Fine Structural Aspects on Auditory Hair Cell Degeneration in the Budgerigar, *Melopsittacus undulatus*, as Induced by Kanamycin

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Summary. The effects of kanamycin, an antibiotic of the aminoglycoside group, on the auditory sensory epithelium of the budgerigar, *Melopsittacus undulatus*, were examined using both scanning and transmission electron microscopes. Results show that the threshold of the auditory brainstem response increased in birds treated with kanamycin 200 mg/kg for 49 days. While the auditory sensory epithelium of the normal budgerigar consists of short and tall hair cells, and supporting cells, following kanamycin administration, the middle to proximal region of the epithelium of the inner ear showed degenerative changes, with the other parts remaining apparently intact. In the damaged region, the short hair cells were flattened, and the tall ones became heterogeneous in shape. Both types of cells contained many dense bodies in their cytoplasm; they were rounded in shape and homogeneously dense. Severely degenerated tall hair cells also contained many large vacuoles with heterogeneous contents. Because the dense bodies and large vacuoles were positive for acid phosphatase reaction, they were respectively judged to be primary lysosomes and secondary lysosomes containing degenerating cell debris. Most supporting cells in the impaired region were lower in cytoplasmic electron density, and their apical surface became enlarged in area. Some flattened short hair cells were situated on the apical part of the swollen supporting cells. This finding suggests that the short hair cells are pressed toward the scala media by the supporting cells.

MATERIALS AND METHODS

Twenty-three adult budgerigars, *Melopsittacus undulatus*, 25-40 g in body weight, were used in the...
present study. Thirteen budgerigars were given kanamycin (200 mg/kg body weight/day) solved in saline by intramuscular injection once a day for 49 days. The other ten animals were given the same volume of saline as controls. After the 49 days of administration, the birds were treated for physiological and subsequent morphological analyses as described below.

**Auditory brainstem response (ABR)**

In all the animals used, ABR was measured under general anesthesia by an intramuscular injection of pentobarbital (35 mg/kg). The reference electrode was placed on the vertex, and a non-reference one on the skin beneath the orifice of the ear canal. The averaging computer (Nihon-Koden MEM-4104) recorded 100 ms of post-stimulus time for ABR (filter bandpass 50–3000 Hz). ABR recording was the averaged response to clicks (0.1 ms duration) presented at a rate of 100 times. Responses were obtained from each ear in 10 dB increments from threshold to 90 dB SPL.

**Scanning electron microscopy (SEM)**

After ABR was recorded, the birds were perfused via the left cardiac ventricle with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.4) for 10 min. The bilateral inner ears were removed from the temporal bones, and immersed in the same fixative for 3 h. The right inner ear was used for SEM and the left one for transmission electron microscopy (TEM). The right-side specimens were post-fixed in 1% buffered OsO₄ solution at 4°C for 2 h, stained en bloc with 3% aqueous uranyl acetate solution, and dehydrated in graded concentrations of ethanol. Freeze-drying was performed in a Vacuum Device VFD-20 freeze-drying apparatus after the specimens were immersed in t-butyl alcohol (INOUÉ and OSAKA, 1988). The tegmentum vasculosum and tectorial membrane were carefully removed from the freeze-dried sample. The samples exposed were coated with platinum in an Eiko IB-5 ion coater, and examined in a Hitachi S-800 scanning electron microscope.

**Transmission electron microscopy (TEM)**

The left inner ears from 10 kanamycin administered budgerigars and from 10 controls were fixed, dehydrated as described above, and embedded in Epon epoxy resin. Ultrathin sections cut with a Leichert-Nissei ultramicrotome were stained with uranyl acetate and lead citrate, and examined in a JEOL 1200EX transmission electron microscope.

Three animals given kanamycin were used for acid phosphatase reaction. They were fixed by perfusion with 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The basilar papilla removed were immersed in the same fixative for 1 h, and rinsed thoroughly with 0.1 M cacodylate buffer containing 5% sucrose. They were incubated at 37°C for 3 h in a modified medium for acid phosphatase activity (GOMORI, 1952; NOVIKOFF, 1963), consisting of 3% Na-β-glycerophosphate, 0.05 M acetate buffer (pH 5.0), 1% Pb(NO₃)₂ and 3% sucrose. After incubation in this medium, the tissues were post-fixed in 1% OsO₄ solution at 4°C for 2 h, dehydrated, and embedded in Epon epoxy resin.

**RESULTS**

**Control budgerigars**

The ABR pattern of the control budgerigars was composed of three positive large waves with a total duration time of 3 ms (Fig. 1a). It was difficult to detect other waves because of their small amplitude and instability. Because the first wave is highest and most stable, it is generally used as an index of the hearing threshold and amplitude. The threshold level of the normal budgerigar’s ABR was 30 dB SPL.

Fig. 1. Auditory brainstem responses (ABR) obtained from control budgerigars (a) and from birds after 49 days of kanamycin administration (b). ABR in budgerigars consists of three positive waves (I, II, III). The threshold of wave I is 30 dB SPL in the controls (a) and 70 dB SPL in the kanamycin-treated birds (b).
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While the short hair cell showed a pitcher-like shape (Fig. 3a), the tall hair cell was columnar in shape (Fig. 3b). Cuticular plates composed of abundant microfilaments lay closely beneath the apical plasma membrane in both hair cells. Core microfilaments in the stereocilia took root in the cuticular plate. The cytoplasm of the hair cells, which was more electron-dense than that of the supporting cells, was occupied by various kinds of organelles, such as mitochondria with lamellar cristae, tubular smooth and rough endoplasmic reticulum, Golgi apparatuses and a number of small vesicles (Fig. 3c, d). Nerve endings formed synapses with the base of the hair cell (Fig. 3a, b).

The supporting cell was interposed between the hair cells (Fig. 3a, b). Its narrow apical surface with plentiful microvilli faced the scala media. The cytoplasm of the supporting cell was electron-lucent, and contained many kinds of organelles: a few mitochondria, small and large vesicles, tubular or vesicular smooth and rough endoplasmic reticulum, Golgi apparatuses, pleomorphic lysosomes, and multivesicular bodies. The hair cell and neighboring supporting cell were joined by a junctional complex (Fig. 3a, b).

Kanamycin-treated budgerigars

The threshold of ABR of the kanamycin-treated birds was 70 dB SPL, which was much higher than that in the controls (Fig. 1b). In addition, the amplitude of each wave was smaller than that in the controls. It is thus concluded that kanamycin-administration induced hearing loss in budgerigars.

SEM revealed severe damage in hair cells in the middle to proximal region of the auditory sensory epithelium of the kanamycin-treated birds (Fig. 4). In a few cells, hair bundles were completely lost, while in most other cells they were disturbed in arrangement and shape. The apical surface of the hair cells...
Fig. 3. Legend on the opposite page.
whose hair bundles were either completely lost or strongly damaged was enlarged; also, part of the supporting cells with numerous microvilli were enlarged in apical surface.

TEM of thin sections also showed degenerative changes in hair cells and swelling in supporting cells (Figs. 5, 6). The short hair cells were lower in height, attenuated, and their apical surfaces enlarged (Fig. 5). Their cytoplasm revealed highly electron-dense bodies, 0.2-2 \( \mu \)m in diameter (Fig. 5a-c). These bodies were bounded by a limiting membrane, and showed a positive acid phosphatase activity (Fig. 7a). A number of dense bodies were seen in the pitcher-like short hair cells as well as flattened ones (Fig. 5b). Other organelles, including endoplasmic reticulum, mitochondria and Golgi apparatuses, were preserved in the short hair cells (Fig. 5c).

The tall hair cells also possessed many highly

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**Fig. 4.** SEM image showing the middle portion of the auditory sensory epithelium after the administration of kanamycin. Stereocilia of some hair cells have disappeared completely, and those of the other hair cells are disordered and distorted. The apical surface of the hair cells is enlarged (\( \star \)). Microvilli on the widened apical surface of the supporting cells are markedly increased in number (\( \ast \)). \( \times 2,300 \)

**Fig. 3.** TEM images of the auditory sensory epithelium in the control budgerigar.  
(a). A normal short hair cell with stereocilia (S) and a cuticular plate (C), and a supporting cell (SC) with microvilli (M). They are sealed by junctional complexes (arrows). The short hair cell forms a synapse with a nerve ending (N) containing numerous synaptic vesicles. \( \times 5,500 \).  
(b). Normal tall hair cells and supporting cells (SC). Note stereocilia (S), microvilli (M), a cuticular plate (C), a nerve ending (N), and junctional complexes (arrows) between the tall hair cells and supporting cells. \( \times 3,500 \).  
(c). The cytoplasm of the hair cell is occupied by mitochondria and tubular rough endoplasmic reticulum (arrowheads). \( \times 17,000 \).  
(d). Golgi apparatus (G) and numerous small vesicles are observed in the cytoplasm of the hair cell. \( \times 17,000 \)
Fig. 5. TEM images showing the auditory sensory epithelium of the kanamycin-treated budgerigars. a. Short hair cells contain numerous highly electron-dense bodies (arrowheads). SC supporting cells. ×4,600. b. Flattened short hair cells containing a highly electron-dense body (arrowhead). SC supporting cells. ×4,600. c. Cytoplasm of the short hair cell showing intact endoplasmic reticulum, mitochondria and Golgi apparatuses (G). ×11,000. d. Swollen supporting cells (SC) occupying the epithelium. Well-developed Golgi apparatuses and numerous vesicles are observed in the supporting cells. ×4,600.
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Electron-dense bodies (Fig. 6a). In addition, the cytoplasm of markedly-deformed tall hair cells contained numerous large vacuoles including variously sized dense bodies, lipid droplets, small vesicles, and disrupted organelles (Fig. 6b). The vacuoles were surrounded by a limiting membrane, and their diameter ranged from 1 to 4 μm. The dense parts of this vacuole also showed a positive activity for acid phosphatase (Fig. 7b).

The supporting cells in the damaged region of the auditory sensory epithelium were swollen, and appeared more lucent in the cytoplasm as compared with normal cells (Fig. 5a, b, d). Flattened hair cells situated on the apical surface of the swollen supporting cells were often recognized (Fig. 5b). In certain regions, we could not find any hair cells. The apical aspect of some supporting cells was enlarged in the area facing scala media. These cells showed also numerous microvilli (Fig. 5d). The membrane organelles, such as multivesicular bodies and small vesicles, were increased in number, and relatively well-developed Golgi apparatuses were localized in the apical cytoplasm of the swollen supporting cells. Junctional complexes tightly sealed the apical part of the lateral plasma membranes between the degenerated hair cell and the adjacent supporting cell, and between the swollen supporting cells (Fig. 5a, b, d).

DISCUSSION

Kanamycin, an antibiotic belonging to the aminoglycoside group which was first synthesized by UMEZAWA (1958), has been widely used for the treatment of bacterial infections. It has been reported that damages in cochlear hair cells are induced by kanamycin administration in cats (HAWKINS, 1959), in guinea pigs (YLIKOSHI, 1974; LIM, 1976, 1986; NAKAI et al., 1981; FORGE, 1985) and in chick embryos (FERMIN and IGARASHI, 1983). In the present study, both hearing loss and hair cell damage were demonstrated in adult
budgerigars. The hearing loss was obviously due to the hair cell damage.

The present study demonstrated that, in adult budgerigars, the administration of kanamycin at 200 mg/kg/day for 49 days is adequate for causing damage to the auditory sensory epithelium, and for inducing hearing loss. STUPP et al. (1967) reported that kanamycin affected the inner ear of the guinea pig at a daily dose of 250 mg/kg for more than 20 days.

In the severely-damaged region of the auditory sensory epithelium, we were able to demonstrate the flattening of the short hair cell, enlargement of the apical surface of the short hair cell, swelling of the supporting cell, and enlargement of the apical surface of the supporting cell. Flattened hair cells were often recognized on the apical surface of the swollen supporting cells. These findings lead us to the following notions about the process of changes in the auditory sensory epithelium. The damaged short hair cells are pushed up toward the scala media by the swollen supporting cells, becoming flat in shape. These flattened short hair cells seem to be ultimately removed from the auditory sensory epithelium; in fact, we could not find any hair cells in some regions. Furthermore, the swelling of the supporting cells was not reported in any of the previous studies mentioned above. Though the mechanism of the swelling is unknown, we consider this phenomenon to be important for the removal of damaged hair cells.

In the present study, the highly electron-dense bodies appeared both in the degenerated short and tall hair cells, and the vacuoles in the degenerated tall hair cells after kanamycin administration. The highly electron-dense bodies were also reported in aminoglycoside antibiotics-treated chick embryos (FERMIN and IGARASHI, 1983) and guinea pigs (YLIKOSHI, 1974). In both studies, the highly electron-dense bodies were regarded as digested mitochondrial debris. The vacuoles were also reported in kanamycin-treated guinea pigs (LIM, 1986). However, the identity of the vacuoles was not established in the previous studies. In the present study, because the highly electron-dense bodies and vacuoles observed in the degenerated hair cells of the kanamycin-treated budgerigar were shown, for the first time, to be positive in acid phosphatase activity, they have been identified as primary and secondary lysosomes. The question now arises as to what these lysosomes are digesting. It is believed that the vacuoles in the damaged tall hair cells are autolysosomes or phagolysosomes, because they contain debris of various membrane organelles. On the other hand, the electron dense bodies seen in the short hair cells and some tall hair cells were relatively homogeneous in electron density, and no debris of membrane organelles was recognized among the contents of the dense bodies. Some of them are there-

Fig. 7. Acid phosphatase staining of the hair cells after the administration of kanamycin. The highly electron-dense bodies (a) and the vacuoles (b) show a positive acid phosphatase activity. a: ×45,000, b: ×30,000
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fore regarded as primary lysosomes. It seems worthwhile to add that such electron dense bodies were never seen in the hair cells damaged by sound overexposure in the same bird species (UMEMOTO et al., 1993b).

Another important question is whether or not the hair cells regenerate after drug administration. We have demonstrated the regeneration of the budgerigar hair cells and their recovery of hearing acuity following acoustic trauma (UMEMOTO et al., 1993b). This fact indicates that the auditory sensory epithelium of the budgerigar has the ability to reproduce new hair cells. Further studies will be necessary to clarify these points.

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REFERENCES


