphatic space of anesthetized chinchillas. The cochleae were then fixed and prepared for examination by phase-contrast, bright-field and transmission electron microscopy (TEM) to determine the distribution of the tracer within the fluid spaces of the cochleae and cells of the OC.

2. Materials and methods

2.1. Tracer

The tracer was a 10% suspension of carbon particles in water (Carbon Dispersion #8, Faber-Castell). The carbon suspension was suction-filtered (Whatman #42 filter paper), placed into sterile vials, and sterilized in an autoclave. Prior to use in experiments, appropriate amounts of crystalline potassium chloride and potassium bicarbonate were added to make a final concentration of 140 mM and 25 mM, respectively. The concentrations of these electrolytes are approximately equal to those in cochlear endolymph (Salt and DeMott, 1997).

2.2. Microelectrodes

Double-barreled glass microelectrodes were made using an electrode puller (Narashige PD-5) and beveled to the appropriate tip size using a diamond-coated sharpening wheel (Narashige EG-40). For measurement of the first-turn EP and carbon injection, the tip diameter was approximately 15–18 μm. This tip size was chosen because it was small enough to avoid significant damage to the stria, and large enough to minimize clogging of the tip by carbon clumps. For measurement of the third-turn EP, a single-barreled electrode with a tip size of 5–7 μm was used.

2.3. Animals

One- to 2-year-old chinchillas (either sex) were purchased commercially (Moulton Chinchilla Ranch, Rochester, MN, USA and Ryerson Chinchilla Ranch, Plymouth, OH, USA) and maintained in a quiet animal facility prior to their use in the study. The study described here was reviewed and approved by Washington University's Animal Studies Committee (Protocols #97100 and #20000131; B.A. Bohne, PI).

Five chinchillas served as controls for EP determination and carbon injection. Five chinchillas were exposed to noise and were then prepared for EP measurement and carbon injection 2–4 h post-exposure (0-day group). Five chinchillas were exposed to noise and then recovered 28 days (28-day group) before undergoing EP measurement and carbon injection.

2.4. Noise exposure

The exposure was an octave-wide band of noise (OBN) with a center frequency of 4 kHz, presented for 1.75 h at a sound pressure level (SPL) of 108 dB (free field). The exposures took place in a reverberant booth. Individual chinchillas were placed in a wire-mesh cage and tethered using an aluminum collar that was attached to a wire coil running to the top of the cage. The tethering prevented the animal from lying on its side and occluding one ear canal, but at the same time, allowed freedom of movement within the cage. The noise level measured 108 ± 2 dB SPL (Brüel and Kjær sound level meter #2203) within the exposure cage.

2.5. EP measurement and carbon injection

Animals were anesthetized with a mixture of ketamine (40 mg/ml), acepromazine (1.0 mg/ml), and atropine (0.04 mg/ml), given intramuscularly at a dosage of 1 ml/kg body weight. Supplemental doses (0.5 ml/kg body weight) were given as needed throughout surgery, EP measurement, and carbon injection.

The left cochlea was surgically exposed through an opening in the ventral bulla. In the first turn, the cochlear bone was gradually thinned, and a 25–30-μm-diameter hole was made over the spiral ligament and stria vascularis at approximately 20% distance from the basal tip of the cochlea. In some cochleae, another hole was made into the endolymphatic space of the third turn.

Once the holes were made, the animal's head was secured in a holder. The EP electrode was inserted into a silver chloride conducting cell (attached to a micromanipulator; Narashige #6475), which was connected to a signal amplifier (WPI, M701 Micro-probe System). The amplifier output was sent simultaneously to a Tektronix 7603 oscilloscope and a Fluke 75 Series II voltmeter. The circuit was completed by a silver chloride ground electrode in contact with a cotton wick soaked with lactated Ringer's solution that was placed in the neck musculature. The EP in the third turn was measured using a single-barreled electrode which was filled with artificial endolymph (Salt and DeMott, 1997). The electrode was slowly advanced into the hole until a positive EP was recorded, and then advanced until a stable plateau was maintained.

For the first-turn recordings, the EP barrel of the electrode was filled with artificial endolymph, and the other barrel was filled with the carbon suspension. The electrode was advanced into the hole until a positive EP was recorded and reached a stable plateau. At this point, 50–150 nl of the carbon suspension was manually injected over a period of 1 min. EP continued to be recorded for 30–45 min after the injection to monitor the effects of the injection and the carbon suspension on EP, and to allow the carbon particles to diffuse through the endolymphatic space.

2.6. Cochlear fixation, processing and dissection

The cochlear fixation and processing techniques were similar to those published previously (Bohne, 1972; Bohne and Harding, 1993). Briefly, the cochleae were fixed by a 5-min perfusion of fixative through scala tympani from base to apex. The fixative was a 1% solution of osmium tetroxide in Dalton's buffer with 1.65% calcium chloride. After removal from the head, the cochleae were immersed in vials of cold fixative for 2 h. They were then washed in cold Hank's balanced salt solution, immersed in 70% ethanol, and stored overnight in a refrigerator.

Prior to dehydration and plastic infiltration (i.e. Durcupan), holes were made in the cochlear bone over scala tympani at the base and over scala vestibuli at the apex. In addition, the stapes footplate and the semicircular canals were removed. These openings into the cochlear fluid spaces allowed adequate fluid exchange during dehydration and embedding. Care was taken to avoid mechanically violating scala media until the cochleae had been embedded and the plastic polymerized. After polymerization of the plastic, the cochlear bone was removed and the cochleae were dissected into flat preparations. All segments of the cochlear duct were examined by phase-contrast and bright-field microscopy (Wild M-20) at 625× or 1250×.

2.7. Preparation of specimens for thin sectioning

Selected segments from control and damaged cochleae were sawed out of the plastic blocks and decalcified in 5% trichloracetic acid for 5 days. After neutralizing in 5% sodium sulfate and washing in 50% and 70% ethanol, the decalcified segments were dried overnight at 60°C. These segments were then re-embedded in Durcupan.

Each segment was semi-thin (2.5–10 μm) and ultra-thin (90 nm) sectioned at a radial angle with an ultramicrotome (MTX, RMC Products). The semi-thin sections were mounted on glass slides and examined by phase-contrast and bright-field microscopy. The ultra-thin sections were mounted on formvar-coated mesh grids and examined by TEM (Hitachi H-7500). None of the sections were stained to avoid masking carbon particles with stain deposits. Because the semi-thin sections were unstained, phase-contrast microscopic evaluation was necessary to see cell membranes and intracellular detail. While carbon was easily seen under the microscope, the contrast on the phase-contrast photomicrographs had to be increased to make the small clumps visible. This digital adjustment produced prominent phase 'halos' and made the photomicrographs appear a little out-of-focus. Bright-field examination of the same sections permitted the identification of intracellular accumulations of carbon.

2.8. Quantitative histological evaluation

In each cochlea, the lengths of all segments of the OC were measured along the junction of the pillar heads using a computer with a video frame grabber board (Data Translations DT2871) and image acquisition software (Foster-Findley). An image of each segment was acquired using a color video camera (Javelin JE3463RGB) attached to a zoom microscope (Nikon

SMZ-U) at approximately 70×. The electrode penetration sites were also located, and their percentage distance from the basal tip of the OC determined.

By phase-contrast microscopy, missing inner hair cells (IHCs), outer hair cells (OHCs), and pillar cells were counted and focal hair-cell lesions identified. A focal hair-cell lesion has been defined as any contiguous region in the OC equal to or greater than 0.03 mm in length in which hair-cell loss is 50% or greater (Bohne and Clark, 1982; Bohne et al., 1990). When present, nerve fiber degeneration in the osseous spiral lamina was also noted. Finally, the base-to-apex distribution of carbon in the endolymphatic space was determined. This information was compiled into a cytocochleogram for each cochlea.

2.9. Qualitative histological evaluation

From the reticular lamina to the basilar membrane, the OC was assessed in the flat preparations by focusing at sequential levels (i.e. optical sectioning) through the organ. The apical surface, cytoplasm, plasma membrane, and nucleus of each hair cell and supporting cell were evaluated for degenerative changes. The nerve fiber bundles within the OC were evaluated for signs of damage, especially swelling and rupture. Special attention was paid to the distribution of carbon within the OC. More detailed information about carbon distribution in the cells and fluid spaces of the OC was obtained from the evaluation of the semi-thin and ultra-thin radial sections.

3. Results

3.1. Tracer

The carbon suspension has several characteristics that made it ideal for this study:

- The particles are small, ranging from 5 to 90 nm in diameter (Fig. 1A).
- The particles are non-toxic in the short term.
- The particles are naturally blue-black in color. Thus, they can be seen by light microscopy without staining or treatment with an incubation medium.
- The particles are electron-dense. Fig. 1A shows a TEM of a dilute solution of carbon particles dried on a formvar-coated grid.
- When making infiltration holes in the cochlear bone, it was noted that the endolymphatic space in the basal turn was very dark. After dehydration and infiltration, the endolymphatic space was not as dark, but was considerably darker than the non-carbon-injected cochleae. Thus, some carbon that was free-floating in the endolymphatic space was washed away during processing. However, much carbon is retained throughout the histological processing technique. In an unpublished study, a solution of carbon particles in artificial perilymph was perfused through scala tympani for 2 h. The cochlea was then fixed, dehydrated, embedded in plastic, thin-sectioned and examined by TEM (Fig. 1B). The 'processed' carbon particles (Fig. 1B) looked very similar to the dried carbon particles (Fig. 1A) and tended to cluster in small aggregations.
- The carbon does not form large, irreversible aggregations in artificial endolymph. When a drop of the carbon suspension in artificial endolymph was viewed by light microscopy, the small carbon aggregations were observed to be in constant Brownian motion. The aggregations adhered to one another briefly (mediated by van der Waals forces) and then split apart.

In a preliminary experiment, the carbon suspension was placed in a beaker of artificial endolymph, and a DC electric field was established by placing positive and negative electrodes on opposite sides. Carbon was deposited on the negative electrode, indicating that the particles had a positive charge. Thus, carbon injected into the endolymphatic space was expected to adhere to surfaces and structures with a negative charge (e.g. tectorial membrane; Thalmann et al., 1993).

3.2. Microscopic identification of carbon

Because individual carbon particles are only 5–90 nm in diameter, they cannot be resolved by phase-contrast or bright-field microscopic examination of either flat preparations or radial sections. However, small aggregations of carbon particles could easily be seen in the flat preparations and in semi-thin radial sections by phase-contrast microscopy. Examination of this material by bright-field microscopy augmented the view of carbon because the visibility of the cells was attenuated while the carbon appeared very black.

The extent of carbon spread in the endolymphatic space was determined in two ways. First, the cochleae were examined under the dissection microscope immediately after the fixative was washed out. Carbon was seen to have spread all the way to the basal end of the endolymphatic space and sometimes filled the saccule. Second, the dissected segments of the cochlear duct were examined as flat preparations by phase-contrast microscopy after the cochleae had been dehydrated and embedded in plastic. The extent of carbon spread towards the apex was determined by noting how far apically carbon could be seen on the upper surface of the tectorial membrane. In cochleae in which EP was measured in the first turn only, the carbon was found at about 50% distance from the apical end of the endo-

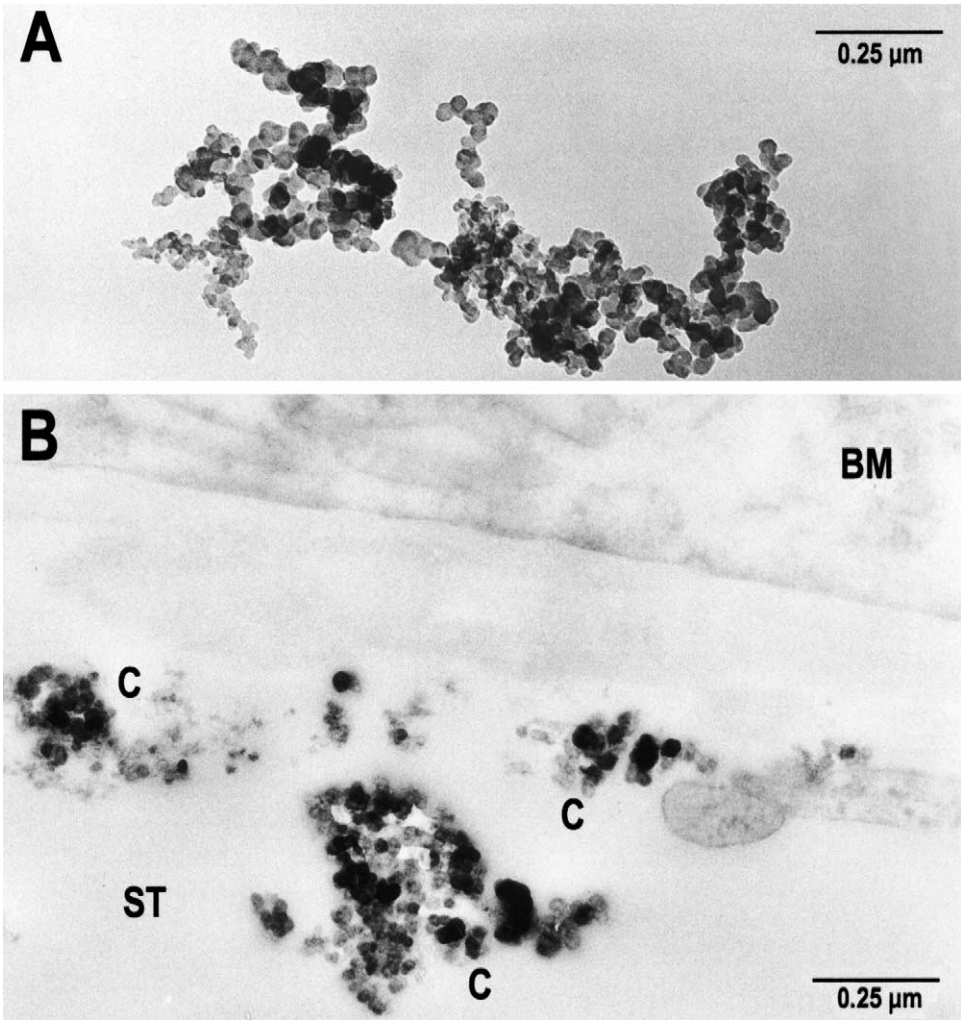

Fig. 1. (A) TEM showing carbon particles dried on formvar-coated grid. (B) TEM showing carbon particles ('C') below the basilar membrane (BM) after the cochlea was fixed, dehydrated, plastic-embedded and thin-sectioned. The dried carbon particles in A are variable in size, ovoid, and electron-dense. The 'processed' carbon particles in B in scala tympani (ST) have a similar appearance.

lymphatic space, presumably spreading from the injection site (i.e. 80% distance from the apex) by passive diffusion. In cochleae in which EP was determined in both the first and third turns, carbon was found at 5–17% distance from the apical end of the endolymphatic space, presumably because the injected solution displaced endolymph.

The distance that carbon diffused in the endolymphatic space prior to fixation cannot be determined. Likewise, the question of whether or not carbon shifted locations during fixation, dehydration and plastic infiltration cannot be answered unequivocally. However, it is unlikely that much carbon relocation occurred during these processing steps because of the difference in carbon distribution between cochleae with one electrode hole in the endolymphatic space versus two holes. All cochleae were dehydrated and embedded in plastic in the same fashion, yet carbon distribution in the endolymphatic space differed considerably between cochleae with one versus two EP recording sites.

3.3. Control group

The EP remained fairly stable throughout the 30–45 min recording session, indicating that the carbon solution was not toxic. The mean EP in the first turn of the cochlea was 72 ± 5 (S.D.) mV (upper third of Table 1). These cochleae had low-level loss of hair cells that was scattered throughout the OC. From the injection site in the first turn, carbon was found to have spread basally and apically over approximately 50% of the endolymphatic space.

In the flat preparations, the sensory and supporting cells of the OC had appearances typical of unexposed cochleae. The stria vascularis had no gross abnormalities. All the nerve fibers were intact. There were no

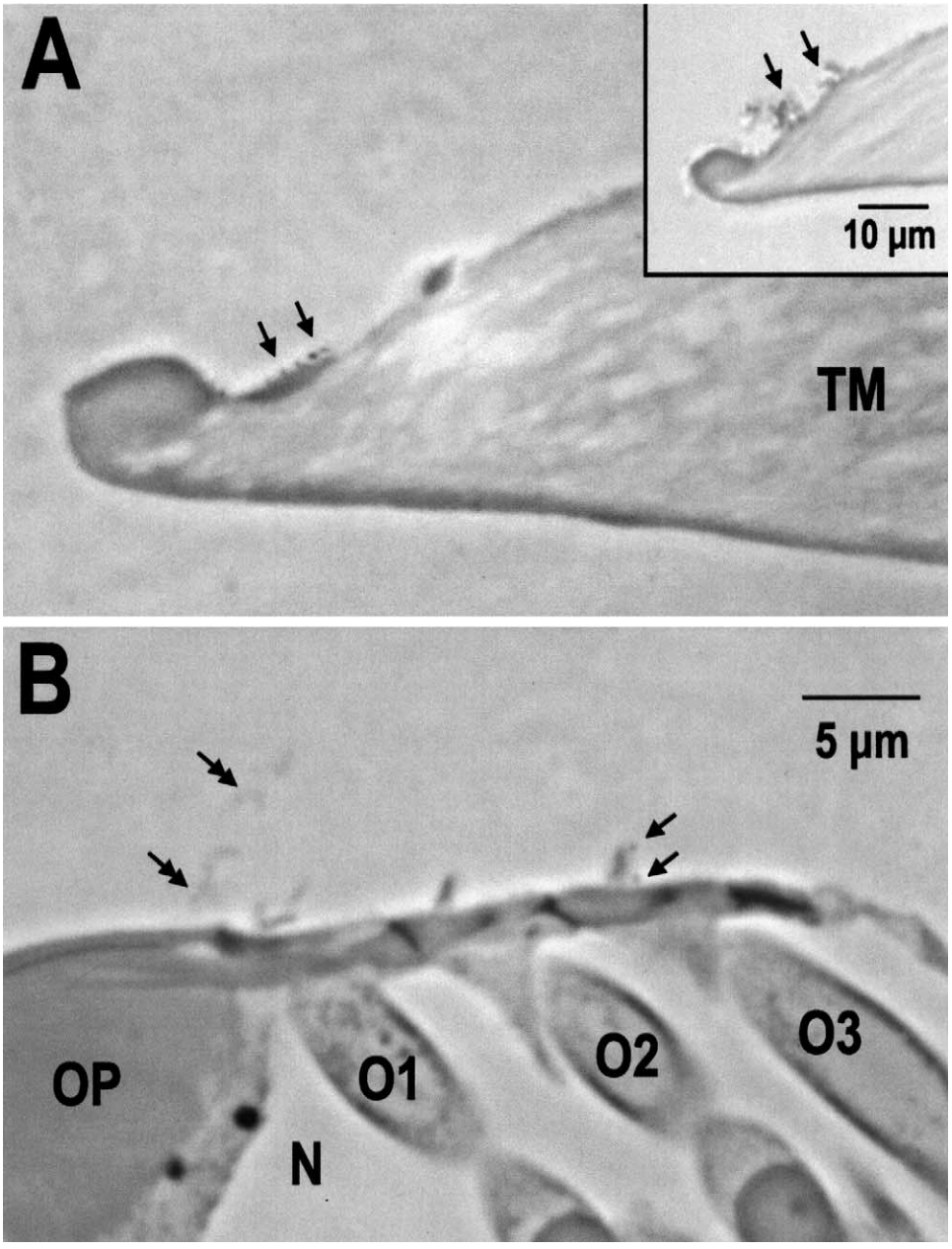

Fig. 2. Phase-contrast photomicrographs of 2.5-μm-thick radial sections (unstained) of the cochlear duct from control chinchilla MA 7L at 78% distance from apex. (A) Arrows point to several small clumps of carbon (i.e. dark dots) on the upper surface of the tectorial membrane (TM). The inset shows a 10-μm-thick section in which more carbon (arrows) is visible. (B) Single arrows point to two small carbon clumps attached to the stereocilia on the third OHC (O3). Several clouds of carbon (double arrows) are out-of-focus in the endolymphatic space. No carbon is seen within the Nuel (N) spaces of the OC. O1, O2 – first and second row OHCs, respectively; OP – outer pillar head.

abnormalities in the OC at the injection site. Carbon was attached to the tectorial membrane, on the inner surface of Reissner's membrane, on the reticular lamina, and on the endolymphatic surfaces of inner sulcus and Claudius' cells.

In radial sections, the pillar bodies were occasionally slightly distorted but they had normal nuclei. The sensory cells were entirely normal. Carbon was seen within the endolymphatic space, especially on the upper surface of the tectorial membrane (Fig. 2A), but not within the OC fluid spaces (Fig. 2B). Because most of the sections viewed by light microscopy were 2.5-μm thick, only a small amount of carbon was visible in any given semi-thin section. By TEM, cell membranes at the reticular lamina were intact (Fig. 3). Carbon particles were seen on the endolymphatic surface of the reticular lamina (Fig. 3), but not within the cells or between cell junctions.

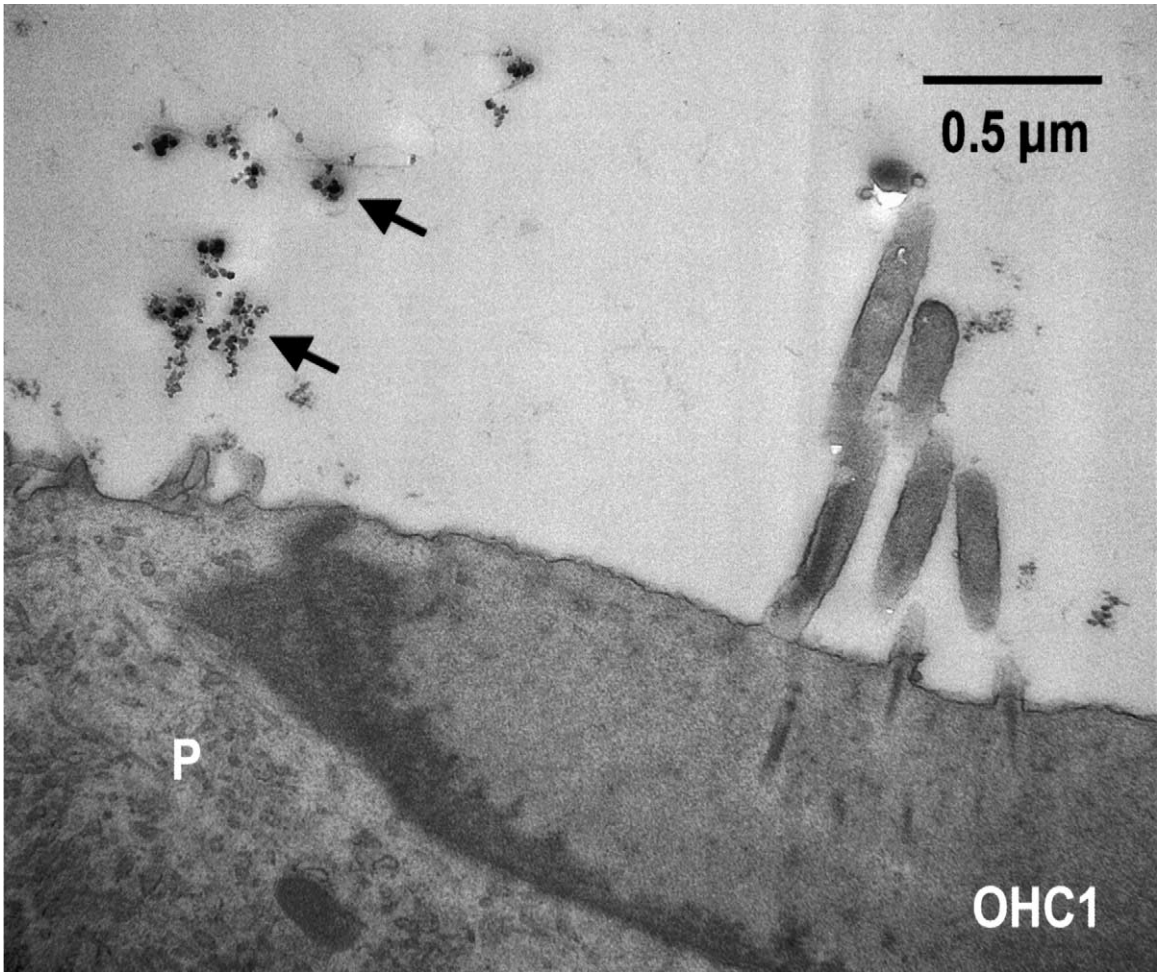

Fig. 3. TEM of control cochlea MA 7L at same location as Fig. 2 showing the surface membrane and three stereocilia on the first outer hair cell (OHC1) and the phalangeal process (P) of an outer pillar cell. Carbon particles (arrows) are visible in the endolymphatic space. The cell membranes bounding the endolymphatic space are continuous.

3.4. 0-Day group

In four cochleae, the first-turn EP averaged 30 ± 6 mV. In MA 11L, the EP was comparable to that in the control group (see histopathological explanation below). If the EP from MA 11L is averaged with that of the other 0-day cochleae, the mean is 39 ± 21 mV (middle third of Table 1), which is still significantly decreased compared to controls ($P = 0.010$). The apical EP in MA 18L was found to be lower (i.e. 35 mV) than the apical EP (i.e. 55 mV) in one control (MA 17L).

Fig. 4 is a cytocochleogram from 0-day cochlea MA 12L that shows the site of EP measurement and carbon injection (i.e. downward-pointing arrow in StV bar), the distribution of carbon in the endolymphatic space (i.e. grayed region in ES bar) and the location(s) of carbon below the reticular lamina (i.e. asterisks in OC bar). Inner and outer hair-cell losses and regions of nerve fiber degeneration (i.e. MNF loss bar) are also shown. There were five focal lesions consisting of 74–100% OHC loss in the basal half of this cochlea. Carbon had penetrated into the OC only at the sites of these focal lesions, as determined in the flat preparations.

Three cochleae (MA 12L, MA 14L, MA 16L) had several focal OHC lesions in the basal half of the OC. The number of focal lesions ranged from four to six, and their sizes ranged in length from 0.04 to 0.90 mm. MA 18L had one confluent 6.59-mm OHC lesion and carbon was seen in the OC along its entire length. MA 11L was different from the others in the group in that it sustained only one 0.28-mm OHC lesion in the base. Carbon was seen in the OC only at the locus of the focal lesion. None of these cochleae had an OC wipe-out.

Except for MA 18L, the OHC lesions in the 0-day cochleae were focal and discrete. Areas of particularly severe OHC loss often had missing outer pillar cells. In a few sites, several contiguous OHCs, outer pillars, inner pillars, and IHCs were missing, resulting in large

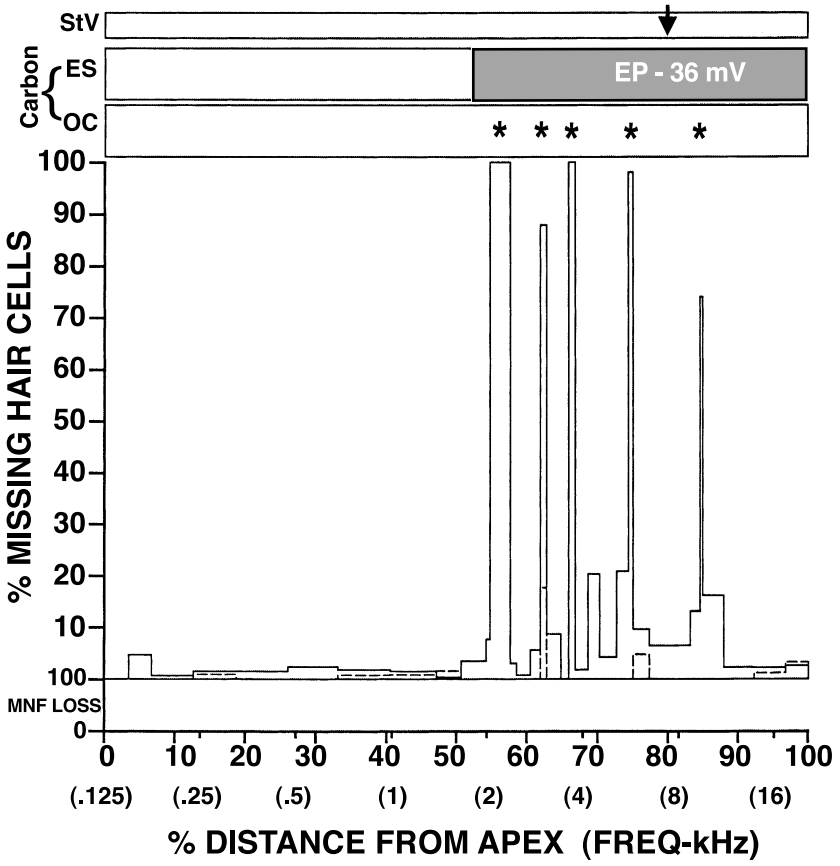

Fig. 4. Cytocochleogram depicting function and structure of 0-day cochlea MA 12L. The five *y*-axes show from top to bottom: (1) StV – Stria vascularis. Downward-pointing arrow indicates site of EP measurement and carbon injection; (2) ES – Endolymphatic space. The grayed region indicates the extent of carbon spread from the injection site; (3) OC – Asterisks indicate location(s) of carbon within the organ of Corti; (4) % missing hair cells – IHCs (dashed line) and OHCs (solid line); (5) MNF LOSS – Percentage loss of myelinated nerve fibers in the osseous spiral lamina. The *x*-axis shows percentage distance from the OC apex and its corresponding frequency-place (Eldredge et al., 1981). There were five focal OHC lesions ranging in size from 0.07 to 0.55 mm. Carbon had penetrated into the OC at each of these lesions. Carbon spread throughout the basal half of the endolymphatic space. No myelinated nerve fibers were missing.

lesions (i.e. holes) in the reticular lamina (Fig. 5A). The OHCs that were immediately adjacent to these lesions were quite swollen and had enlarged, pale-staining nuclei. The radial tunnel fibers were ruptured and the radial afferent nerve fibers beneath the IHCs were swollen. Optical sectioning showed carbon from the rim of the lesion at the level of the reticular lamina down to the basilar membrane, including surrounding swollen radial afferents (Fig. 5B). Swollen nerve fibers beneath the IHCs were confirmed in radial sections. This type of lesion is characteristic of developing degeneration that is secondary to the initial noise damage (Bohne and Harding, 2000).

Radial sections from areas between focal hair-cell lesions revealed damaged OHCs that were swollen, distorted and/or displaced. The inner and outer pillars were moderately buckled, resulting in flattening of the OC. No carbon was seen in the OHCs by light microscopy at these locations. Sections from focal lesions showed OHCs that were necrotic or had ruptured basolateral membranes. Occasionally, the cuticular plate of one or more of these cells had been displaced, which resulted in the formation of OHC-sized holes in the reticular lamina. Many of the displaced cuticular plates were covered with carbon. Debris from these cells was present in the OC fluid spaces and above the reticular lamina (Fig. 6A). Substantial amounts of carbon could be seen in the OC where the OHCs had degenerated, including inside the degenerating pillars, and attached to cellular debris and damaged radial tunnel fibers (Fig. 6B).

3.5. 28-Day group[2]

In four cochleae, EP in the first and third turns was

[2] In this group, auditory brainstem response threshold shifts and distortion-product otoacoustic emission level shifts were determined at 0, 7 and 28 days post-exposure. These results are included in Harding et al. (2002).

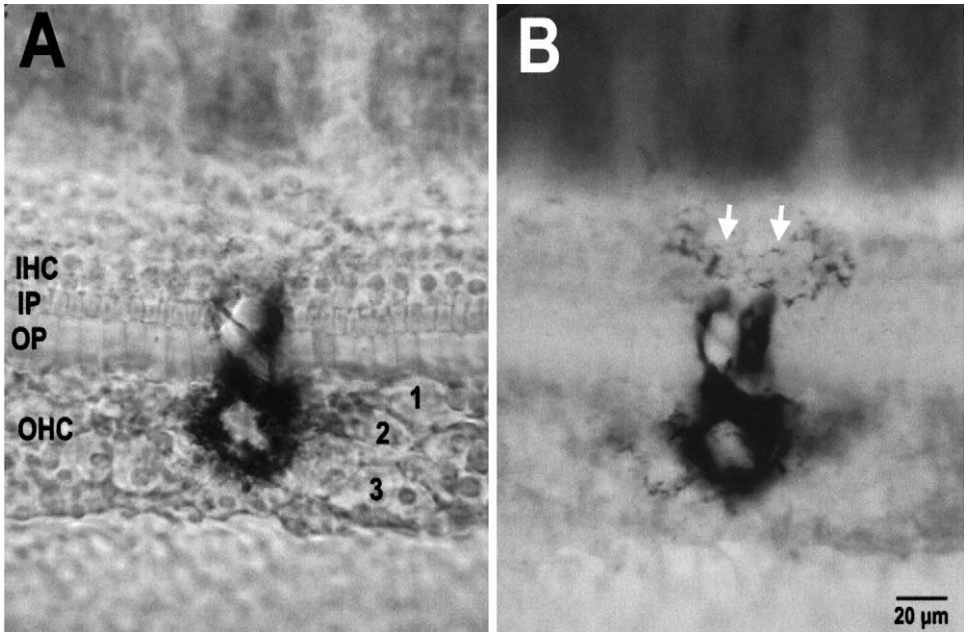

Fig. 5. (A) Phase-contrast photomicrograph from 0-day cochlea MA 12L at 61.9–62.8% distance showing a lesion in the reticular lamina in which outer hair cells (OHC), inner hair cells (IHC), inner pillars (IP) and outer pillars (OP) are missing. The lesion is surrounded by necrotic outer hair cells (1, 2, 3). (B) Bright-field photomicrograph of the same lesion showing carbon distribution in and around the large hole in the reticular lamina and beneath the inner hair cells surrounding swollen afferent nerve fibers (white arrows).

similar to that in controls. The mean EP was 65 ± 12 mV in the apex and 70 ± 11 mV in the base, which was significantly greater than in the 0-day cochleae ($P = 0.001$). On the other hand, EP in MA 21L was comparable to the 0-day group (see histopathological explanation below). If the data from MA 21L are averaged with the other four, the mean EP was 60 ± 16 mV in the apex and 65 ± 16 mV in the base (lower third of Table 1), which was not quite significantly greater than in the 0-day group ($P = 0.065$). The mean EP in the base

Table 1
EP and percent missing hair cells in the apex and base

Cochlea	Apex (0–50%)			Base (50.1–100%)		
	EP (mV)	% Missing IHC	% Missing OHC	EP (mV)	% Missing IHC	% Missing OHC
Controls						
MA 6L	–	0.9	3.4	74	0.2	2.0
MA 7L	–	0.8	3.8	79	1.2	1.1
MA 8L	–	2.3	4.7	74	1.5	2.1
MA 9L	–	0.5	3.1	66	0.1	0.8
MA 17L	55	0.6	3.2	67	1.1	1.0
Mean ± S.D.	–	1.0 ± 0.7	3.6 ± 0.7	72 ± 5	0.8 ± 0.6	1.4 ± 0.6
0-Day						
MA 11L	–	1.1	1.5	76	0.6	3.7
MA 12L	–	0.4	1.6	36	0.9	17.1
MA 14L	–	0.2	2.1	34	1.2	32.1
MA 16L	–	0.3	5.0	23	6.4	19.7
MA 18L	35	0.4	4.9	28	6.0	54.5
Mean ± S.D.	–	0.5 ± 0.4	3.0 ± 1.8	39 ± 21	3.0 ± 2.9	25.4 ± 19.1
28-Day						
MA 20L	80	0.4	10.5	66	8.9	71.3
MA 21L	38	0.3	11.3	42	14.0	47.2
MA 23L	53	0.3	10.5	78	18.9	92.2
MA 24L	60	11.1	28.1	57	29.2	95.7
MA 25L	67	0.4	2.4	80	4.7	15.1
Mean ± S.D.	60 ± 16	2.5 ± 4.8	12.6 ± 9.4	65 ± 16	15.1 ± 9.5	64.3 ± 33.6

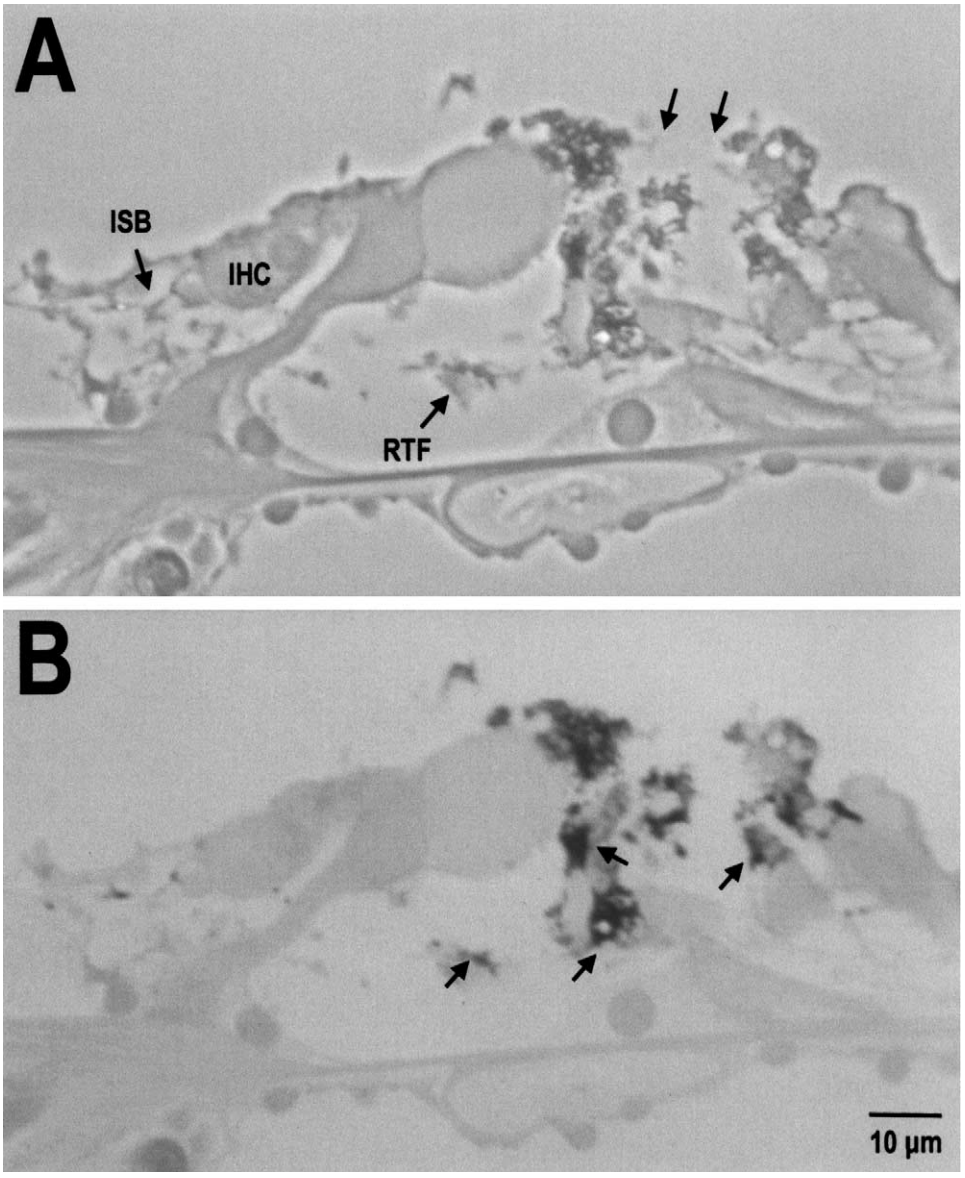

Fig. 6. Photomicrographs of a 2.5-μm-thick radial section (unstained) from 0-day cochlea MA 12L at 62.4% distance from apex. (A) Phase-contrast view shows that the inner hair cell (IHC) and inner pillar are intact. The reticular lamina is ruptured (arrows) where all outer hair cells have just degenerated. Much cellular debris is lateral to the outer pillar head. Radial tunnel fibers (RTF) and nerve fibers beneath the IHC are swollen. (B) Bright-field view shows much carbon (arrows) below the severely damaged reticular lamina, attached to the cellular debris, outer pillar body and radial tunnel fibers. ISB – inner spiral bundle.

of these cochleae was not significantly different from controls ($P = 0.349$).

Because the 28-day cochleae had two electrode holes into the endolymphatic space, carbon had spread to 5–17% distance from the apex. In four of these cochleae, the entire basal half of the OC had substantial hair-cell loss that was much more extensive than in the 0-day cochleae. For example, MA 23L (Fig. 7) had a large number of missing OHCs, several focal losses of IHCs and three OC wipeouts in the base. MA 25L had the least damage, consisting of a 1.2-mm IHC and OHC focal lesion. This lesion was only slightly longer than those in the 0-day cochleae, but it included a 0.35-mm OC wipeout. In four cochleae, the damaged areas were entirely healed. The missing hair cells were replaced in the reticular lamina by phalangeal scars. At OC wipeouts, squamous epithelial cells covered the basilar membrane and sealed the open ends of the tunnel and Nuel spaces. In these cochleae, carbon was seen on the endolymphatic surface of both types of scars, as well as the inner sulcus cells, Claudius cells and the tectorial membrane. No carbon was seen within the OC fluid spaces.

Cochlea MA 21L sustained a 3.99-mm hair-cell lesion that included a 0.37-mm OC wipeout, a 0.13-mm un-

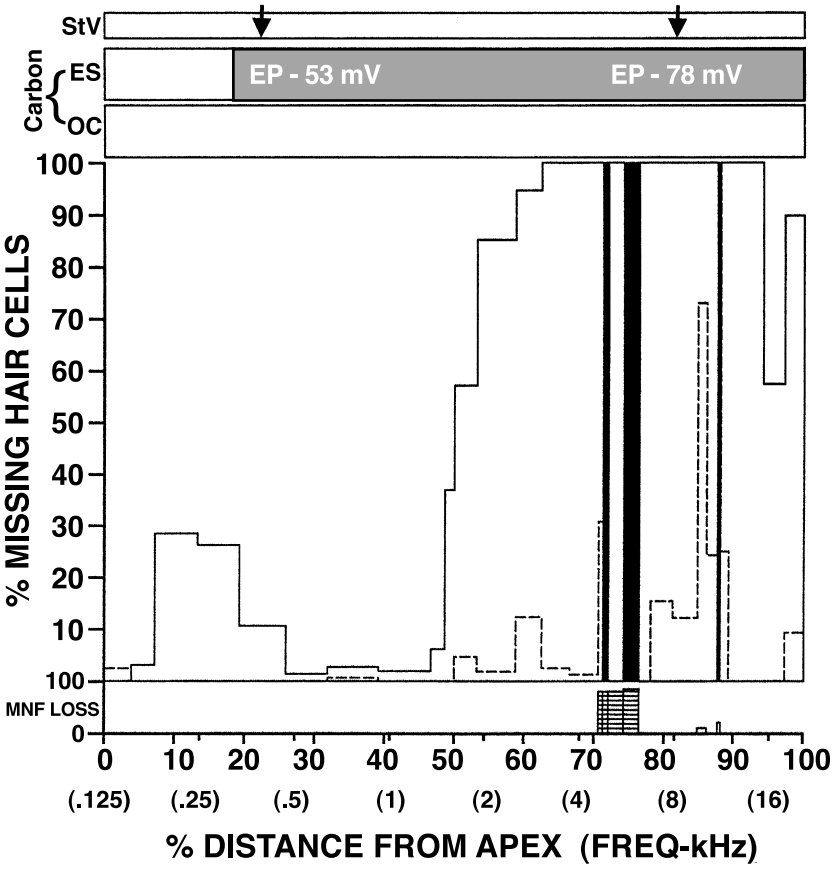

Fig. 7. Cytocochleogram for 28-day cochlea MA 23L. There was one 10.59-mm healed lesion that included three OC wipeouts (tall, black bars). There was no carbon penetration into the OC throughout the damaged area. Downward-pointing arrow at 82% distance indicates site of EP measurement and carbon injection. Downward-pointing arrow at 20% distance indicates site of EP measurement only. Some nerve fibers had degenerated (MNF LOSS – open and striped bars), especially those that innervated hair cells in the OC wipeouts. See Fig. 4 legend for further annotations.

healed lesion, and a 0.29-mm near-wipeout. The OC wipeout had a classic appearance with a layer of squamous epithelium replacing the degenerated OC on the basilar membrane. By optical sectioning, it appeared that carbon was present only on the endolymphatic side of the epithelial scar. By TEM, the surface membranes of the cells forming the scar were continuous, and tight junctions were present between the individual cells. Numerous carbon particles were seen near and attached to the microvilli on the epithelial cells. No carbon could be seen within the tight junctions or between the membranes of adjacent cells (Fig. 8). At the unhealed lesion, all sensory and supporting cells were missing but an epithelial scar had not yet formed. Carbon was seen on the upper surface of the basilar membrane, as well as filling some of the supporting cells that surrounded the lesion.

3.6. Group statistics

The percentages of missing IHCs and OHCs for each animal in the three groups are shown in Table 1 for the apical half and basal half of the OC, respectively. In the apical half of the OC in control cochleae, IHC loss ranged from 0.6 to 2.3% and OHC loss ranged from 3.1 to 4.7%. In the 0-day cochleae, IHC loss ranged from 0.2 to 1.1%, while OHC loss ranged from 1.5 to 5.0%. The difference in IHC and OHC losses between the control and 0-day groups was not significant ($P = 0.177$ and 0.485, respectively). In the 28-day cochleae, IHC loss ranged from 0.3 to 11.1%, and OHC loss ranged from 2.4 to 28.1%. The difference in IHC loss between the control and 28-day groups was not significant ($P = 0.515$), and the difference in OHC loss was not quite significant ($P = 0.068$). The difference in IHC loss between the 0-day and 28-day groups was not significant ($P = 0.376$), and the difference in OHC loss was not quite significant ($P = 0.057$).

In the basal half of the OC of the control cochleae, IHC loss ranged from 0.2 to 1.5%, and OHC loss ranged from 0.8 to 2.1%. In the 0-day cochleae, IHC loss ranged from 0.6 to 6.4%, while OHC loss ranged

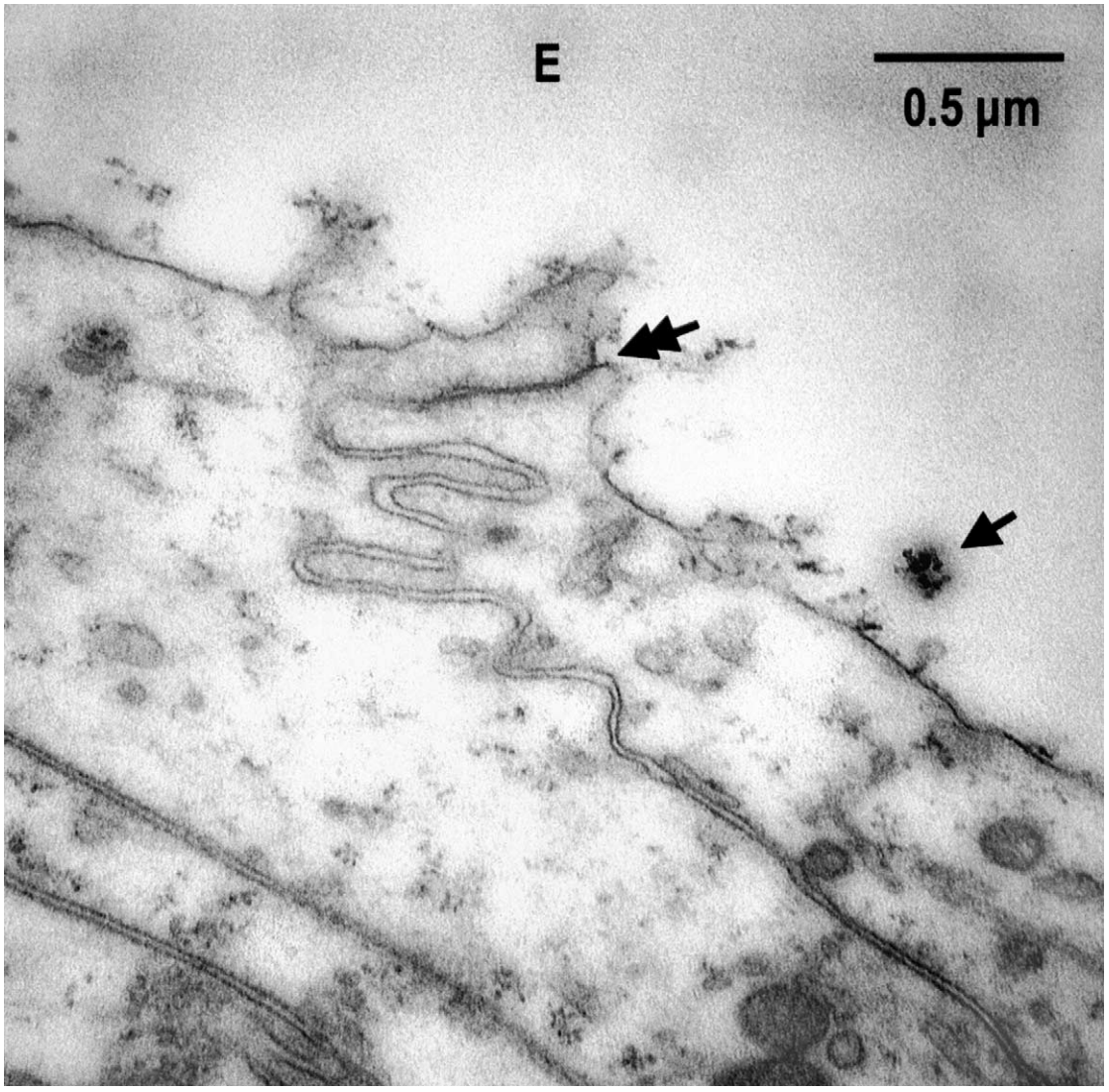

Fig. 8. TEM from cochlea MA 21L of OC wipeout at 74% distance from apex. Squamous epithelial cells cover the basilar membrane (out of photo at lower left) in the OC wipeout. The double arrow points to a tight junction between adjacent cells. The cells' membranes show extensive interdigitation beneath the tight junction. A clump of carbon particles (single arrow) is visible in the endolymphatic space (E). Smaller carbon particles are attached to the surface membranes of the epithelial cells, especially the microvilli. No carbon particles are seen in the cells' cytoplasm or the intercellular spaces.

from 3.7 to 54.5%. The difference in IHC loss between the control and 0-day groups was not significant ($P = 0.138$), but OHC loss was significantly greater in the 0-day group ($P = 0.023$). In the 28-day cochleae, IHC loss ranged from 4.7 to 29.2%, and OHC loss ranged from 15.1 to 95.7%. Hair-cell losses in the 28-day group were significantly greater than in controls ($P = 0.010$ for IHCs; $P = 0.003$ for OHCs). IHC loss in the 28-day group was significantly greater than that in the 0-day group ($P = 0.026$). The difference in OHC loss between the 28-day and 0-day groups was not quite significant ($P = 0.055$). However, the pattern of damage in one cochlea from each group differed substantially from the others in the group. In the 0-day group, MA 18L had one broad OHC lesion in the base rather than one to six focal lesions (e.g. Fig. 4). In the 28-day group, MA 25L had a single, narrow lesion at 4 kHz rather than a broad lesion encompassing most of the base (Fig. 7). If these two cochleae are not included in the *T*-test, the difference in OHC loss between the 0-day and 28-day groups is highly significant ($P = 0.004$).

3.7. *Discontinuities in the apical membranes of hair cells and supporting cells*

Membrane discontinuities were not seen in any cells in control cochleae. In all 0-day cochleae, scattered OHCs were found in which carbon appeared to have

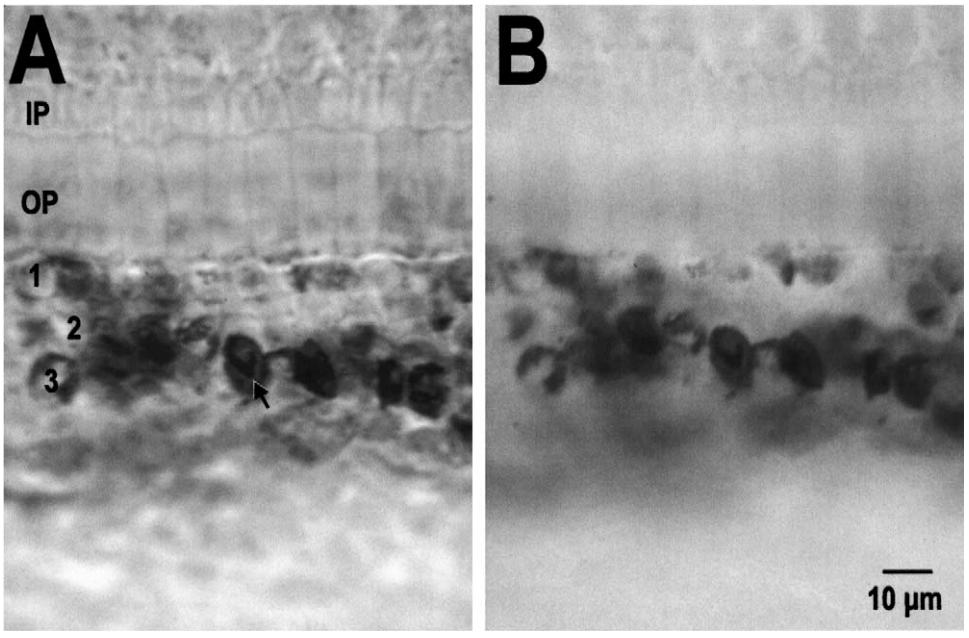

Fig. 9. (A) Phase-contrast photomicrograph from 0-day cochlea MA 12L at 66–67% distance from apex showing carbon in the apices of degenerating OHCs (1, 2, 3). Stereocilia (arrow) covered with carbon can be seen on one third-row outer hair cell. The inner pillar cells (IP) and outer pillar cells (OP) are intact. (B) Bright-field photomicrograph of the same region showing carbon in the OHC apices and in 'clouds' within the OC.

penetrated their apical membrane and accumulated in the cuticular plate (Fig. 9). Sections through these cells revealed that the cuticular plates appeared abnormal (i.e. paler and thinner; Fig. 10A). Although the reticular lamina did not have visible holes by light microscopy, dense collections of carbon were seen surrounding swollen OHC bodies and tracking down toward the basilar membrane (Fig. 10B). Evaluation of these same areas by TEM revealed that there were small discontinuities in the apical membranes of the OHCs (Fig. 11). Carbon particles could be seen on the endolymphatic surface of the OC, within and beneath the cuticular plates of these OHCs and between the inner pillar headplate and outer pillar head. The membrane discontinuities were located where carbon was seen in and around the OHCs (Fig. 9), but frank holes in the reticular lamina were not visible (Fig. 10). Finally, membrane discontinuities were not present in the four 28-day cochleae that had a normal EP (lower third, Table 1).

One 28-day cochlea (MA 21L) had a low EP. Radial sections of this cochlea indicated that cell degeneration may still have been ongoing at 28 days. In the region near the unhealed lesion, most of the OHCs were necrotic. The reticular lamina appeared intact by light microscopy, but carbon was seen within the cytoplasm of the outer pillars and OHCs (Fig. 12). These findings suggest that some sensory and supporting cells had developed defective apical membranes shortly before the carbon injection at 28 days post-exposure.

4. Discussion

4.1. Integrity of the reticular lamina after noise- or drug-induced hair-cell loss

A number of studies have examined whether or not the integrity of the reticular lamina is disrupted after exposure to noise or ototoxic drugs. Some researchers have stated that the phalangeal processes of supporting cells form scars in the reticular lamina at the same time that the hair cells are degenerating (e.g. Hunter-Duvar et al., 1982; Forge, 1985; McDowell et al., 1989; Raphael and Altschuler, 1991). These studies reported that the cuticular plate and surface membrane of degenerating hair cells were intact after their basolateral membranes had ruptured. By scanning electron microscopy, TEM and/or immunohistochemistry, the reticular lamina never appeared to be disrupted after damage from noise (Lim and Melnick, 1971; Hunter-Duvar et al., 1982; Raphael and Altschuler, 1991) or ototoxic drugs (Forge, 1985; McDowell et al., 1989). Instead, the phalangeal processes of the surrounding supporting cells expanded into the space left by the degenerating hair cells and formed new tight junctions. The cuticular plate and surface membrane of the degenerating hair cells were expelled into the subtectorial space only after the tight junctions had formed. Degeneration in the OC was thought to be a controlled sequence of events that is geared toward maintaining the endolymphatic boundary (McDowell et al., 1989; Raphael and Alt-

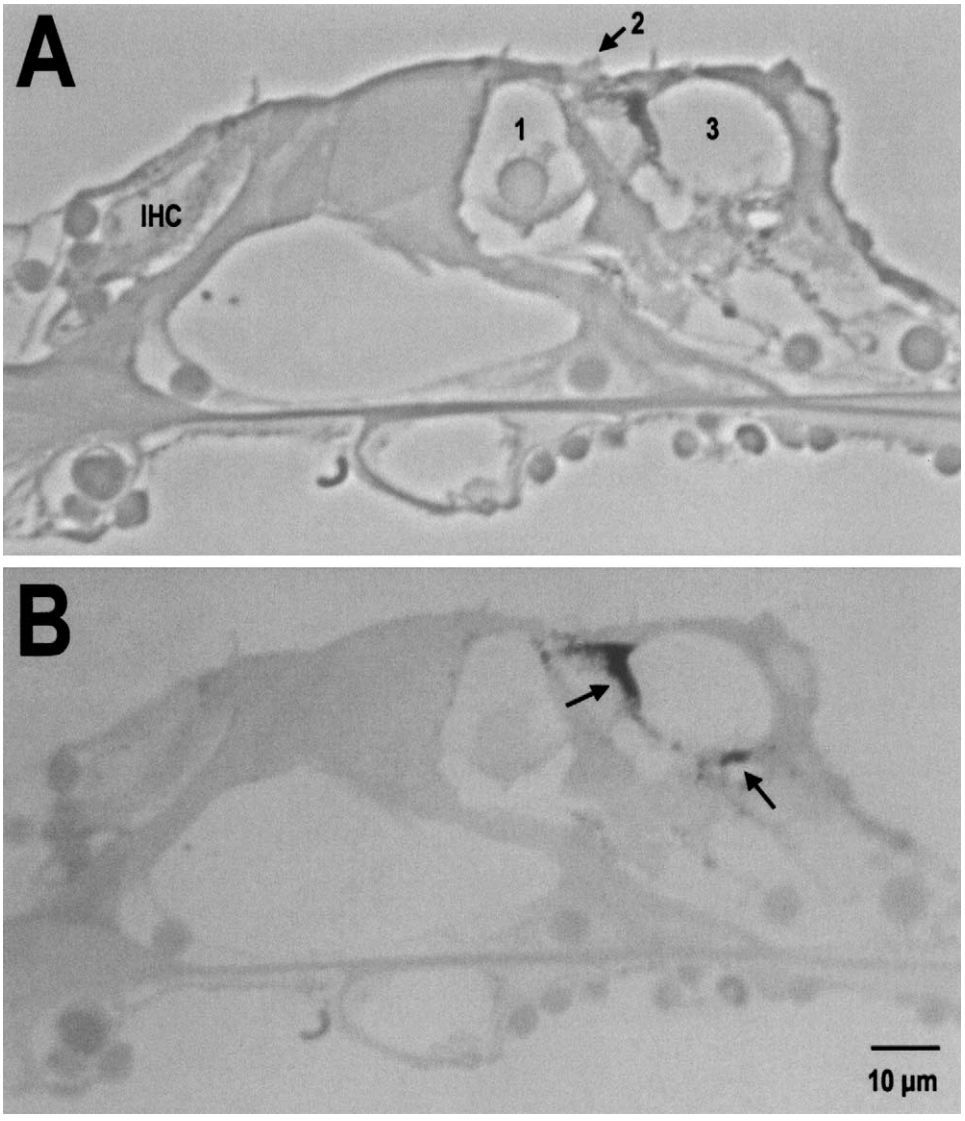

Fig. 10. Photomicrographs of a radial section (unstained) from 0-day cochlea MA 12L at 66% distance from apex, just to left of area in Fig. 9. (A) Phase-contrast view shows grossly swollen OHCs (1, 2, 3), buckled pillar bodies and partially collapsed Deiters' cells. The inner hair cell (IHC) is intact. (B) Bright-field view shows carbon (arrows) has penetrated into the OC from the endolymphatic space through a seemingly intact reticular lamina.

schuler, 1991). It is noteworthy that none of these reports addressed the issue of the integrity of the reticular lamina as OC wipeouts are forming.

Some researchers have found holes in the reticular lamina that persisted for varying periods of time after noise damage (Bohne, 1971, 1976a; Lim, 1976; Bohne and Rabbitt, 1983; Fredelius et al., 1988). These reticular lamina holes ranged from hair-cell sized that healed within days of the exposure (Bohne, 1971, 1976a; Lim, 1976; Bohne and Rabbitt, 1983) to 0.5-mm or larger holes that required 16–32 days to heal (Bohne, 1971) and resulted in the formation of OC wipeouts. Radial sections of developing OC wipeouts at 5–12 days post-exposure revealed that a large area of the OC, as well as the reticular lamina, was missing. No cells or parts of cells were seen separating the endolymphatic space from the fluid spaces of the OC. Thus, it was concluded that during the formation of OC wipeouts, there is an intermixing of cochlear fluids and, as a consequence, cell degeneration spreads beyond the area that was initially damaged (Bohne, 1971; Fredelius et al., 1988).

Possible explanations for the incongruities in the published literature include differences in the mechanisms of OC damage induced by noise and aminoglycosides, variations in hair-cell loss patterns at the cochlear base and apex, species differences and variations in experimental methods. In the present study, the use of a tracer unequivocally demonstrated the temporary breakdown of the boundary between cochlear fluid

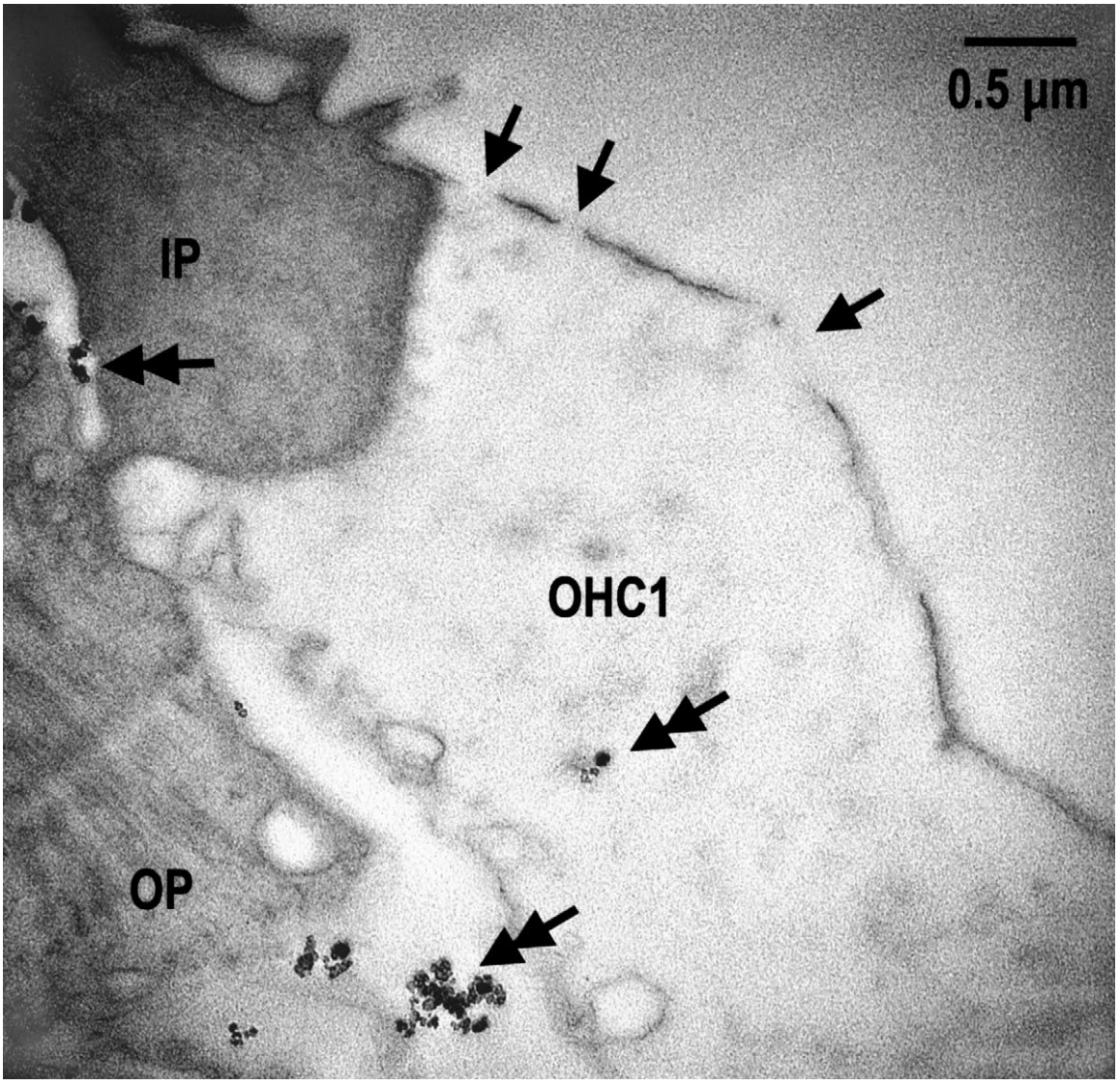

Fig. 11. TEM of area adjacent to that in Fig. 10. The inner pillar headplate (IP) and the outer pillar head (OP) are visible. The first OHC (OHC1) is degenerating. There are several discontinuities (arrows) in its apical membrane. Carbon particles (double arrows) are seen within and beneath the cuticular plate of the hair cell and between the IP headplate and OP head.

compartments after noise damage. In the 0-day cochleae, both flat preparations and radial sections revealed that carbon injected into the endolymphatic space entered the OC only at sites of focal hair-cell loss or damage. Because carbon particles cannot pass through intact plasma membranes or through the tight junctions between the cells forming the reticular lamina, it must be concluded that the particles or small aggregations of particles passed through holes in the damaged reticular lamina to enter the OC fluid spaces and through apical membrane discontinuities to enter injured sensory and supporting cells. In four 28-day cochleae, carbon injected into the endolymphatic space did not enter the OC fluid spaces despite the massive losses of hair cells and the formation of OC wipeouts. In these cochleae, phalangeal scars had replaced missing hair cells and squamous epithelial scars had replaced degenerated regions of the OC. Thus, by 28 days post-exposure, the endolymphatic boundary was usually re-established.

4.2. EP and the effects of noise

In the present study, the average EP in the first turn of non-noise-exposed cochleae was similar to that found in previous studies of control chinchillas (Benitez et al., 1972; Morizono et al., 1980; Ikeda and Morizono, 1988). Our control cochleae had a low-level loss of hair cells that is typical of aging (Bohne et al., 1990). The missing hair cells had been replaced by mature phalangeal scars, indicating that the cells had not de-

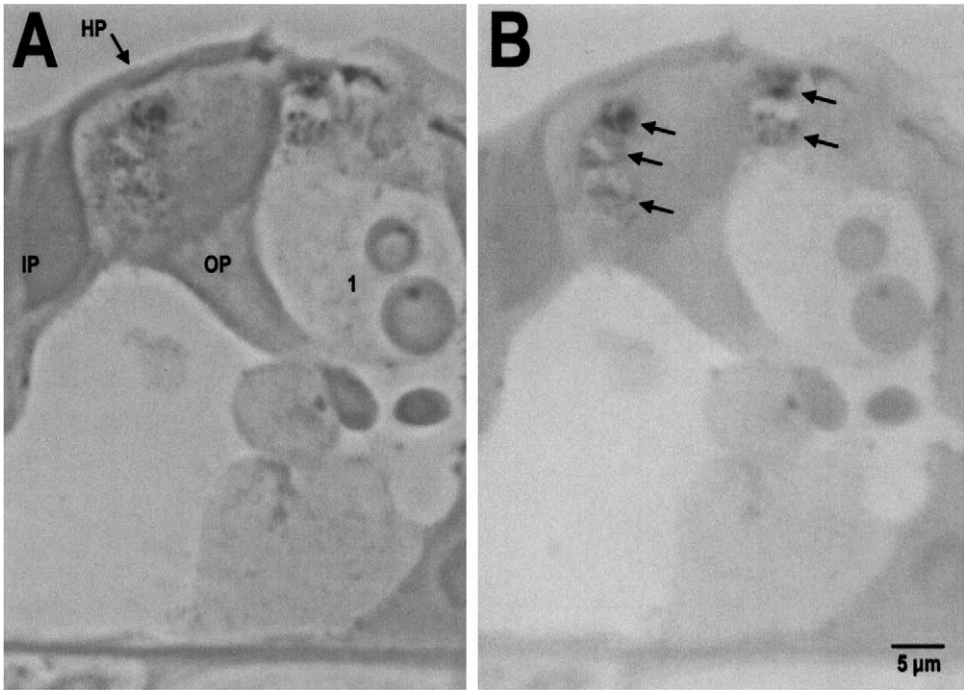

Fig. 12. Photomicrographs of a radial section (unstained) from 28-day cochlea MA 21L at 73% distance from apex. (A) Phase-contrast view shows a necrotic first-row OHC (1). The cell is grossly swollen, has lost cytoplasm and has an enlarged nucleus. The inner pillar (IP) and outer pillar (OP) heads and inner pillar headplate (HP) appear relatively normal. (B) Bright-field view shows carbon (arrows) within the outer pillar head and the subcuticular region of the OHC.

generated recently. Therefore, it was concluded that neither the injection technique nor the carbon solution caused acute injury to the cochlea.

Previous studies examined the effect of noise on EP and found contradictory results. Some studies found no change in EP after a variety of low- to moderate-level exposures (e.g. Benitez et al., 1972; Boettcher and Schmiedt, 1995; Ma et al., 1995). Other studies, employing higher level exposures, found moderate to severe decreases in EP during and shortly after exposure, with EP recovering to normal levels with longer times post-exposure (e.g. Melichar et al., 1980; Syka et al., 1981; Vassout, 1984). The histopathological appearance of the cochleae was assessed in three of these studies immediately post-exposure. Benitez et al. (1972) found minimal hair-cell loss and no change in EP. Melichar et al. (1980) and Syka et al. (1981) found severe OC damage, including rupture of the reticular lamina and detachment of the OC from the basilar membrane, and a greatly reduced EP. Thus, changes in EP immediately post-exposure seem to be dependent on the magnitude and pattern of acute OC damage.

In the present study, four of the 0-day cochleae had a reduced EP and multiple focal losses of OHCs in the basal turn. One 0-day cochlea with only one small OHC lesion had an EP similar to that of the controls. By light microscopy, there were no obvious structural alterations in the stria vascularis. The reduction in EP in the four cochleae with moderate to large OHC losses could be the result of the shunting of current through the damaged reticular lamina. If current were to flow from the endolymphatic space into the OC fluid spaces, one would expect the potential in the endolymphatic space to decrease. Further support for the current-shunting hypothesis is provided by the 28-day cochlea with a persistent unhealed hole in the epithelial layer on the basilar membrane. It had a low EP similar to that in four of the 0-day cochleae.

4.3. Effects of elevated potassium in the OC fluid spaces

Several studies have demonstrated reduced cochlear responses (e.g. cochlear microphonics or whole nerve action potentials) when fluids with a higher than normal potassium concentration are perfused through the perilymphatic spaces (Tasaki and Fernandez, 1952; Salt and Stopp, 1979). Zenner et al. (1994) exposed isolated OHCs, cochlear explants, and intact OC to elevated potassium concentrations and determined the effects on OHC function and morphology. Normally, OHCs undergo dynamic changes in length in response to stimulation, modulating the position of the reticular lamina. When exposed to elevated potassium, isolated OHCs contracted and began to swell. When artificial endolymph was perfused through guinea pig cochleae in vivo, the OHCs were depolarized in a dose-dependent

manner. The OHC effects were temporary if exposure to elevated potassium was brief. However, when the exposure to elevated potassium exceeded 5 min, the OHCs were irreversibly damaged.

Cody et al. (1980) used a 20-μm-diameter micropipette to create a mechanical lesion in the OC. The OC damage 4–43 days post-lesioning was much more extensive than the size of the micropipette. Similarly, Duvall et al. (1969) have shown that the cells and nerve fibers of the OC do not survive if the reticular lamina is mechanically ruptured. Bohne (1976b) showed that brief perfusion of scala tympani with an endolymph-like solution caused gross swelling of OHCs and clumping of axoplasm in radial tunnel fibers. These cytological changes were strikingly similar to those seen in cochleae that had been exposed to noise and analyzed shortly after termination of the exposure.

Cell loss data also support the hypothesis that leakage of endolymph into the OC causes additional loss of hair cells and supporting cells. Four of five 0-day cochleae had very discrete OHC lesions. However, after 28 days of recovery, the OHC loss nearly encompassed the entire basal half of the OC. Also, there were sizable losses of IHCs and supporting cells and OC wipeouts appeared. These data suggest that the noise exposure caused discrete losses of OHCs and that some other process(es) led to the degeneration of additional OHCs as well as IHCs and supporting cells. The end result seems to have been the merging of several discrete lesions into one or two larger lesions.

Previous studies demonstrated the importance of containing endolymph in the endolymphatic space and the role of the reticular lamina in maintaining the boundary of the OC. These findings, combined with those from the current study, provide incontrovertible evidence that a severe noise exposure damages the reticular lamina, allowing endolymph to enter the OC. Acute disruption of the reticular lamina and the apical membranes of sensory and supporting cells appears to be a major mechanism to account for degeneration in the OC that spreads or continues for days to weeks post-exposure.

4.4. Membrane discontinuities after noise damage

Mulroy et al. (1998) hypothesized that microlesions form in hair-cell membranes after noise exposure. The fluorescent marker, Lucifer yellow, was placed into the perilymphatic space of alligator lizards that had been exposed to a temporary threshold shift-producing noise. Lucifer yellow can pass through the basilar membrane into the sensory epithelium but can only enter intact hair cells by endocytosis. When non-noise-exposed cochleae were incubated with the marker, it was only seen in discrete endocytotic vesicles within the hair cells' cytoplasm. In noise-exposed cochleae, some hair cells had a homogeneous distribution of fluorescence in their cytoplasm. It was thought that the fluorescent dye entered the cytoplasm of the cells through microlesions in their plasma membranes. Because the marker was still present in the cytoplasm of some hair cells 24 h after being placed in scala tympani, it was hypothesized that the microlesions were repaired over a 24 h period. If the microlesions had persisted, the marker would have leaked back out of the cells before the cochleae were fixed.

Geyer et al. (1978) perfused horseradish peroxidase into the perilymph of noise-exposed guinea pigs. Histological examination revealed that single hair cells and some supporting cells were stained due to in vivo penetration of the horseradish peroxidase. This staining was found in both necrotic cells with intact cuticular plates and in some cells that appeared only slightly damaged. It was hypothesized that the damaged hair cells had lost portions of their membranes, allowing the marker to enter the cytoplasm.

The existence of microlesions in the plasma membrane of injured hair cells was hypothesized by Geyer et al. (1978) and Mulroy et al. (1998) but was not demonstrated because neither study involved TEM. In the 0-day cochleae in the present study, TEM revealed discontinuities (i.e. microlesions) in the apical membranes of injured hair cells (Fig. 11). In the 28-day cochlea with an unhealed OC lesion, the apical cytoplasm of hair cells and supporting cells around the lesion contained carbon (Fig. 12). Presumably, carbon entered these latter cells through discontinuities in their plasma membranes that may have appeared at about the time the carbon solution was injected into the endolymphatic space. The long-term fate of hair cells and supporting cells with injured plasma membranes is unknown. If the discontinuities are repaired, the cells would survive, thereby limiting the post-exposure expansion of the lesion. If the discontinuities are not repaired, the cells would degenerate, thereby increasing the post-exposure size of the lesion. These two possibilities may be one distinguishing feature between susceptible and resistant cochleae.

The first sign of a developing noise-induced permanent threshold shift (PTS) is degeneration of OHCs, focal losses in the cochlear base and scattered loss in the apex (Bohne, 1976c; Bohne and Clark, 1982). The PTS itself is a consequence of degeneration that is secondary to the initial OHC loss (Bohne and Harding, 2000; Nordmann et al., 2000). The cause of the initial OHC degeneration from noise has been attributed to mechanical damage (e.g. Spoendlin, 1985), metabolic exhaustion (e.g. Lim and Dunn, 1979), ischemia (e.g. Hawkins, 1971; Quirk et al., 1992) and/or the generation of free oxygen radicals (e.g. Yamane et al., 1995;

Ohlemiller et al., 1999) but has never been unequivocally identified. By using appropriate noise exposures along with the carbon tracer technique, it should be possible to determine if membrane discontinuities are responsible for the initial noise-induced loss of OHCs.

5. Summary and conclusions

1. Our method for the injection of carbon into the endolymphatic space caused minimal structural damage to the stria vascularis. Neither the injection nor the carbon suspension had an adverse effect on cochlear function or morphology.
2. In non-damaged cochleae, carbon injected into the endolymphatic space did not cross the reticular lamina and gain access to the OC fluid spaces. Thus, the carbon suspension can be used to determine the integrity of the endolymphatic boundary in the cochlea.
3. 2–4 h after the severe noise exposure, carbon injected into the endolymphatic space was found between the reticular lamina and basilar membrane in regions of focal OHC loss. The only way the carbon could have entered the OC fluid spaces was through holes in the reticular lamina.
4. Carbon injected into the endolymphatic space at 28 days post-exposure did not enter the OC fluid spaces. Thus, the phalangeal scars that replaced degenerated hair cells and the epithelial scars that replaced missing regions of the OC re-established the endolymphatic boundary.
5. During or shortly after the noise exposure, sub-μm-sized discontinuities appeared in the apical membranes of some injured sensory and supporting cells. The presence of holes in the reticular lamina and discontinuities in cell membranes resulted in a decrease in EP throughout the cochlear duct, probably due to the shunting of current through the damaged OC.
6. The presence of discontinuities in the apical membranes of sensory and supporting cells appears to lead to the intracellular accumulation of carbon. Thus, the initial noise-induced loss of OHCs may be due in part to these membrane discontinuities.

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