DEGENERATIVE OTOTOXIC CHANGES IN THE COCHLEA AS SEEN IN MICRODISSECTIONS AND SURFACE PREPARATIONS

R.A. TANGE

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- Tange, R.A. & Vuzevski, V.D. Changes in the stria vascularis of the guinea pig due to cis-Platinum.
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- Tange, R.A. & Huizing, E.H. Hearing loss and inner ear changes in a patient with severe Gentamicin ototoxicity.
  Arch. Otorhinolaryngol. 228: 113-121 (1980).
- Tange, R.A. & Bernard, J.L. A cochlear vascular anomaly in a patient with hearing loss and tinnitus.
  Arch. Otorhinolaryngol. 233: 117-125 (1981).

CONTENTS

Chapter 1

GENERAL INTRODUCTION

Anatomical and pathological studies of the structures of the inner ear have always played an important role in the investigation of the causes of sensorineural hearing loss. In 1851 Alfonse Corti (marchese di San Stefano Bello) described the anatomy of the hearing organ. Since then this name has been attached to the hearing organ (organ of Corti). Almost thirty years later, in 1884, a Swedish histologist Gustaf Retzius perfected the dissection technique of Corti as the basis for his work on the inner ear structures in vertebrates[1].

Although the name of Retzius is no longer identified with any specific cochlear structure, he was the one who has given the most detailed graphic representation of the cochlea structures. His work "Das Gehörorgan der Wirbelthiere (1884)" contains beautiful drawings of the inner ear anatomy as visible in dissections and surface preparations.

After Retzius brilliant work, the research technique of dissection and surface preparations of the inner ear began to fall into oblivion. The so-called midmodiolar section technique came to dominate inner ear research. In 1914 even Retzius regretted the fact that he had not used this technique to re-do his monographic work of 1884[2]. In the first part of this century the midmodiolar section technique was the only method used for inner ear research.

Guild in 1921 and later Schuknecht in 1953 developed techniques for graphically reconstructing of the organ of Corti[3,4].

In 1950 and 1954 Neubert revived the largely forgotten microdissection method and surface preparations used by Retzius for viewing the organ of Corti as a spiral band. In his "Häutchenpräparat" stained with "Saürealirarin" with hematoxylin, the cell nuclei were the only features clearly seen[5,6].

Engström and his colleagues (1962) used osmium tetraoxide for staining the inner ear structures. They re-introduced this stain, which colours the entire membranous labyrinth perfectly, as was shown by Retzius in 1884. Phase-contrast microscopy was used to study the surface preparations[7].

In the last decade three new research methods have contributed greater clarity to the difficult anatomy and pathology of the inner ear. Scanning electron microscopy (S.E.M.), transmission electron microscopy (T.E.M.)

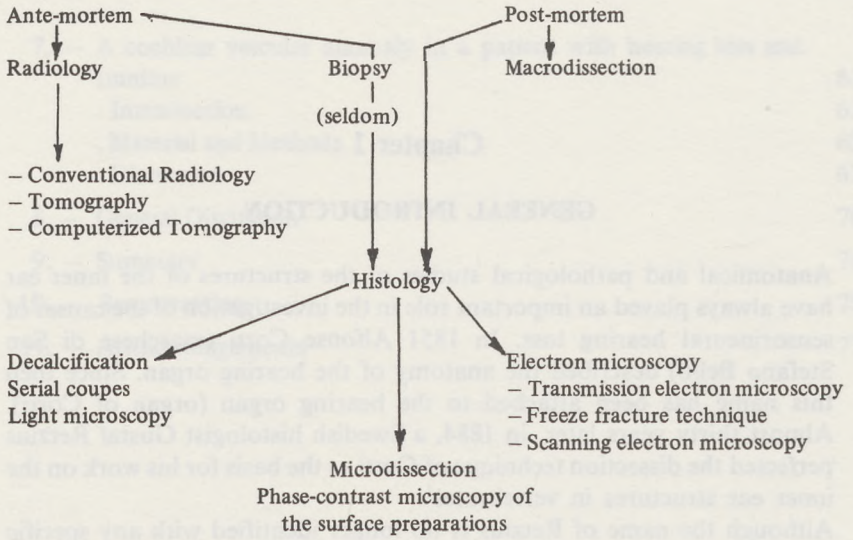

Fig. 1. Inner ear research methods.

and freeze fracture electron microscopy have now given more information on the ultrastructure of the inner ear[8,9,10]. Fig. 1 attempts to illustrate diagrammatically the development of inner ear research methods.

In our study we applied the microdissection technique according to Hawkins and Johnsson[11].

The surface preparations were studied by phase-contrast microscopy and interference microscopy. This last technique is a relative new method for studying surface preparations.

In some cases the research was extended with transmission electron microscopy.

We studied the morphological degenerative changes in the organ of Corti of the guinea pig and the hearing loss as a function of the duration of the administration of two ototoxic drugs: gentamicin and cis-diammine-dichloroplatinum II (DDP).

The purpose of these studies was to find possible differences in the cochlear degeneration patterns induced by these ototoxic drugs. Special attention was given to the starting point of the degenerative process.

The thesis was completed by examining two cases of degenerative changes in human cochlea by microdissection and surface preparations. In one case, severe gentamicin ototoxicity had destroyed the organ of Corti during life. In the other case a vascular anomaly was found in a patient who had used ototoxic drugs.

Using the microdissection technique it was possible to study the degenerative changes in both cochleae.

Chapter 2

MATERIAL AND METHODS

Material

Before starting the experiment a pilot study was performed, in which the material of Carriere[12] was used, in order to obtain experience with microdissection and surface preparations.
This pilot study encouraged us to start our ototoxicity experiments. Fourthy-one healthy adult guinea pigs were used for these experiments. Fifteen guinea pigs (strain CGP-GP.A.165, mean weight 889 g) received 100 mg/kg of gentamicin i.p.; each animal once a day for a period varying from 7 to 17 days. Twenty six guinea pigs (strain CPB-G.A.165, mean weight 472 g) received 1,5 mg/kg DDP i.p. once a day for a period varying from 5 to 20 days.
Two human temporal bones were obtained from autopsies, carried out at the University of Leiden (chapter 6) and the University Hospital (Dijkzigt) at Rotterdam (chapter 7).
Both patients had received ototoxic drugs, their history was known and several audiograms were available. In both cases the timelapse between death and inner ear fixation was less then 24 hours.

Methods

The temporal bones were fixated with formalin immediately after removal. For guinea pigs specimen 20%, for human material 40% solution was used. Except for the specimen used for transmission electromicroscopy, the inner ears were fixated in a solution of glutaraldehyde-formaldehyde (see page 16).

Removal and staining of the temporal bones

Guinea pig material

Before the removal of the temporal bones of the guinea pig, the animals were anaesthetized with a letal dose of Nembutal® i.m. They were then sacrificed by decapitation with stout scissors. The two temporal bones were taken from the skull and the ventral surface of the auditory bulla was exposed; the middle ear cavity was opened with a small forceps. The cochlea and middle ear were inspected and with the aid of a small hook

the stapes was extracted and a small hole was made in the apex of the cochlea.
By means of a curved Pasteur pipette cold OsO_4 (Zetterquist solution) was gently injected into the oval and round window to fixate and colour the inner ear tissues. The temporal bones were then placed in a plastic jar with OsO_4 solution. The bones were then placed in a refrigerator for one hour.
The OsO_4 solution was removed from the cochlea with a Pasteur pipette and replaced with rising percentages of methyl-alcohol to 70% (15 min.-35%; 15 min.-50%).
The bones, now darkened by the OsO_4, were kept in the 70% methylalcohol until they were dissected.

Human material

The two most recommended techniques for removing human temporal bones are:
1. The Block Method
2. The Bone Plug Method
Both methods are well described in the brochure of the temporal bone banks program for ear research[13]. In our studies we used the block method. With a rocker-type oscillating saw four cuts were made in the human temporal bone (fig. 2). The first two cuts were made at right

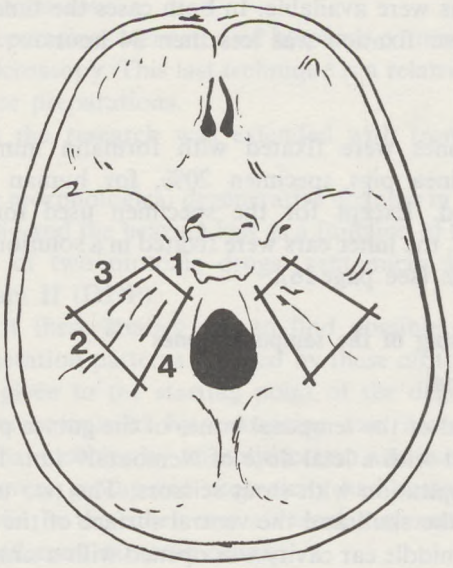

Fig. 2

Temporal bone block method.

angles to the superior angle of the petrous pyramide (1,2). The third cut (3) was made in the floor of the middle cranial fossa about 2 cm in the petrous ridge and laterally as close as possible to the skull cortex. The fourth cut (4) was made in a horizontal plane and as near as possible to the floor of the posterior cranial fossa.
The temporal bone blocks could then be easily removed. Sometimes saw cuts had to overlap to make the removal of the bone blocks easier. The Bone Plug Method, as described by Schuknecht[14], was not used for our material. For staining and postfixating OsO_4 (Zetterquist-solution) was used. Using a Pasteur pipette this solution was injected in the round window and aspirated from the open oval window. After one hour the OsO_4 solution was replaced from the inner ear by methylalcohol using the same methods as described for the guinea pigs.
Before dissecting the cochlea all surrounding tissue was removed.

Microdissection

Under microscopical view and a continuous drip of 70% methylalcohol to wash away the bone dust; the otic capsule was reduced to a thin shell by means of fraising burrs (fig. 3).

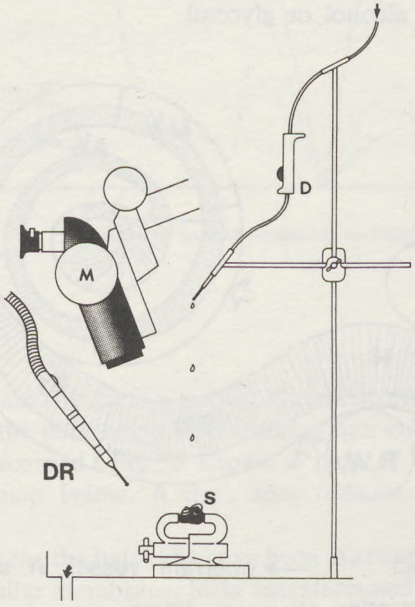

Fig. 3

Drawing up of microdissection.
M = Microscope. D = Drip of 70% Methylalcohol. DR = Drill. S = Specimen.

The thin bony shell was then carefully removed. Our motion picture shows this action of the microdissection clearly[15].
For both guinea pig and human material the following procedure was used. By means of sharp curved stapes needles, gouges and capsule knives, the thin otic bone shell was picked away with as little damage to the membranous structure as possible.
After removal of the thin otic shell, the stria vascularis, Reissner's membrane, organ of Corti and the afferent and efferent nerve fibres of the cochlea nerve are all clearly visible. The entire length of the osseous spiral lamina with Corti's organ and spiral ligament was dissected out, preferably one turn or more at a time. Most labyrinthine tissues were thin and translucent and suited for surface preparations, which were mounted on a slide with glycerol. All dissected parts of the cochlea were recorded and mapped on a special designed cochlear map. The dissected cochlear parts were classified in apical parts (A_1, $A^1/_2$), middle turn parts (M_1, $M^1/_2$), for guinea pig (T_1, $T^1/_2$), basal turn parts (B_1, $B^1/_2$) and hook area (H). Deviations were recorded in these maps. As an example of this method of mapping dissected cochlea, the observations of the case described in chapter 7 are given in fig. 4. All preparations were examined and photographed through a stereo microscope with the specimens immersed in 70% alcohol or glycerol.

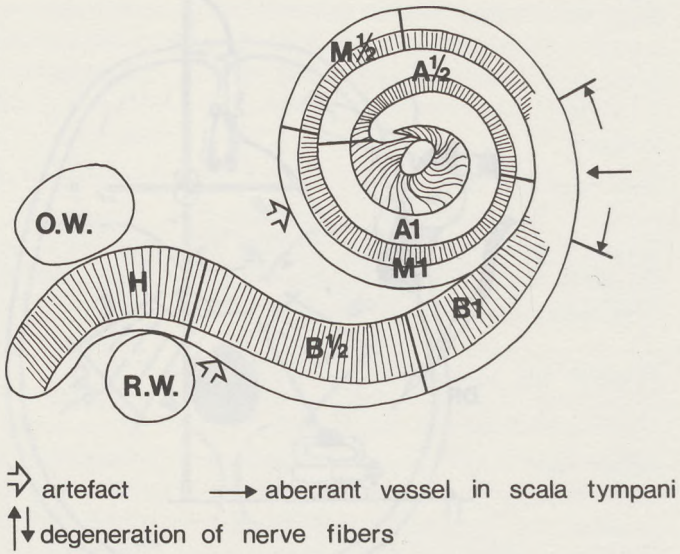

Fig. 4

A example of a cochlear map with the observations of the case described in chapter 7.

Cytocochleogram

The surface preparations were studied by phase-contrast and interference microscopy. By focussing up and down it is possible to perform a "optical section" of the organ of Corti. The presence or absence of each hair-cell was noted and the pattern of pathological changes in the cochlea was plotted in a cytocochleogram.

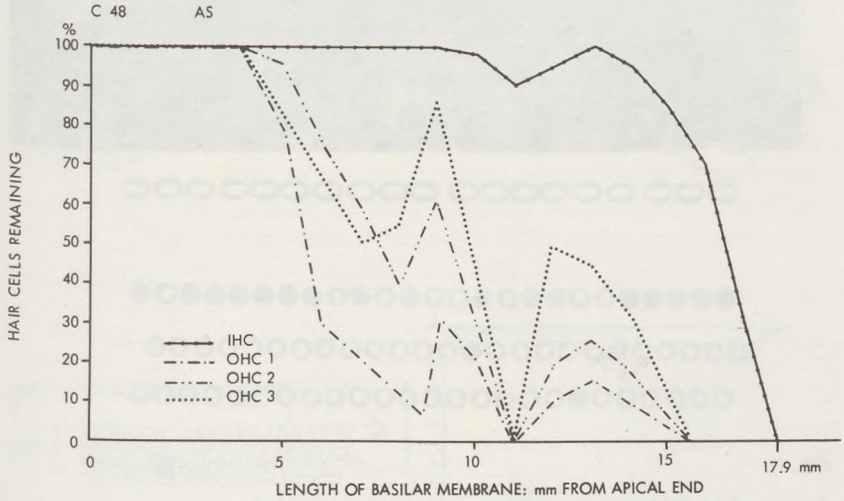

Fig. 5

A cytocochleogram.

The cytochleogram (fig. 5) is a graphical reconstruction of the organ of Corti in which the percentage of remaining hair-cells along the basilar membrane is recorded. Fig. 6 shows a part of the cochlea with a reconstruction map below. A dark spot indicates that a hair-cell is missing.

In all our specimens the hair-cells have been examined over the complete length of the basilar membrane. Stria vascularis and Reissner's membrane were also investigated by phase-constrast and interference microscopy. With this method it was possible to investigate the surfaces and intracellular structures of the cells of these parts of the inner ear. The vestibular system was not studied in this thesis.

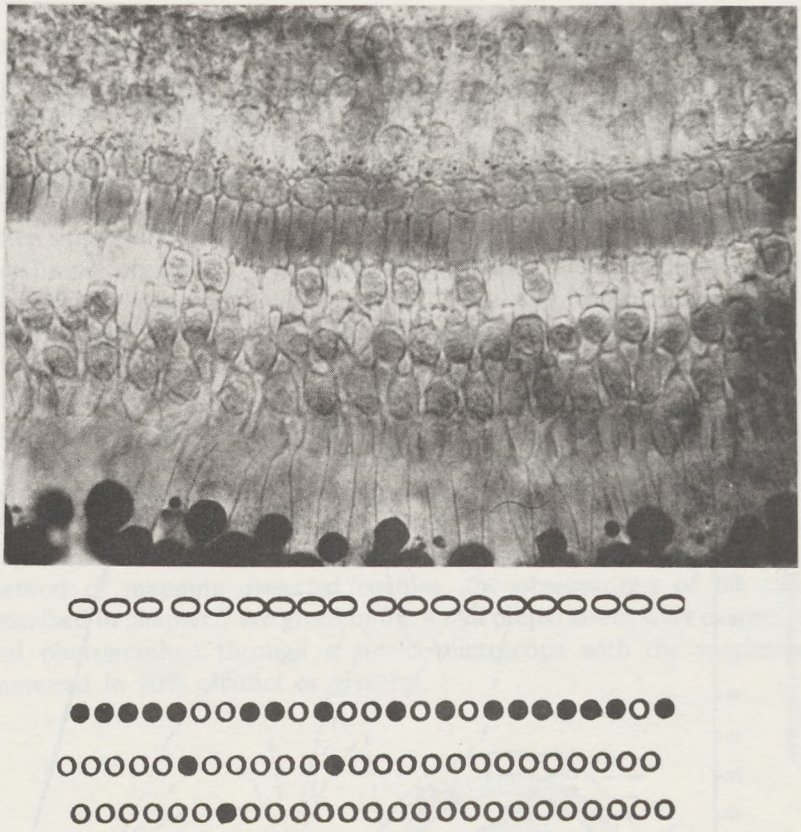

Fig. 6

A part of a cochlear turn with a reconstruction map below.

Transmission electron microscopy

In two studies[16,17] we applied transmission electron microscopy. The inner ears were fixated in a solution of gluraldehyde-formaldehyde (4 CF-1G), dehydrated with acetone and post fixated with OsO_4 (4°C). The specimen were then embedded in Epon. Ultrathin 1μ sections (computerized LBK-ultratome) were stained with uranyl acetate (20 min. at 40°C) and lead citrate. The sections were observed and photographed with a Philips 201 electron microscope.

Audiometry

Before the start of the experiments the hearing threshold was measured. Also 7 days after the termination of the administration of two ototoxic

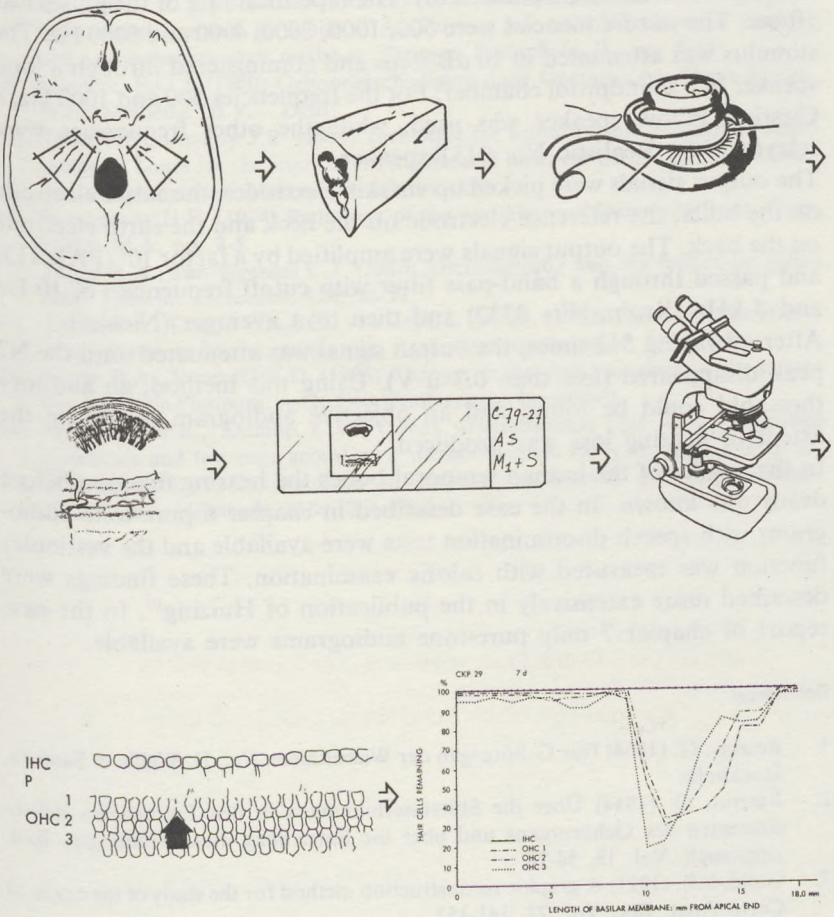

Fig. 7

A diagrammatically expression of the stages in the microdissection.

drugs the hearing threshold was measured by the following method. Since movements of the guinea pigs can influence the stimulus[18], the animals were anesthized before audiometry. Anesthesia has the additional advantage that possible muscle activity which could interfere with the electrocochleography is eliminated. The anesthesia was obtained with Ketalar (0,8 ml/kg) i.m. and Rompun 2% (0,2 ml/kg) i.m.
The acoustic stimuli used consisted of tone pulses (generated by an HP 3300 A function generator) with a duration of 5 ms which were switched

on at zero transition (risetime zero). The repetition rate of the pulses was 10/sec. The test frequencies were 500, 1000, 2000, 4000 and 8000 Hz. The stimulus was attenuated in 10 dB steps and administered through a loud speaker in a soundproof chamber. For the frequencies 500 and 1000 Hz, a Clestion Pillow speaker was used, while the other frequencies were relayed via a Realistic No. 9131 speaker.
The output signals were picked up via skin electrodes, the active electrode on the bulla, the reference electrode on the neck and the earth electrode on the back. The output signals were amplified by a factor 10^4 (PAR 113) and passed through a band-pass filter with cutoff frequencies of 10 Hz and 4 kHz (Krohn-Hite 3332) and then to a averager (Nicolet).
After averaging 512 times, the output signal was attenuated until the N2 peak disappeared (less than 0.3 u V). Using this method, an auditory threshold could be found and an objective audiogram indicating the extent of hearing loss was produced.
In the studies of the human temporal bones the hearing function before death was known. In the case described in chapter 6 pure-tone audiograms with speech discrimination tests were available and the vestibular function was measured with caloric examination. These findings were described more extensively in the publication of Huizing[19]. In the case report of chapter 7 only pure-tone audiograms were available.

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Chapter 3

Pattern of Gentamicin-induced Cochlear Degeneration in the Guinea Pig

A Morphological and Electrophysiological Study

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Durch Gentamyzin verursachte Degeneration in der Schnecke des Meerschweinchens

Eine morphologische und elektrophysiologische Studie

Zusammenfassung. Die durch Gentamyzin verursachte Degeneration in der Kochlea des Meerschweinchens wurde mit dem Zytokochleogramm sowie mit Phasenkontrast- und Interferrenzmikroskopie der Stria vascularis und der Reissnerschen Membran dargestellt. Der erste Haarzellenverlust wurde 6–8 mm vom runden Fenster entfernt festgestellt. Von diesem „Degenerationspunkt" aus schreitet der Haarzellverlust rasch zum runden Fenster und langsam spitzenwärts fort. In der Stria vascularis war keine Degeneration zu erkennen, aber die Reissnersche Membran zeigte eine intrazelluläre Vakuolisierung der endolymphatischen Zellen. Der Hörverlust wurde mit der Elektrokochleographie bestimmt und mit den histologischen Befunden verglichen.

Schlüsselwörter: Gentamyzin-induzierte Kochleadegeneration – Degenerationspunkt – Vakuolisierung der Reissnerschen Membran – Elektrokochleographie

Summary. Gentamicin-induced cochlear degeneration in the guinea pig was studied by complete hair-cell counting (cytocochleograms) and phase-contrast and interference microscopical examination of the stria vascularis and Reissner's membrane. Gentamicin (100 mg/kg/day) was administered over a period of 7–17 days. The first loss of hair cells (OHC) occurred in a region 6–8 mm from the round window. From this 'degeneration point', the loss of haircells progressed towards the round window (fast) and the apex (slowly). The stria vascularis showed no signs of degeneration. Reissner's membrane,

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on the other hand, showed intracellular vacuolization of the endolymphatic cells over the complete length of the cochlea after 12 or more days' intoxication. Hearing loss was measured by electrocochleography with skin electrodes. The histologic findings were compared with the objective audiograms.

Key words: Gentamicin-induced cochlear degeneration – Degeneration point – Reissner's membrane vacuolization – Skin-electrode-evoked response audiometry

Introduction

The ototoxic effect of the aminoglycoside antibiotic gentamicin has been extensively studied by various workers. Many of these investigations were concerned with the damage caused by gentamicin to the hair cells of the organ of Corti. Lundquist and Wersäll [15] were the first to demonstrate the toxic effect of gentamicin on the hair cells by means of electron microscopy. Hawkins et al. [9], Wright [24], and Federspil [6] studied the effect with the aid of phase-contrast microscopy, while Ylikoski [27] and Darrouzet and Guilhaume [5] made use of interference-contrast light microscopy and transmission electron microscopy. In a scanning-electron-microscopy study, Theopold [20] demonstrated abnormalities in the hair cells after gentamicin intoxication. Harpur and Bridges [8] evaluated the normal organ of Corti and the same organ after damage by gentamicin in the guinea pig by means of scanning and transmission electron microscopy.

These studies showed that gentamicin initially damages the first row of outer hair cells (OHC I) in the basal turn of the cochlea. The damage progresses in the apical direction, and to the second and third outer hair cells (OHC II and OHC III). The inner hair cells (IHC) remain intact for a long time. The degeneration of the IHC, when it does occur, also seems to proceed from base to apex. In the above-mentioned studies, the hearing loss caused by gentamicin was measured by the disappearance of the Preyer reflex [6, 8, 24] by operant conditioning [25, 26], and by cochlear microphonic response changes and action potential changes of the cochlear nerve [9].

In the present study, both the degenerative changes in the organ of Corti and the hearing loss are studied as a function of the duration of administration of gentamicin. The hearing loss is compared with the degenerative pattern. Special attention is given to the starting point of the degeneration process. The stria vascularis and the Reissner membrane are also investigated.

Material and Methods

Dosage

Fifteen healthy adult guinea pigs (strain: CGP-GP, A165-mean: 889 g and average 835.8 g) were used for the experiment. Each animal was given 100 mg/kg of gentamicin once a day for a period

varying from 7 to 17 days. The duration of the medication was determined at random beforehand. In certain cases, however, the gentamicin had to be stopped earlier than planned because of the poor general condition of the animals.

Audiometry

Since movements of the guinea pigs can influence the stimulus [21], the animals were anesthetized before audiometry. Anesthesia has the additional advantage that possible muscle activity which could interfere with the electrocochleography is eliminated. The anesthesia was performed with Ketalar (0.8 ml/kg) i.m. and Rompun 2% (0.2 ml/kg) i.m.

The acoustic stimuli used consisted of tone pulses (generated by an HP 3300 A function generator) with a duration of 5 ms which were switched on at a zero transition. These tone pulses were sent at a frequency of 10 Hz. The test frequencies were chosen as 500, 1,000, 2,000, 4,000, and 8,000 Hz. The stimulus was attenuated in 10-dB steps and relayed via a speaker in a soundproof chamber. For the frequencies 500 and 1,000 Hz, a Celestion Pillow speaker was used, while the other frequencies were relayed via a Realisitic No. 9131 speaker.

The output signals were picked up via skin electrodes, the active electrode on the bulla, the reference electrode on the neck, and the earth electrode on the back. The output signals were amplified by a factor 10^4 (PAR 113) and passed through a band-pass filter with cutoff frequencies of 10 Hz and 4 kHz (kron-Hite 3332) and then to an averager (Nicolet).

After averaging 512 times, the output signal was attenuated until the N2 peak disappeared (less than 0.3 μV). Using this method, an auditory threshold could be found and an objective audiogram indicating the extent of hearing loss was produced. The thresholds were determined before the start of the treatment and 7 days after its termination.

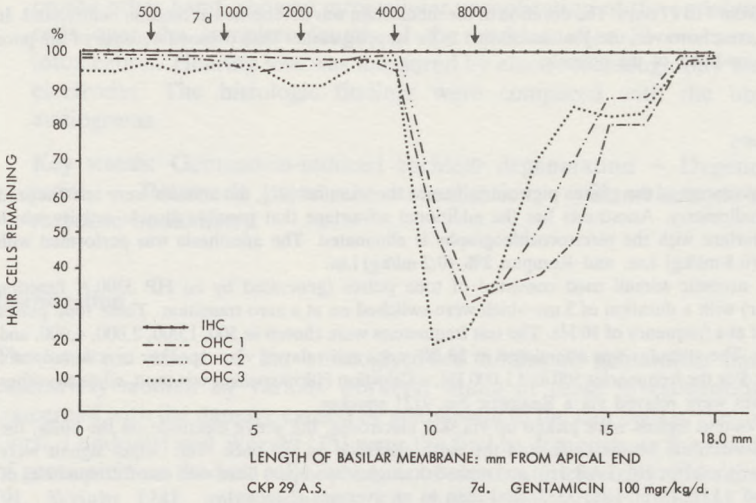
Histological Examination

After the last audiometric investigation, the guinea pigs were anesthetized with Nembutal i.m. and decapitated. The temporal bones were removed, and the cochleae fixed in 20% formalin. The cochleae were then stained with OsO_4 (Zetterquist) and surface preparations were made as described by Hawkins and Johnsson [11]. In each case, the whole cochlea was studied. All hair cells were counted with the aid of phase-contrast and interference-contrast microscopy and cytocochleograms were made. The stria vascularis and the Reissner's membrane were also investigated by phase-contrast interference microscopy.

Results

After 7 days of gentamicin administration, audiometry revealed no hearing loss; histologic examination, on the other hand, showed a slight degeneration of the OHCs in the basal turn. After 17 days of intoxication, both the audiological and the histologic investigations showed serious changes. Striking evidence of the progressive course of the lesions, both histologically and audiologically, was provided by the guinea pigs that were killed at intermediate phases. Figure 1 shows the cytocochleograms and the corresponding audiograms.

It is clear that the hair-cell loss (OHC) starts in the basal turn. The degeneration did not begin, as is often assumed, directly in the corner by the round window, but at 6–8 mm from the round window. From this point, the degeneration process continues in both apical (slowly) and basal (fast)



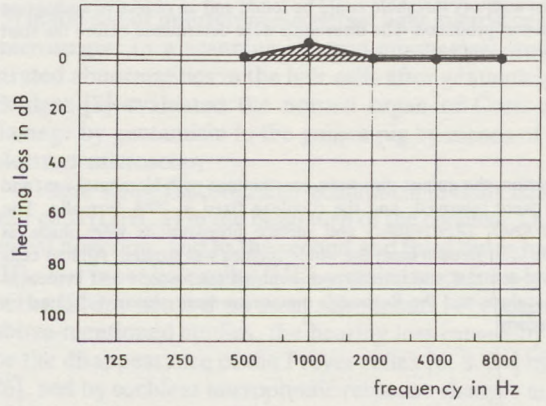
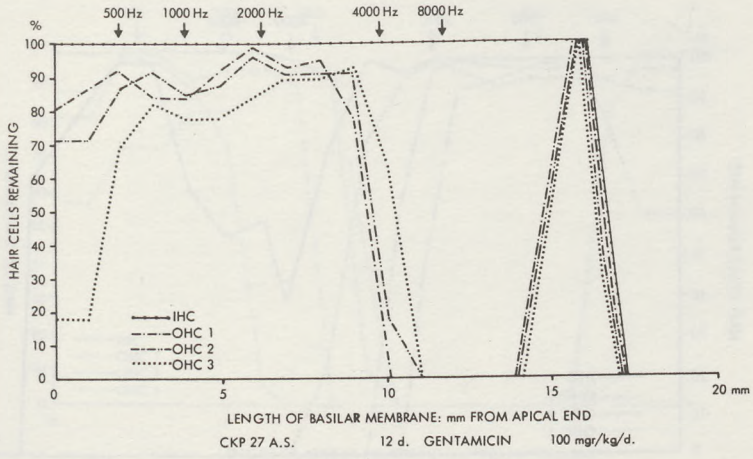


Fig. 1a

Fig. 1a–d. Cytocochleograms and audiograms of different stages of increasing intoxication

directions). In our study, degeneration of the IHCs was observed from day 15 after intoxication. The IHC degeneration also started at the same 'degeneration point' and proceeded in both a basal (toward round window) and an apical direction. Figure 2 shows the hair cell loss in the cochlea plotted as a function of the duration of administration. The loss of OHCs is greater than that of the IHCs. As we have mentioned, degeneration of IHCs does not start until day 15.

The electrode-audiological studies showed no drop in the threshold when only a few OHCs were missing. A threshold loss (of 30 dB on average) did not appear until all OHCs between the degeneration point and the round window



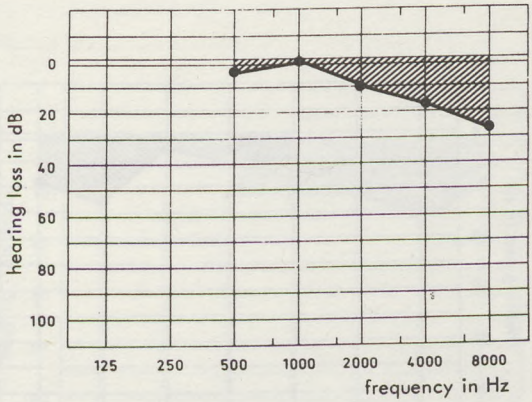
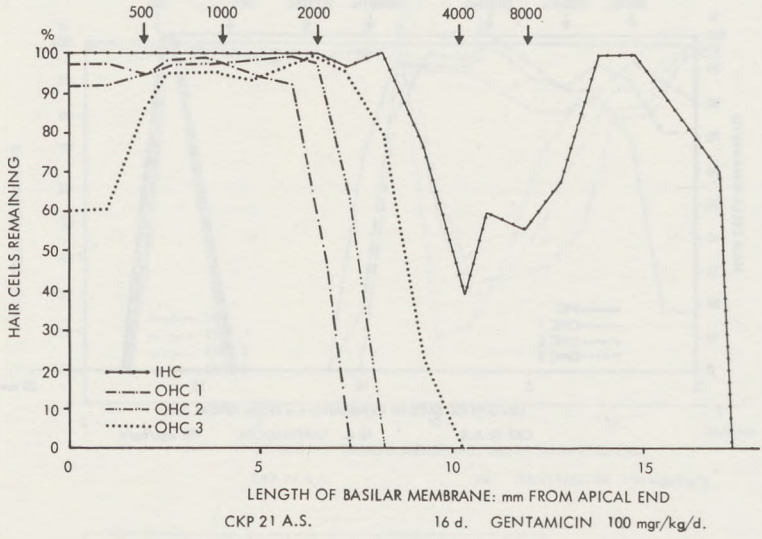


Fig. 1b

had disappeared. In those cases where IHC loss was also observed, the threshold was found to fall by a further 5 dB. However, in none of these cases studied in our experiment was total loss of IHCs observed between the degeneration point and the round window. The hearing loss increased with the duration of the treatment. We applied linear regression lines determined for the measured frequencies. The results are presented in Fig. 3. The figure shows that high frequencies induce more hearing loss than the lower ones and that the hearing loss increases with the number of days of gentamicin treatment.

Statistical evaluation of the results shows a significant difference between the various test frequencies (Friedman, less than 0.01). A positive correlation was



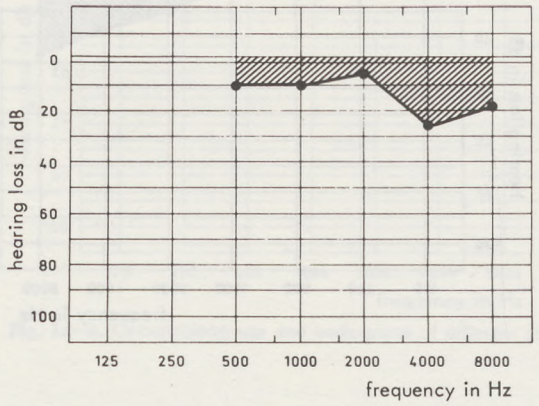
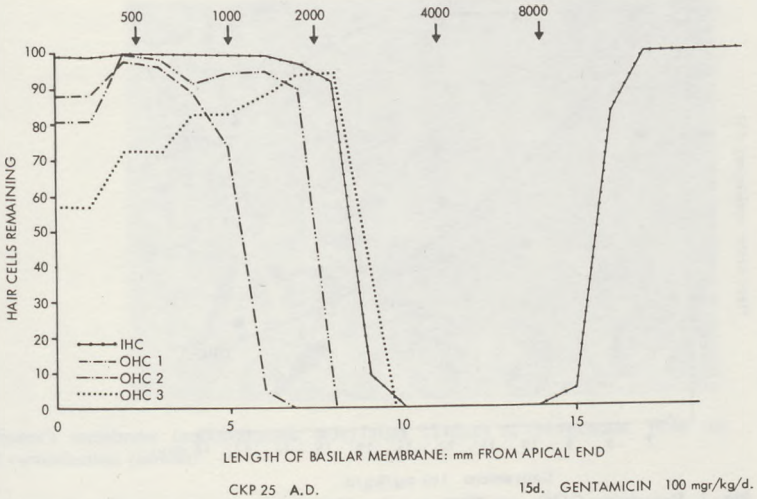


Fig. 1c

also found between hearing loss and the dose (average value of $\alpha = 1.58$, SD = 0.72).

Study of the stria vascularis showed no signs of abnormalities in the form of vasoconstriction of vacuolization in any of the cochleae. Inspection of Reissner's membrane, however, did show appreciable changes: in those animals which had received gentamicin for 12 days or more, large intracellular vacuoles were found in the cells on the endolymphatic side of the membrane over the whole length of the cochlea (see Fig. 4).



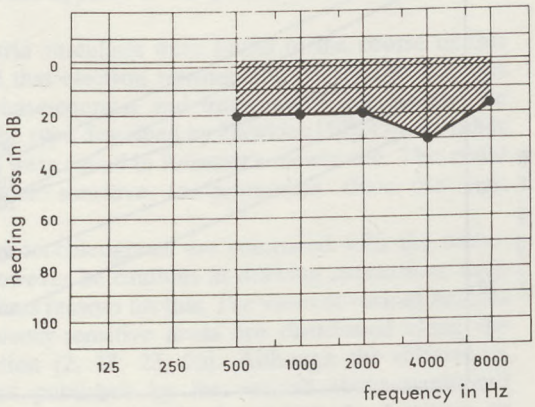


Fig. 1d

Discussion

The first signs of hair cell loss due to gentamicin were found in the OHC I of the basal turn. The degeneration then progresses toward the apex of the cochlea and toward the OHC II and OHC III. Subsequently, the IHCs start to degenerate, the degeneration pattern is also running from the base to apex. The last structures to show degeneration are the connective tissue, the nerve tissue, and the stria vascularis [1, 7, 12, 14, 18, 19, 25].

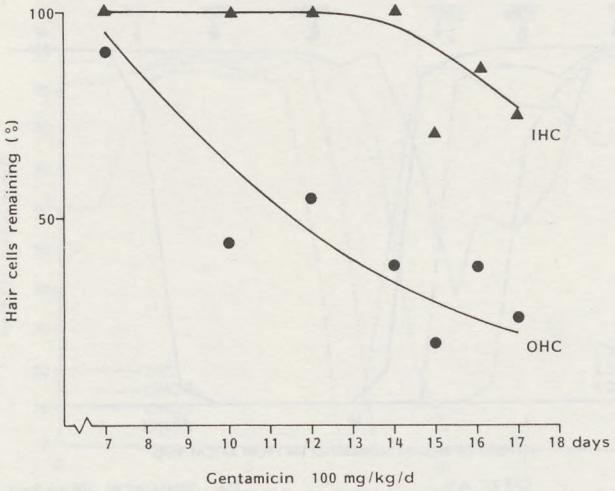


Fig. 2. Remaining OHCs and IHCs as a function of duration of gentamicin administration

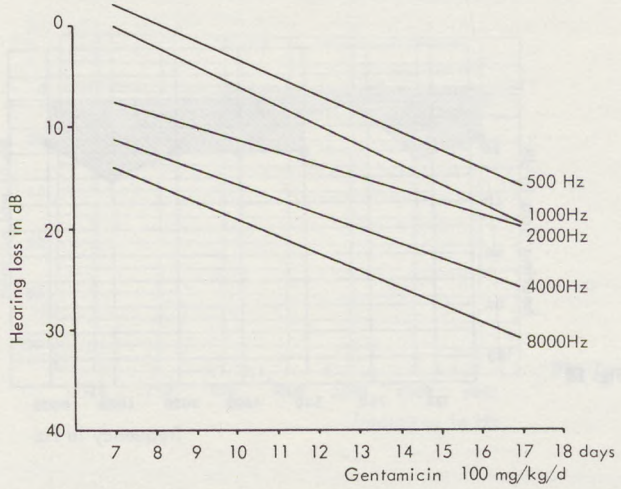


Fig. 3. Linear regression lines showing the hearing loss as a function of the duration of gentamicin administration for the frequencies 500, 1,000, 2,000, 4,000, and 8,000 Hz

Ylikoski et al. [25] reported that the toxic effect of gentamicin starts to manifest itself in the upper part of the basal turn and that the degeneration proceeds quickly in the basal direction. However, he did not confirm this finding in a later study, nor did he mention precisely where the initial hair cell lesion due to gentamicin intoxication was located. The results of our study confirm the

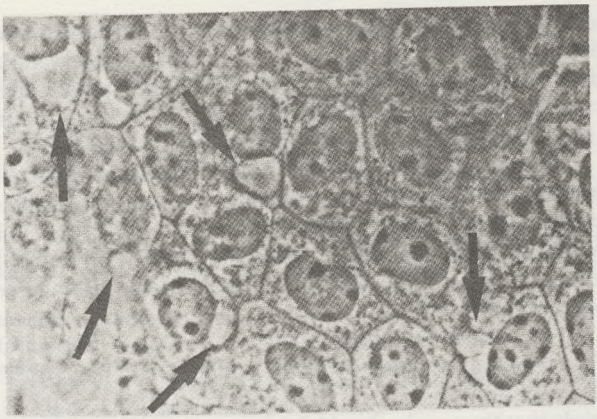


Fig. 4. Reissner's membrane (endolymphatic side) after 12 days of intoxication. Note the intercellular vacuolization *(arrows)*

findings of Ylikoski et al. [25], and furthermore provide evidence that the initial degeneration point for gentamicin is located 6–8 mm from the round window. This part of the basilar membrane appears to be specifically more sensitive to gentamicin.

No abnormalities of the stria vascularis were found in the course of this study. It should be mentioned that electron transmission microscopy was not used here. However, using phase-contrast and interference microscopy, we observed no abnormalities of the type described by Hawkins [10]. On the other hand, abnormalities (vacuoles) were found in Reissner's membrane. This could mean that the latter is more sensitive to gentamicin than the stria vascularis.

In Fig. 1, the individual cytocochleograms are compared with the audiograms obtained. We should however be cautious in drawing conclusions from this comparison. There are various reasons for this. The views of various authors on how the specifically frequency-sensitive areas are distributed along the cochlea show a certain variation [2, 13, 23, 26]. Although the differences between the frequency curves published by the various above-mentioned authors seems quite small, it will be clear that they do form a barrier to unambiguous interpretation, especially in the basal region of the cochlea. In view of the variation in the length of the basilar membrane from animal to animal, the relatively simple technique used for quantification of the complex aural function of the ear and the choice (for practical reasons) of a relatively low number of tests frequencies by no means increase the reliability of the interpretation.

The cytocochleograms for the early phase of the gentamicin lesion in our study show a very sudden degeneration in all rows of OHCs in the form of a sharp 'dip'. Under these circumstances, it is surprising that even when the estimated proportion of OHCs remaining intact is only 30%, the audiological

measurement showed no hearing loss at all. However, it should be noted that we did not measure above 8,000 Hz. Wersäll [22] observed that at least 20% of the hair cells present would have to be damaged before clinical signs of deafness could be demonstrated in the guinea pig. He stressed the great importance of the intact OHCs in the direct neighborhood of the lesion.

Even when there was 100% loss of OHCs and intact IHCs, we only found a hearing loss of 25–30 dB in our study. This is lower than the value found by most other authors. Ylikoski [26] found a loss of 42 dB for guinea pigs under these conditions. For chinchillas, Ryan and Dallos [16] found 40 dB and Dallos and Harris [4] 35–50 dB. Stebbins et al. [17], who used guinea pigs and monkeys, found 50 dB.

The spread in these values is probably largely due to the different experimental animals used in the various studies and the different definitions of the auditory threshold in the various measuring methods. It was found in our study that those animals which had lost the IHCs as well as the OHCs suffered an extra hearing loss of 5 dB on average. This agrees well with the findings of other authors [3, 17, 26].

Our histologic investigations showed the existence of a restricted lesion ('degeneration point') early in the gentamicin intoxication process. This point could not be detected by audiometry; indeed, the lesion could not be confirmed audiometrically until the late stage of its development. This point has already been touched on by other authors. For example, Hawkins et al. [9] stated that audiometric investigations often gave problems in ototoxicity tests. It was for this reason that we looked for a simple, objective test method.

The method we chose for our study can best be described as electrocochleography with skin electrodes. While the data from our study agree well with those obtained from other ototoxicity tests, our method has a number of advantages compared with other audiometric test methods, which can be summed up as follows: The method is relatively simple, can be carried out often, and does not cause any audiological trauma in the experimental animal. Moreover, it is objective and is more directly applicable to ototoxicity testing than brain-stem-evoked response.

The present study has shown that the data obtained from skin-electrode-evoked response audiometry are very useful for ototoxicity tests. Combined with histologic investigation by means of microdissection and surface preparations, and if necessary with scanning and transmission electron microscopy, this method can give a good impression of the ototoxicity of the medicine under test. It would be advisable, however, to increase the number of frequencies at which measurements are made. In particular, detailed examination of the frequency range between 5,000 and 8,000 Hz would probably be useful for tracking down the initial stages of the lesion.

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Chapter 4

The Cortitoxic Effects of Cis-Platinum in the Guinea Pig*

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Der ototoxische Effekt von Cis-Platin beim Meerschweinchen

Zusammenfassung. Untersuchung der Cis-Platin-Wirkung auf das Cortische Organ des Meerschweinchens mittels Zytocochleogramm und Elektronenmikroskopie. Die Schädigung begann stets an den äußeren Haarzellen der Basalwindung. Der Beginn der Degeneration konnte nicht exakt bestimmt werden. Tiere mit Hörverlust zeigten einen Verlust des Körpergewichtes. Vielleicht trägt der letztere zur Verstärkung des ototoxischen Effektes bei.

Schlüsselwörter: Cis-Platin – Schädigung der äußeren Haarzellen – Degenerationsmuster – Hörverlust – Gewichtsverlust

Summary. Cis-diammine-dichloroplatinum-II (DDP)-induced cochlear degeneration in the guinea pig was studied by complete hair-cell counting (cytocochleograms) and transmission electron microscopy. The DDP (1.5 mg/kg/day) was administrated over a period of 5–20 days. The degeneration of the organ of Corti started sporadically in almost every outer hair-cell (OHC) with a strong prevelance in the OHC 1 in the basal turn.

No distinct starting point for the degeneration of the organ of Corti could be found. It seemed that the ototoxic effects of DDP are rather different from the ototoxic changes due to aminoglycoside antibiotics.

This study showed that the animals with hearing loss due to DDP also had a clear loss of body weight. Perhaps DDP induces toxic effects (loss of body weight) which can amplify the ototoxic effects.

Key words: Cis-diammine-dichloroplatinum-II (DDP)-induced cochlear degeneration – Sporadically injured outer hair-cells – Degeneration pattern – Hearing loss – Loss of weight

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Introduction

Rosenberg et al. (1965) observed that a bacterial cell division can be inhibited by a platinum-containing chemical substance. These platinum compounds also showed antitumor activity (Rosenberg et al. 1969). Cis-diammine-dichloroplatinum II (DDP) had the strongest antitumor activity and has for several years been tested in many types of human cancers (Dixon et al. 1971; Merrin 1978; Gralla et al. 1979; Peppard et al. 1980). These and other publications mentioned good results with DDP but, as every drug, DDP has its toxic effects. The major toxicity caused by DDP is nephrotoxicity, though it also provokes neurotoxicity, ototoxicity, nausea, myelosuppression, and anaphylactic-like reactions (Williams and Whitehouse 1979). Ototoxicity was originally thought to occur only occasionally but recent studies of patients receiving DDP frequently have shown hearing loss during and after therapy (Piel et al. 1974; Helson et al. 1978; Rybak 1981).

In contrast to many studies on aminoglycoside-induced ototoxicity, we found only a few animal studies on DDP ototoxicity in the literature. The hearing loss in these studies was measured by disappearance of the Preyer reflex (Fleischman et al. 1975), operant conditioning (Stadnicki et al. 1975), and by cochlear microphonic response changes (Komune et al. 1981). In these studies, histologic changes in the inner ear due to DDP were evaluated with surface preparations (representative segments) and midmodiolar sections.

In the present study, both the degenerative changes in the organ of Corti and the hearing loss are studied as a function of duration of the administration of DDP. The inner ears are studied in surface preparations, complete hair-cell counting, and some with transmission electron microscopy. The hearing loss is measured by electrocochleography with skin electrodes. Special attention is given to the starting point of the degeneration process.

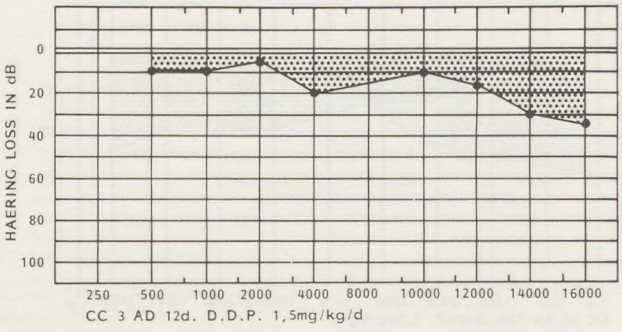
Material and Methods

Dosage

Twenty-six healthy adult guinea pigs (strain CPB-GD.A.165; mean weight – 472 g) were used for the experiment. Every animal was given 1.5 mg/kg of DDP IP once a day for a period varying from 5 to 20 days. The duration of the medication was determined at random beforehand but in certain cases, however, DDP had to be stopped earlier than planned because of the poor general condition of the animal.

Audiological Examination

The animals were anesthetized with Vetalar (40 mg/kg IM) and Rompun 2% (0.2 ml/kg IM) before audiometry. Free-field audiometry was carried out in a sound-proof chamber. The acoustic stimuli used consisted of tone pulses with a duration of 5 ms, which were switched on at zero transition. These tone pulses were presented with a repetition frequency of 10 Hz. As test frequencies we used 0.5, 1, 2, 4, 10, 12, 14, and 16 kHz (HP 3300 AA function generator). The output signals were picked up via skin electrodes, amplified, and passed through to an averager (Nicolet). After averaging 512 times, objective audiograms indicating the extent of hearing loss were produced. This method is described more extensively in an earlier publication (Tange et al. 1982). The hearing thresholds were determined before the onset of the treatment and 7 days after its termination.



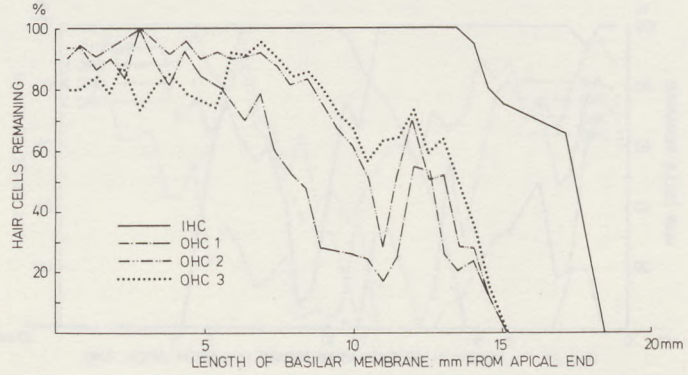


Fig. 1A

Fig. 1A–C. Cytocochleograms and audiograms of different stages of increasing intoxication

Histologic Examination

Surface preparations from the organ of Corti were prepared according to the technique of Hawkins and Johnsson (1975). The whole cochlea was studied. All hair-cells were counted with the aid of interference-contrast microscopy and cytocochleograms were made.

Some cochleas were investigated with transmission electron microscopy. In such cases, the inner ears were fixed in a solution of glutar-di-aldehyde-formaldehyde (4CF-1G) and postfixated with OsO4 1% (4° C) and aceton dehydration. The specimens were embedded in Epon. Ultrathin sections (computerized LBK-ultratome) were stained with uranyl acetate (20 min at 40° C) and lead citrate and were observed with a Philips 201 electron microscope.

Results

In the first 7 days of DDP administration no significant hearing loss ($\leq$ 10 dB) was found. After 7 days, increasing hearing loss especially in the high frequencies was measured. The low frequencies were less affected (Fig. 1).

Histologic investigation showed that the ototoxic lesion of DDP started in the basal turn of the cochlea and progressed towards the apex. We did not find a specific

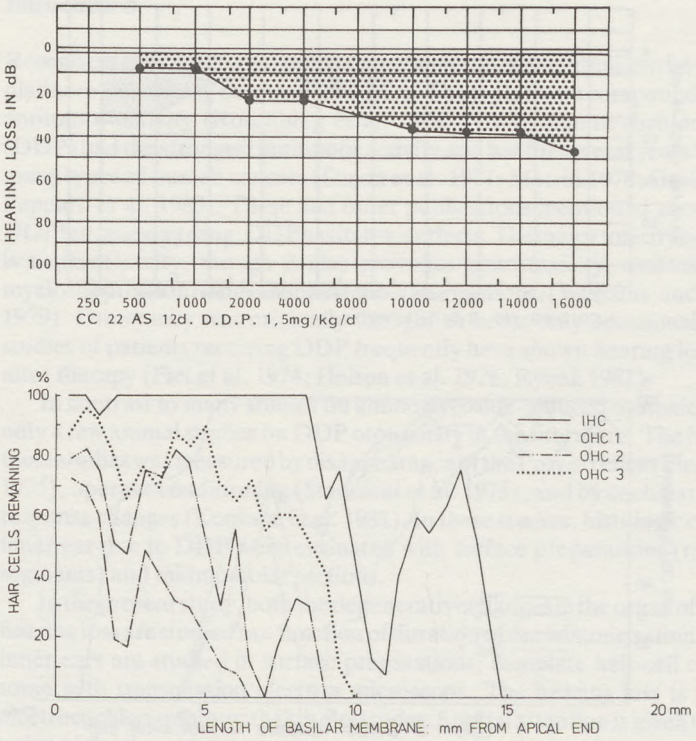


Fig. 1B

starting point of the degeneration of the hair-cells on the basilar membrane. Although the basal turn was more affected, we found in almost every turn sporadic spots with hair-cell loss (Fig. 2). The first cells that collapsed were the outer hair-cells near the tunnel of Corti (OHC 1), followed by the OHC 2 and OHC 3. A the same time we saw changes in the outer pillar cells of the tunnel of Corti (Fig. 2). The inner hair-cells (IHC) were the last cells to degenerate.

Electron microscopic study showed that the mitochondria were the first organelles to change during DDP administration. Loss of internal membranes and the accumulation of osmiophilic inclusion bodies were seen especially in the OHC. The normal shape also changed (Fig. 3). In the cells of Claudius, we found mitochondrial changes in the form of internal membrane vacuolization (Fig. 4) as a result of DDP ototoxicity.

In cases with profound degeneration, we found that almost the complete OHC had disappeared and that only a small piece of the cuticular and subcuticular region was left. The stereociliae had also disappeared (Fig. 5).

There was no clear relationship between the total hearing loss and the cumulative DDP dosage. We found a great variation in the hearing loss of the animals tested which could indicate an individual sensitivity to DDP. Concerning

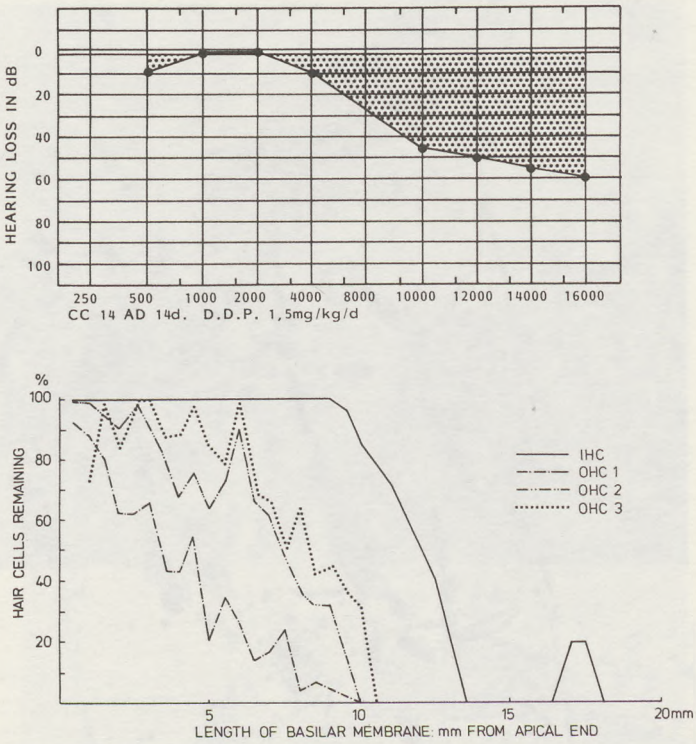


Fig. 1C

Fig. 2. Surface preparation of the middle winding of a cochlea (12 days DDP). *Curved arrows,* hair-cell loss (OHC); *straight arrows,* loss of outer pillar cells. Note: the inner cells are intact

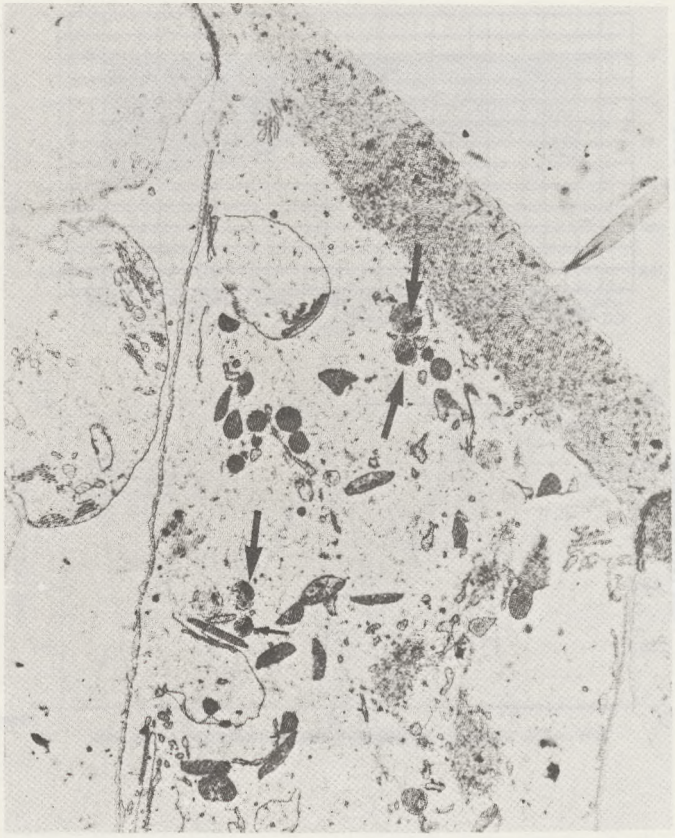


Fig. 3. The upper parts of an hair-cell (OHC II). Note: the changes in the mitochondria *(arrows)* with osmiophilic inclusion bodies (transmission electron microscopy). 18 000×

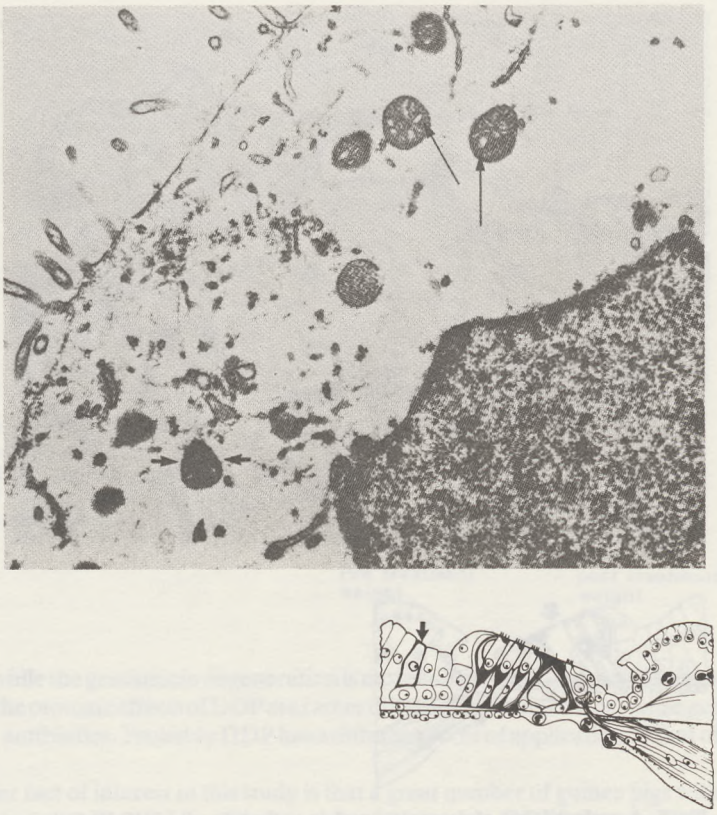


Fig. 4. A Claudius cell with internal membrane vacuolization in the mitochondrium *(long arrow)* (transmission electron microscopy) 27 000×

the mean body weight, we found that the animals with hearing loss (> 30 dB loss in three frequencies or more) also had a significantly ($\alpha < 0.01$) larger loss of weight than the animals without hearing loss (≤ 10 dB loss) (Fig. 6).

Discussion

This present study on the ototoxic effects of DDP confirms the findings of Fleischman et al. 1975, Stadnicki et al. 1975, and Komune et al. 1981. The treatment with a standard dose of DDP (1.5 mg/kg/day) leads to a high-tone sensorineural hearing loss in guinea pigs. With the complete hair-cell counting method it was possible to find a DDP-induced cochlear degeneration pattern.

Fig. 5. A profound case of degeneration of an outer hair-cell (OHC 1). Only a small part of the cuticular and subcuticular region is left. The stereociliae are gone (transmission electron microscopy) 27 000×

The degeneration of the organ of Corti starts in almost every OHC over the complete length of the cochlea with a strong prevelance in the OHC 1 and the basal turn. At the same time, a number of outer pillar cells disappear and ultrastructural changes are seen in the cells of Claudius. The degeneration progresses toward the OHC 2, OHC 3, and IHC from the round window toward the apex.

The audiological data of the degeneration process due to DDP are quite in agreement with these histologic data. The first signs of hearing loss appear in the high frequencies.

In contrast to the findings in aminoglycoside (AG) studies (Ylikoski et al. 1973; Tange et al. 1982), we could not find a distinct starting point for the degeneration of the organ of Corti due to DDP. The pattern of the degeneration of the hair-cells seems to be different from that we found in gentamicin-intoxicated guinea pigs (Tange et al. 1982). The degeneration process of the hair-cells (OHCs) is more

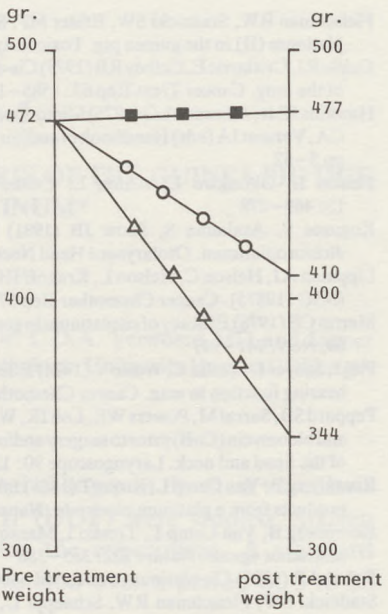


Fig. 6. Mean body weights before and after DDP administration. −○−○− all animals; −■−■− animals with hearing loss ≤ 10 dB (in 3 frequencies); −△−△− animals with hearing loss ≥ 30 dB (in 3 frequencies)

sporadic, while the gentamicin degeneration is more continuous. It seems from this study that the ototoxic effects of DDP are rather different from the ototoxic changes due to AG antibiotics. Probably DDP has a different point of application to that of the AGs.

Another fact of interest in this study is that a great number of guinea pigs with hearing loss also had a clear loss of body weight. As is known, DDP has toxic effects on the blood, the kidneys, and the digestive tract, which could mean that these side effects of DDP were responsible for the loss of weight.

A direct relation between ototoxicity and the other toxic effects of DDP was not found in the literature (Lippman et al. 1973; Piel et al. 1974). However ototoxicity never occurred without other toxicity. Perhaps DDP induces other toxic effects which can amplify the ototoxic effects. More study on this aspect seems advisable for the future.

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Chapter 5

CHANGES IN THE STRIA VASCULARIS OF THE GUINEA PIG DUE TO CIS-PLATINUM*

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Summary

The microscopical and ultramicroscopical changes in the stria vascularis due to cis-diamminedichloroplatinum II (DDP) were studied. Sixteen healthy adult guinea pigs were used for the experiment. A standard dosage DDP (1.5 mg/kg/d) was administered over a period of 5 to 20 days.

A clear degeneration pattern was found (no changes to cystic degeneration with protrusion of the marginal cells followed by loss of marginal cells). DDP seems to be especially toxic for marginal cells in the stria vascularis in the guinea pig.

Introduction

Cis-platinum (cis-diamminedichloroplatinum II=DDP) is known to be an effective drug against many types of human cancers[1,2,3,4]. These and other publications mentioned good results with DDP but, as every drug, DDP has its toxic effects. Ototoxicity has been reported as a toxic effect, along with nephrotoxicity, neurotoxicity, myelosuppression and nausea[5]. Ototoxicity was originally thought to occur only occasionally but recent studies of patients receiving DDP frequently showed hearing loss during and after therapy[6,7,8].

In contrast to the many studies on aminoglycoside-induced ototoxicity we found in the literature[9,10,11,12] only a few animal studies on DDP

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ototoxicity. The authors of these studies showed only the damage done to the haircells by DDP without paying attention to the morphological changes in the stria vascularis.
Only Nakai et al.[13] described very mild changes in the stria vascularis after DDP administration, observed with transmission electron microscopy.
In the present study we studied the degenerative changes by DDP in the stria vascularis of the guinea pig.

Material and Methods

Dosage

Sixteen healthy, adult guinea pigs (strain CPB-GD.A.165, mean weight: 472 g) were used for the experiment. Each animal was given 1.5 mg/kg of cis-diamminedichloroplatinum i.p. once a day for a period varying from 5 to 20 days. Beforehand the duration of the medication was determined at random, but in certain cases DDP had to be stopped more early than planned on account of the poor general condition of the animal.

Audiological examination

Free field audiometry was carried out in a sound-proof box. The acoustic stimuli used consisted of tone pulses with a duration of 5 ms, which were switched on at zero transition. The output signals were picked up via skin electrodes, amplified and passed through to an averager. By averaging 512 times, objective audiograms indicating the extent of hearing loss were produced. This method was described more extensively in an earlier publication[14]. Before the onset of the treatment and 7 days after its termination, the hearing thresholds were determined.

Histological examination

After the last audiometric investigation, the guinea pig were anesthetized with Nembutal® intramuscular and decapitated. Twentysix cochlea's were prepared according the technique of Hawkins and Johnsson[15]. The whole cochlea was studied by surface preparations. All striae vascularis were investigated with interference-contrast microscopy.
Six cochlea's were examined with transmission electron microscopy (T.E.M.). In these cases the inner ears were fixated in a solution of glutaraldehyde-formaldehyde (4CF-1G) and post fixated with OsO4 1% (4°C) and aceton dehydration. The specimens were embedded in Epon. Ultrathin sections (computerized LBK-ultratome) were stained with uranyl acetate (20 min at 40°C) and lead citrate and were observed with a Philips 201 electron microscop.

Table 1.

No pathology in the stria vascularis.

guinea pig	Audiometry	Days DDP intoxication
CC17	–	5
CC18	–	5
CC16	–	5
CC21	–	7
CC26	–	7
CC25	–	8
CC11	–	10

– = no change in the hearing threshold

Table 2

Groups of striae with only a few cystic degenerative changes near Reissner's membrane and with swelling and protrusion of the marginal calls.

guinea pig	Audiometry	Days DDP intoxication
CC24	+	8
CC 1	+	10
CC10	+	10
CC23	+	12
CC27	+	16

+ = hearing threshold dropped over 30 dB or more

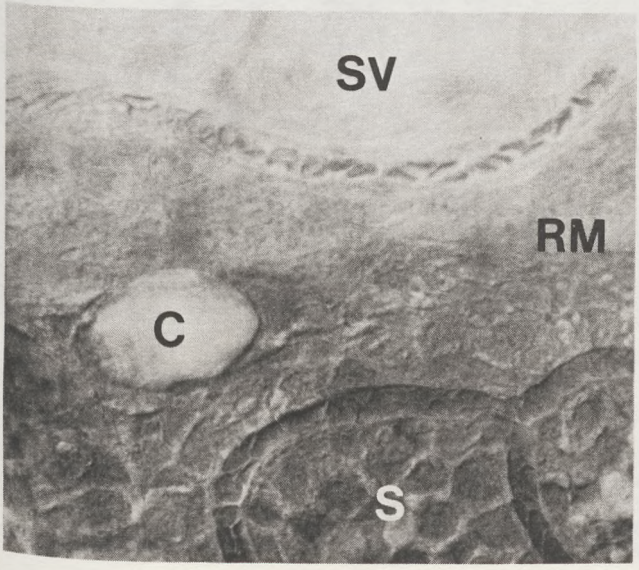


Fig. 1

Phase-contrast interference microscopy of a part of the stria vascularis of the basal turn. Cystic degeneration (C) is seen near Reissner's membrane (RM).
S.V. = Scala Vestibuli
S = Stria vascularis

In the control group, 4 cochlea's were studied by T.E.M. while the other cochlea's were investigated by surface preparations with interference contrast microscopy.

Results

Using our histological investigation we could distinguish two types of degenerative changes in the stria vascularis due to DDP. We formed three groups of investigated cochleae.

The first group had no changes in the stria vascularis (table 1). There was no change in the hearing threshold and the duration of the DDP administration was 5 to 10 days.

The second group of striae (table 2) showed only a few cystic degenerative changes in the basal turn near Reissner's membrane (fig. 1) and some-

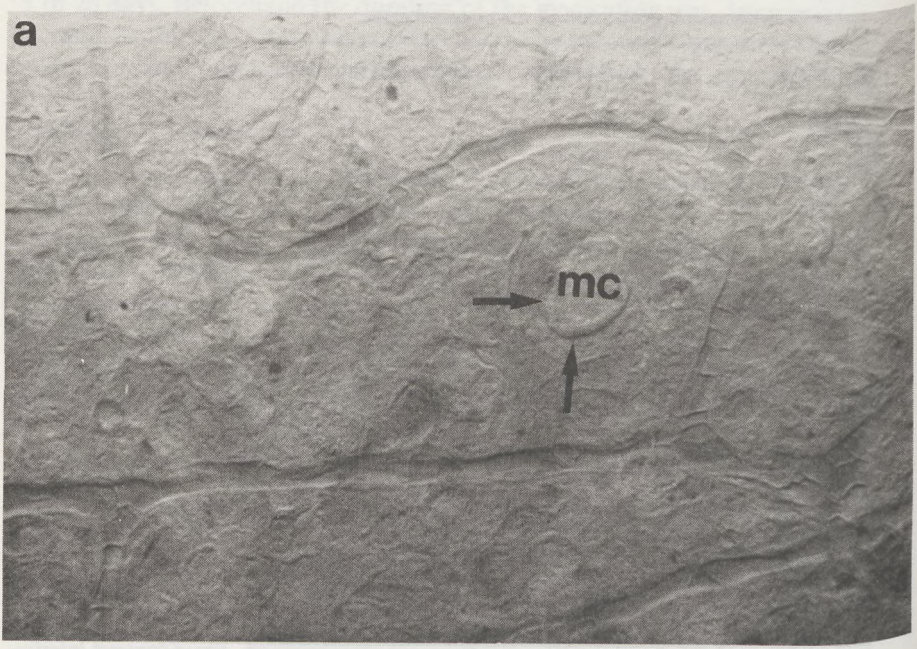


Fig. 2

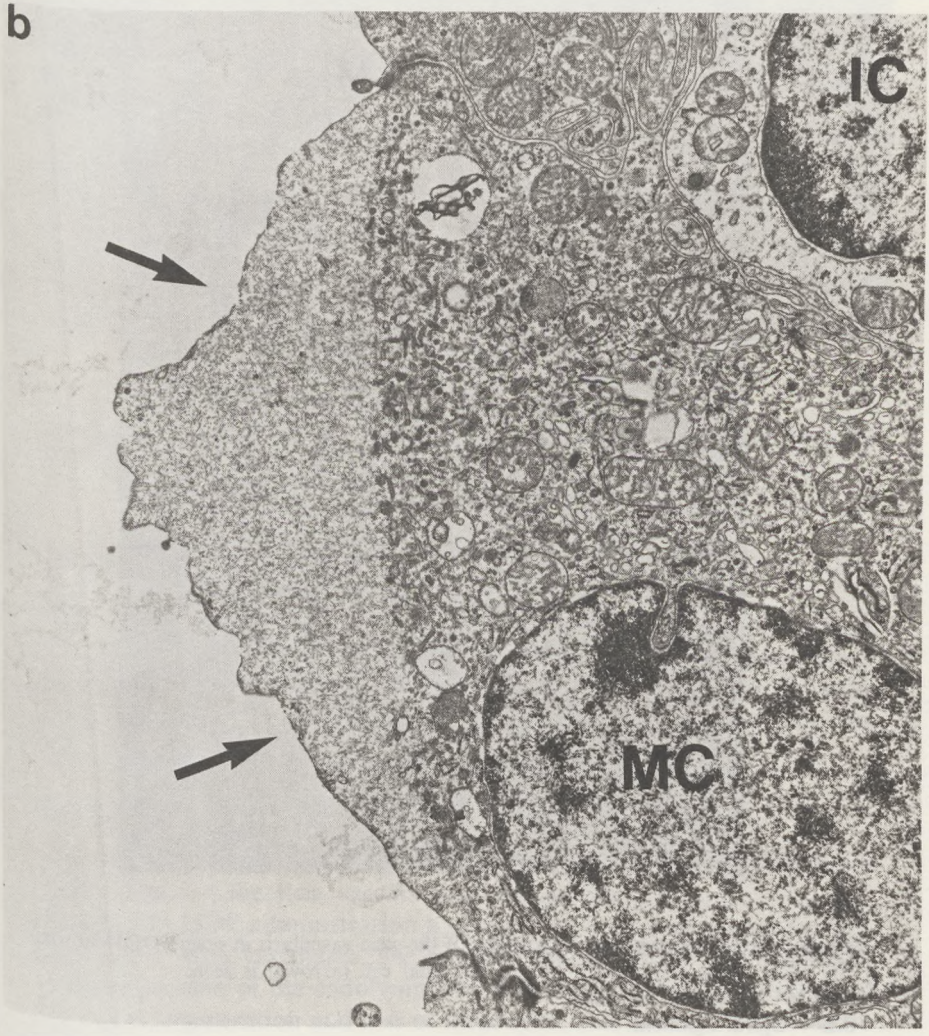
Phase-contrast interference (a) and transmission electron microscopy (b) of the stria vascularis.
Both methods show the swelling and protrusion of the marginal cells (arrows) into the endolymphatic space.
M.C. = Marginal cell
I.C. = Intermediate cell.

Table 3

Group of striae with severe degeneration changes.

guinea pig	Audiometry	Days DDP intoxication
CC 3	+	12
CC22	+	12
CC14	+	14
CC 2	+	20

+ = hearing threshold dropped over more than 30 dB



times swelling and protrusion of the marginal cells into the endolymphatic space.
We could find this phenomenon in both T.E.M. and phase-contrast interference preparations (fig. 2). The swelling seemed to be a result of a toxic process in the endolymphatic border of the marginal cell.
The last group of striae (table 3) showed severe degenerative changes with cystic deformation and loss of marginal cells over the complete length of the cochlear duct (fig. 3).

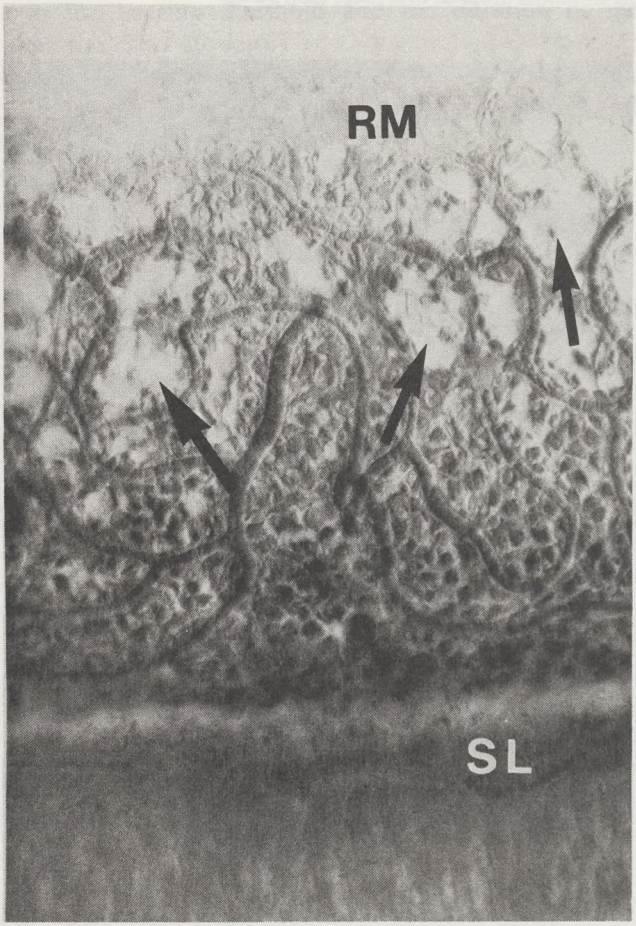


Fig. 3

Phase-contrast interference microscopy of the stria vascularis in severe DDP ototoxicity cystic degeneration and loss of marginal cell (arrows) is seen.
R.M. = Reissner's membrane
S.L = Spiral ligament.

Fig. 4

Transmission electron microscopy of the cystic deformations (arrows) in the upper part of the stria vascularis near Reissner's membrane (R.M.).

Within the cystic deformations near the Reissner's membrane only a few denatured mitochondria were seen (fig. 4).
The electrode-audiological study showed no drop of the hearing threshold until the 8th to 10th day of DDP intoxication. A threshold loss of 30 dB or more did appear after that period of intoxication. In those cases we found the cochlea's (group two and group three) with the described changes in the stria vascularis.

Discussion

The present study showed that, contrary to other findings[9,10], DDP has a toxic effect on the stria vascularis of the guinea pig.
During the DDP administration a progressive degeneration pattern was seen in the stria vascularis. The ototoxic changes in the stria vascularis were protrusion of the endolymphatic side of the marginal cell followed by cystic degeneration and loss of these cells. This ototoxic degenerative

process seemed to start both in the basal turn of the cochlea and in the area near Reissner's membrane.
Stria vascularis pathology, as in this study is seldom mentioned in ototoxicity studies. Only in very late stages of aminoglycoside ototoxicity the same stria pathology was seen[16,17]. In atoxyl intoxication damage to the marginal cells in the form of loss of microvilli and protrusion of the cell surface was seen, but mainly in the apical turn[18]. No edema of the stria vascularis was found in this study. Stria edema is often seen in ethacrynic acid intoxication[19,20,21].
Our study showed that DDP has a special affinity to the endolymphatic side of the marginal cells in the basal turn of the cochlea. The important inner ear enzyme adenylate cyclase is concentrated along the endolymphatic surface of the marginal cell[22]. DDP significantly (60% ± 20 S.D., N=7) inhibits the activity of this adenylate cyclase from the stria vascularis[23].
In conclusion it may be said that DDP is a toxic agent for the inner ear and especially toxic for the haircells and the stria vascularis. This morphological study showed a progressive characteristic degeneration pattern of the stria due to DDP. Further research into the possible reversibility of the toxic effects of DDP to the stria vascularis seems to be important.

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The Bristol-Myers Company offered the cis-Platinum (Platinol) for this experiment. The authors also thank Mrs. D. de Jong-v.d. Werf for her help in preparing this manuscript.

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Chapter 6

Hearing Loss and Inner Ear Changes in a Patient Suffering From Severe Gentamicin Ototoxicity

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Hörverlust und Innenohrveränderungen bei einem Patienten nach hochdosierter Gentamyzin-Applikation

Zusammenfassung. Die histologischen Veränderungen an der menschlichen Kochlea nach acht Jahre zurückliegender hochdosierter Gentamyzin-Applikation bei an Taubheit grenzender Schwerhörigkeit, werden aufgezeigt. Folge der ototoxischen Wirkung von Gentamyzin ist zunächst eine Zerstörung der Sinneszellen im Cortischen Organ, denen eine solche der Stützzellen folgt. Dann verschwinden die Nervenfasern und die Zellen im Ganglion spirale und letztlich bleibt eine dünne Zellage auf der Basilarmembran. In der Stria vascularis sind dann bei Bildung von Zysten keine Gefäße mehr nachzuweisen.

Schlüsselwörter: Gentamyzin-Ototoxizität – Hörverlust – Innenohrveränderungen

Summary. The long-term histological effects of gentamicin ototoxicity could be studied in a human being in relation to the audiometric impairment. The possible sequence of degeneration of hair cells, supporting cells, nerve fibers, stria vascularis, spiral ganglion cells, and vascular supply is discussed.

Key words: Gentamicin ototoxicity – Hearing loss – Inner ear changes

The inner ear pathology produced by aminoglucoside administration has been the subject of extensive studies by many authors. Most data were obtained in animal studies (Hawkins et al. 1969; Wersäll et al. 1969; Ylikoski et al. 1973), reports on inner ear lesions in human beings are still rare (Benitez et al. 1962; Lindsay et al. 1960; Lowry et al. 1973). In most experimental animal studies the classical inner ear cross-section technique has been used (Schuknecht 1953).

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The present study gives a report of the audiometric data and the inner ear findings in a young human patient affected with a severe gentamicin intoxication obtained by means of the s.c. surface technique (Hawkins and Johnsson 1975; Johnsson 1979).

Clinical Data

The patient, a young woman aged 24, bodyweight 31 kg, with a congenital low-thoracic meningocele and paralysis of the lower limbs developed a serious pseudomonas osteomyelitis of the pelvis and right femur in August 1970. She was treated by surgery and relatively high doses of gentamicin (180 mg daily). As the disease exacerbated immediately when, after 2 weeks, gentamicin was changed for other (non-ototoxic) antibiotics, the gentamicin administration was resumed. The disease was ultimately cured by repeated surgery and a prolonged cure of gentamicin in decreasing dosage (Fig. 1).

During the treatment the kidney function was temporarily impaired.

By the end of the therapy it was noted that a severe deafness had developed. On audiometry the patient's left ear appeared to be totally deaf and on the right side a severe high-tone sensory neural impairment and speech discrimination loss was measured. Eight months later a considerable increase of the hearing impairment was found. Since that moment hearing remained stable.

On caloric examination, the vestibular function was found to have completely ceased bilaterally.

As the patient received no other ototoxic drugs and her hearing was normal before, the lesion was attributed to gentamicin ototoxicity. Her case was described before by Huizing (1972).

Since this illness the condition of the patient remained stable until she developed an urosepsis and peritonitis in December 1978, of which she died within a few days. During her fatal illness she received 180 mg tobramicin i.v. per day for 5 days. No other ototoxic drugs were administered.

Histological Data

The right ear was microdissected and the cochlea was studied by means of the surface technique and phasecontrast microscopy. The modiolus was cross-sectioned into serial slices and was studied with light microscopy.

In the middle ear a congenital deformation of the stapes was found. The crura were considerably thickened and partially fused. The middle ear was otherwise normal and a normal stapedius tendon was present.

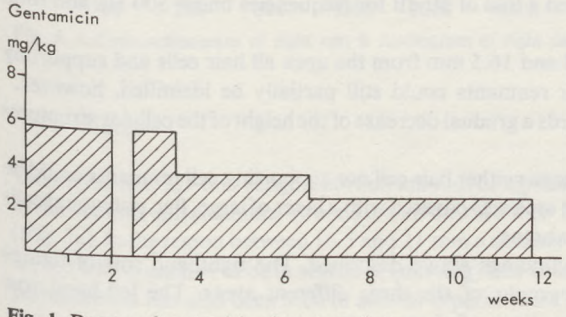


Fig. 1. Dosage of gentamicin given to patient

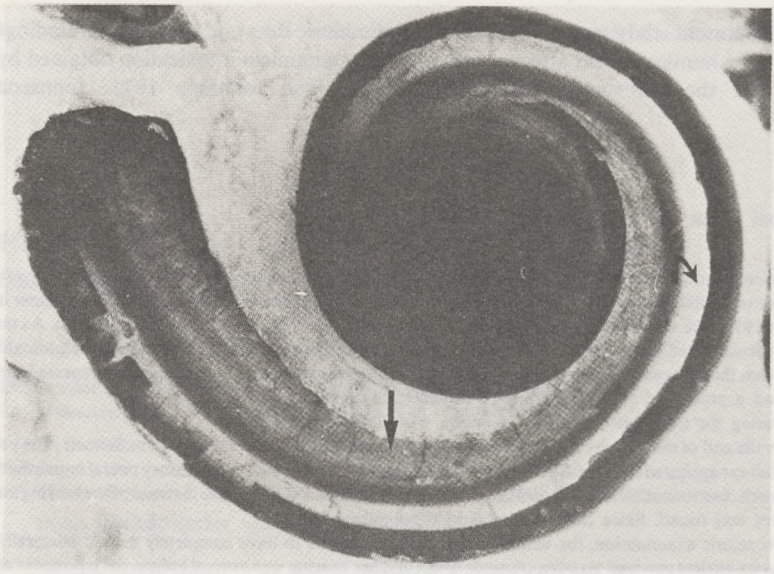


Fig. 2. Microdissected right cochlea of patient. Straight arrow: no myelinated nerve fibers in osseous spiral lamina; curved arrow: absence of spiral organ

In the inner ear a severe degeneration of almost all parts was observed in all cochlear windings (Fig. 2).

Hair Cells

Only in the first 3.3-mm range from the apex some of the hair cells and supporting cells were found intact. The percentage of intact outer and inner hair cells are given in Fig. 3a and b in combination with the threshold audiogram.

The outer hair cells had almost completely disappeared except for a very small percentage in the first 3.3 mm. About 60% of the inner hair cells were still present in this area. The audiogram showed a loss of 50 dB for frequencies under 500 Hz and total deafness above 8,000 Hz.

In the area between 3.3 and 16.5 mm from the apex all hair cells and supporting cells were destroyed. Their remnants could still partially be identified, however.

From the apex downwards a gradual decrease of the height of the cellular structures was observed.

Beyond the 16.5-mm range neither hair cell nor supporting cell remnants could be discovered. The spiral organ area was covered with a layer of large, flat, polygonal cells resting on the basilar membrane.

Figure 4 illustrates the findings above described. The righthand row of figures shows characteristic photographs of the three different areas. The left-hand row consists of drawn reconstructions of these parts.

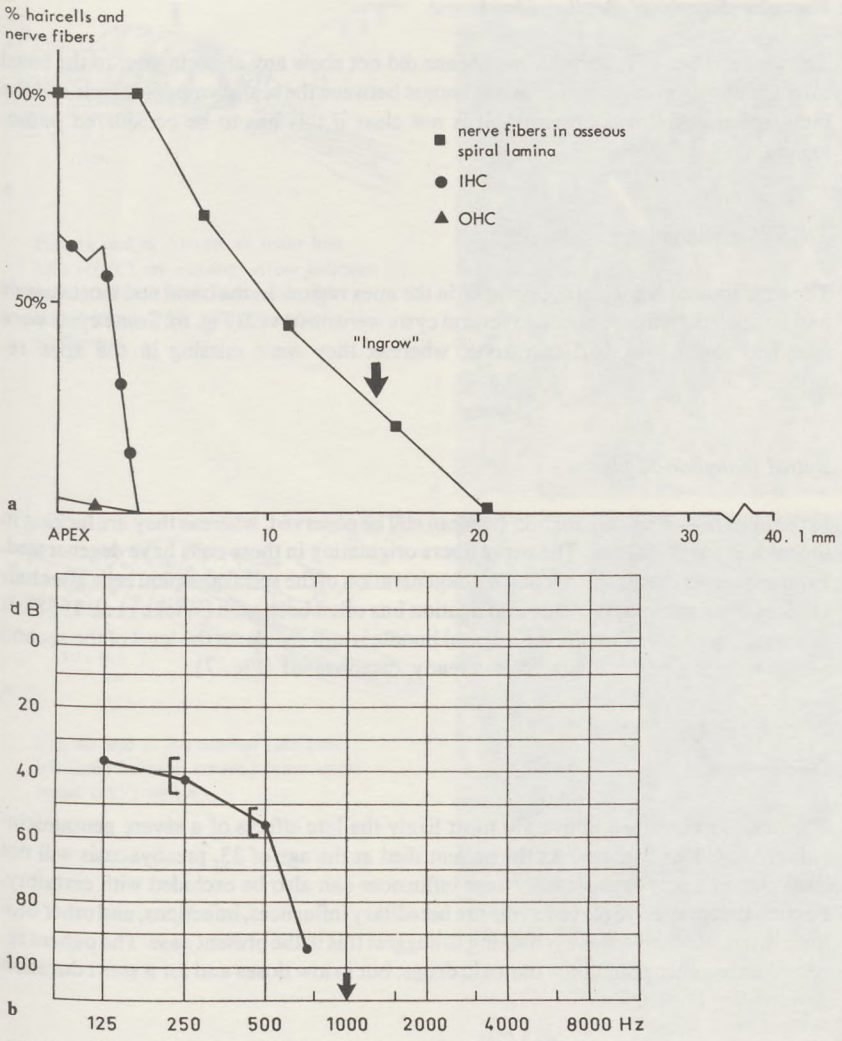


Fig. 3. a Cytocochleogram of right ear; b Audiogram of right ear

Nerve Fibers

Within the first 3.3-mm range a normal amount of myelinated nerve fibers were visible in the osseous spiral lamina. From 3.3 mm onward their number gradually diminished (Fig. 3a). In the region between 16.5 and 21 mm a number of myelinated fibers were observed which seemed to be in search of (missing) hair cells (see Fig. 5). This interesting phenomenon has also been seen in animal experiments (Terayama et al. 1977, 1979). Beyond 21 mm no nerve fibers were present.

Vascular Supply of Basilar Membrane

The vascular bed of the basilar membrane did not show any abnormality. In the basal turn a relatively great numer of anastomoses between the scala tympani vessels and the outer spiral vessel was observed. It is not clear if this has to be considered pathological.

Stria Vascularis

The stria showed a normal appearance in the apex region. In the basal coil most vessels had atrophied. In this region also several cysts were observed (Fig. 6). Some cysts were found in the 3.3 to 16.5-mm zone, whereas they were missing in the apex region.

Spiral Ganglion-Modiolus

In the apex region spiral ganglion cells can still be observed, whereas they are lacking in the middle and basal coil. The nerve fibers originating in these coils have degenerated. In animal experiments this secondary degeneration of the spiral ganglion cells after hair cell loss due to aminoglycoside intoxication has often been seen (Wicke et al. 1978). It is interesting to note that the inner spiral bundle is still visible on the level of the second turn where the nerve fibers have already disappeared (Fig. 7).

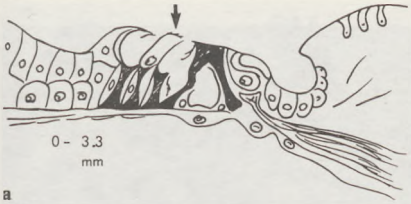
Discussion

The findings described above are most likely the late effects of a severe gentamicin-induced cochlear damage. As the patient died at the age of 33, presbyacusis will not have played a role in this case. Noise influences can also be excluded with certainty. Factors that may have played a role are hereditary influences, infections, and other ototoxic drugs. However, there is nothing to suggest this in the present case. The patient received some other potentially ototoxic drugs, but in low doses and for a short duration only.

Sequence of Degeneration in the Case Described

If we assume that the findings in this case are the result of a gradual degeneration proceeding from the base to the apex, the various parts of the cochlea will represent successive stages of this process. If we accept this hypothesis, the following stages of degeneration might be distinguished:

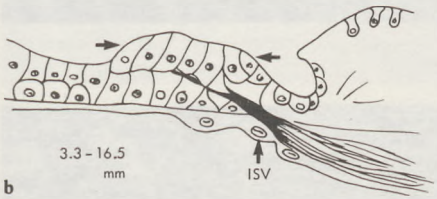
Stage I (0—3.3 mm) – degeneration of hair cells, the outer hair cells first and then the inner hair cells, (immediately) followed by deterioration of the supporting cells. Other structures normal.



a

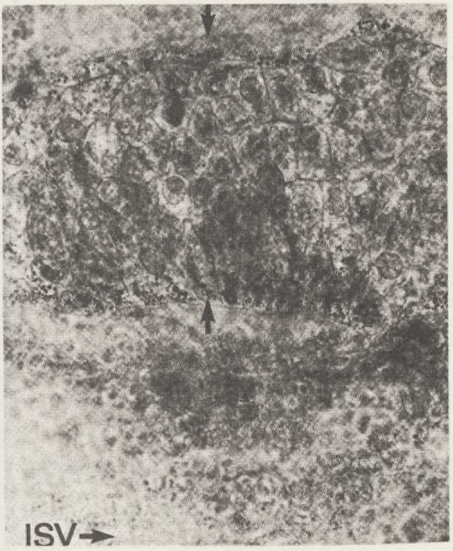
Fig. 4a and d. Almost all outer hair cells (*OHC*) are missing, arrow indicates intact OH-cell. Most inner hair cells (*IHC*) intact. Pilar cells (*P*) partially malformed

d



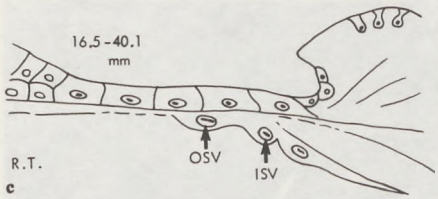
b

Fig. 4b and e. No normal hair cells left (area between arrows) Inner spiral vessel (*ISV*) visible



e

Fig. 4. Photographs (right) and reconstruction (left) of the three areas with different degrees of degeneration



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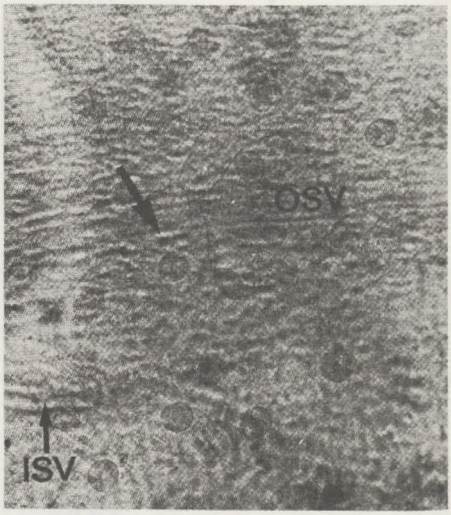

f

Fig. 4c and f. Spiral organ replaced by one layer of large, flat, polygonal cells (arrow). Both the outher spiral vessel (*OSV*) and the inner spiral vessel (*ISV*) can be seen

Fig. 5. Representative part of area between 16.5 and 21 mm *SO:* remnant of spiral organ. *ISV:* inner spiral vessel. *INF:* ingrowing nerve fibers. *L:* limbus (cp. Figs. 3a and 4b)

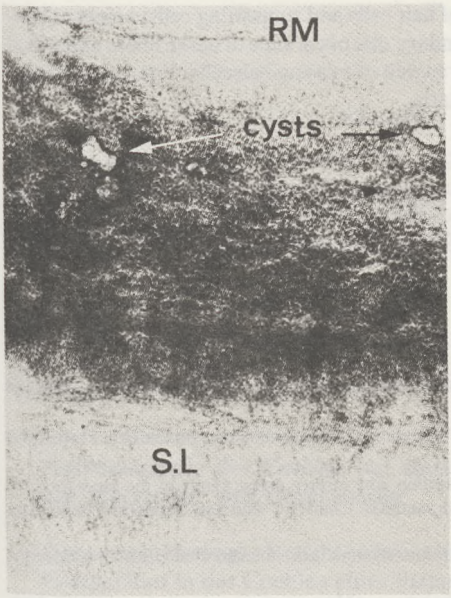

Fig. 6. Stria vascularis in the basal coil. *RM:* Reissner's membrane, *SL:* spiral ligament. Note the cysts

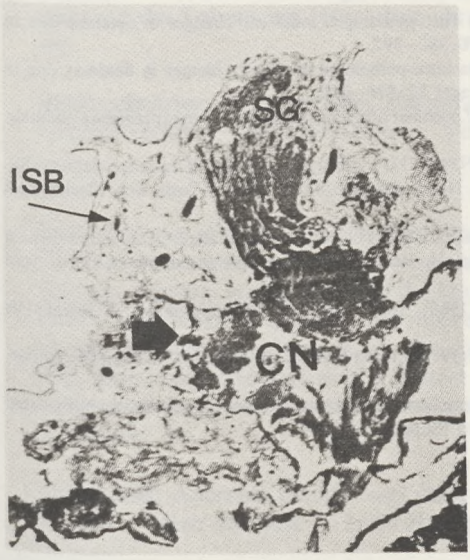

Fig. 7. Modiolus cross section. *SG:* spiral ganglion cells. *CN:* cochlear nerve. *arrow:* degenerated part of cochlear nerves. *ISB:* inner spiral bundle

Stage II (3.3–16.5 mm) – destruction of hair cells and supporting cells; height of sensory epithelium decreased; gradual secondary disappearance of nerve fibers and spiral ganglion cells. Ingrowth of new fibers in search of missing haircells. Vascular atrophy and formation of cysts in stria vascularis.

Stage III (beyond 16.5 mm) – spiral organ area replaced by a layer of large thin cells resting on the basilar membrane. Disappearance of all nerve fibers and ganglion cells. Complete devascularisation of stria with formation of many cysts. Vascular anastomoses between scala tympani vessels and outer spiral vessel.

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Chapter 7

A Cochlear Vascular Anomaly in a Patient with Hearing Loss and Tinnitus*

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Gefäßanomalie der Cochlea bei einem Patienten mit Hörverlust und Ohrensausen

Zusammenfassung. Bericht über einen ungewöhnlichen Verlauf eines Blutgefäßes in der Cochlea eines Patienten. Das Gefäß zog von der Lamina spiralis ossea unter Durchquerung der Scala tympani bis zur lateralen Wand des Ductus perilymphaticus. In der Region dieses abnorm verlaufenden Gefäßes fand sich ein völliger Haarzellverlust. Beim Patienten bestand während des Lebens ein Hörverlust vor allem bei 2000 Hz mit Ohrensausen.

Schlüsselwörter: Aberrierendes Blutgefäß – Scala tympani – Mensch – Haarzellverlust – 2000 Hz-Senke – Tinnitus

Summary. An unusual blood vessel in the cochlea of a patient is reported. The blood vessel derives from the osseous lamina spiralis and crosses straight through the scala tympani toward the lateral wall of the perilymphatic duct. In its course a branch derives from this vessel toward the other spiral vessel. In the region of this aberrant vessel a complete hair-cell loss is present. A high tone perceptive loss with a relative dip and tinnitus was found in the same ear during life. The possible cause and effects of this aberrant vessel are discussed.

Key words: Aberrant blood vessel (suspension vessel) – Scala tympani – Human – Hair-cell loss – 2,000 Hz dip – Tinnitus

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Introduction

The cochlear vasculature in various animals and in man has been studied extensively over the last few years. A considerable contribution toward our present knowledge was made by Axelsson (1968). In his excellent paper he gave a detailed description of the cochlear blood vessel pattern in man and guinea pig. Other studies described the cochlear blood vessel pattern in rabbits (Axelsson and Lind 1973), rhesus monkeys (Axelsson 1974), chinchillas (Axelsson and Lipscomb 1975), and rats (Hornstrand et al. 1980). Miodonski et al. (1978) visualized the vascular structure of the cochlea in rats by means of scanning electron microscopy.

Deviations in vasculature in the form of an unusual course of a vessel in the scala tympani were described in the cavia (Maass 1969; Axelsson 1971) and in the rat (Hornstrand et al. 1980).

In man, such an anomaly occurring in the vascular bed of the cochlea has been reported by Wolff (1935), by Polvogt and Crowe (1937) and more recently by Johnsson (1972). In their studies they did not find any anomalies in the other structures of the cochlea.

This paper reports on a histological study in which a deviating vessel was found in the scala tympani of the right ear of a patient suffering from a bilateral high tone perceptive hearing loss with a relative dip at 2,000 Hz on the right, accompanied by tinnitus in the right ear.

Material and Methods

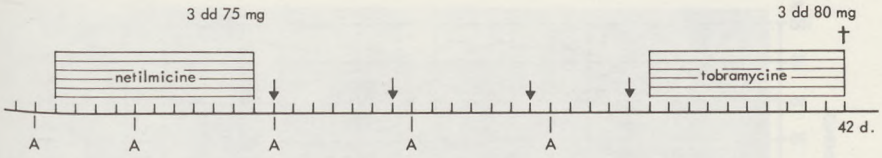
Clinical Data

Our patient (a 66-year-old woman) had been known to suffer from a chronic myeloid leukemia for 10 years, because of which she was regulary treated with the cytostatics Myleran and hydroxy ureum. In May 1979, an exacerbation of her illness occurred. The patient complained of headache and tinnitus in the right ear. She was treated with vincristine (2 mg i.v. once a week). In the course of this therapy, a sepsis (B. pseudomonas) developed, which was cured with netilmicin (10 days 3 dd 75 mg). Before, during and after the netilmicin therapy, the patient's hearing was checked every week, without any changes occurring in the audiogram. Twenty days after the netilmicin treatment, another gram-negative sepsis developed. Tobramicin was chosen as an antibiotic. During the tobramicin medication (3 dd 80 mg for 10 days) no hearing measurements were carried out (Fig. 1).

In spite of the therapy, the patient died of respiratory insufficiency. Dissection was carried out and the ossa temporalia were examined.

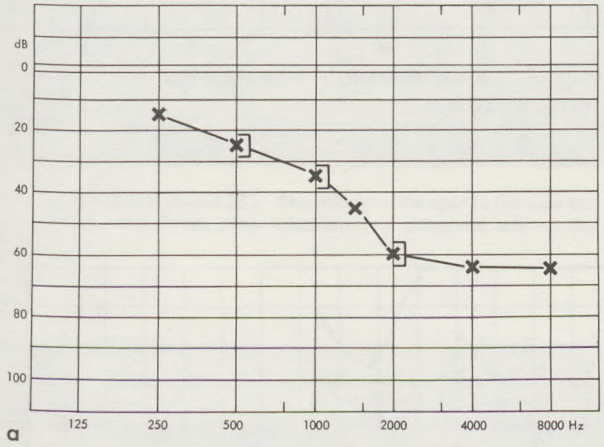
Audiologic Data

The anamnesis did not point to any possible trauma caused by exposure to noise. The patient had never complained of deafness or dizziness. When she was last taken in, she complained of tinnitus in the right ear. Unfortunately, neither its frequency nor its intensity were measured. Both ears had a perceptive hearing loss with a sloping curve and a relative dip at 2,000 Hz on the right side (Fig. 2).



A = audiogram
↓ = vincristine 2 mg i.v.

Fig. 1. Therapy scheme of patient



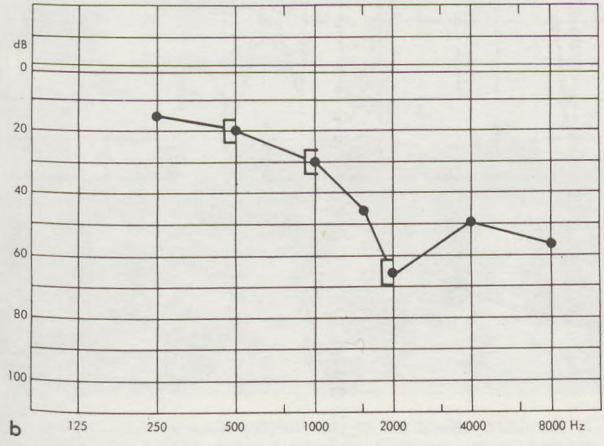
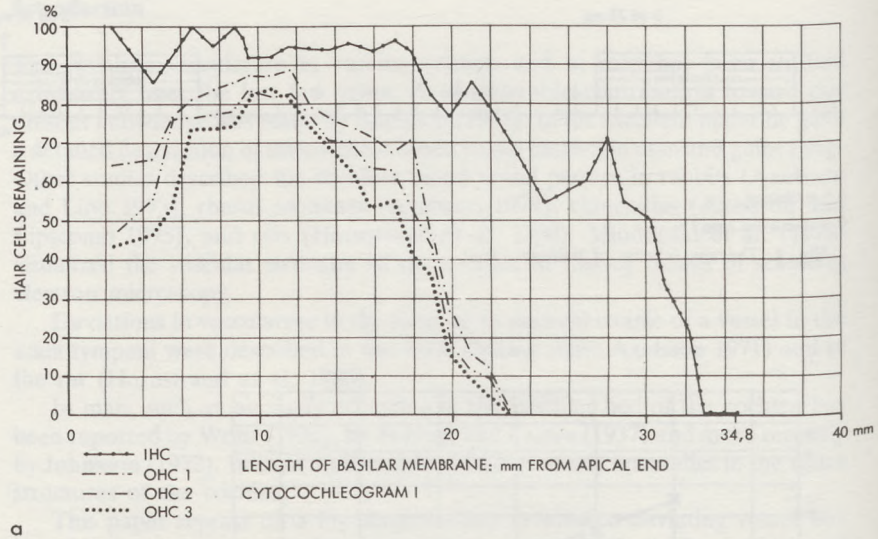
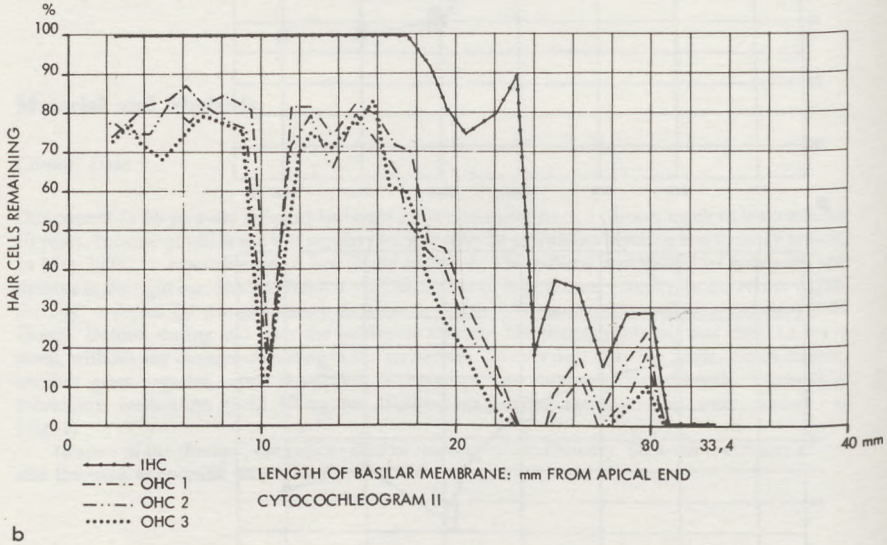


Fig. 2. Tone audiograms of patient



a



b

Fig. 3. Cytocochleograms, **a** left ear, **b** right ear

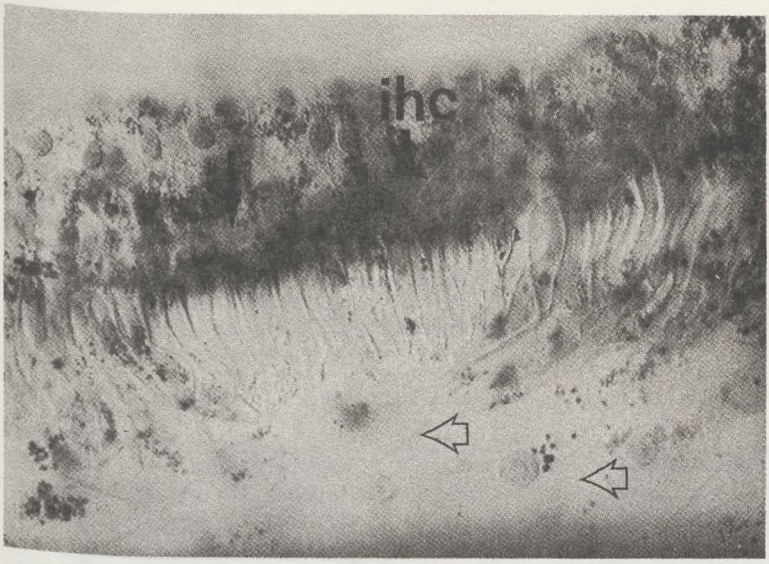


Fig. 4. Hair-cell loss in area 23.1–24.5 mm from the apex in the right ear. IHC = inner hair cell left; *straight arrow* = IHC has gone; *open arrows* = polygonal cells on the place where OHC's have been

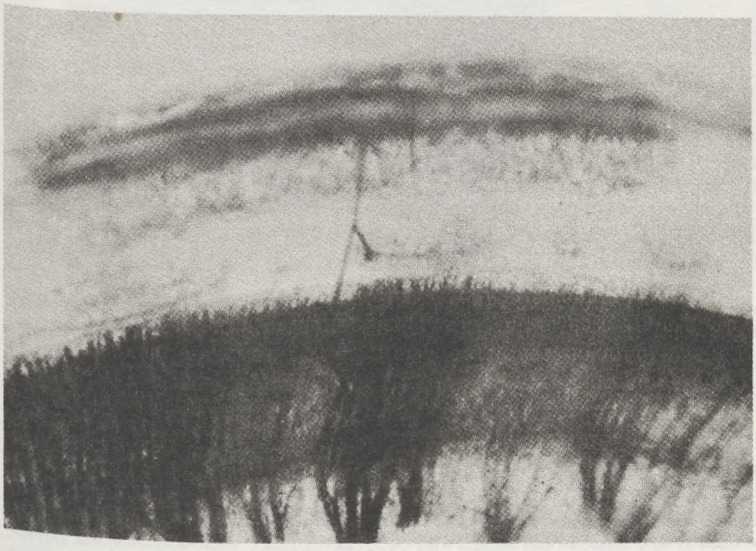


Fig. 5. View from the scala tympani side on the basilar membrane at 21.1–24.1 mm from apex. Aberrant "suspension" vessel is clearly seen. Note the extreme hair-cell and nerve-fiber loss round the vessel

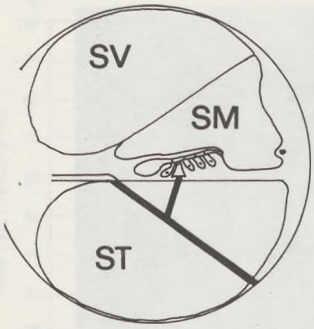


Fig. 6. Schematic drawing of the suspension vessel. SV = Scala Vestibuli, SM = Scala Media, ST = Scala Tympani

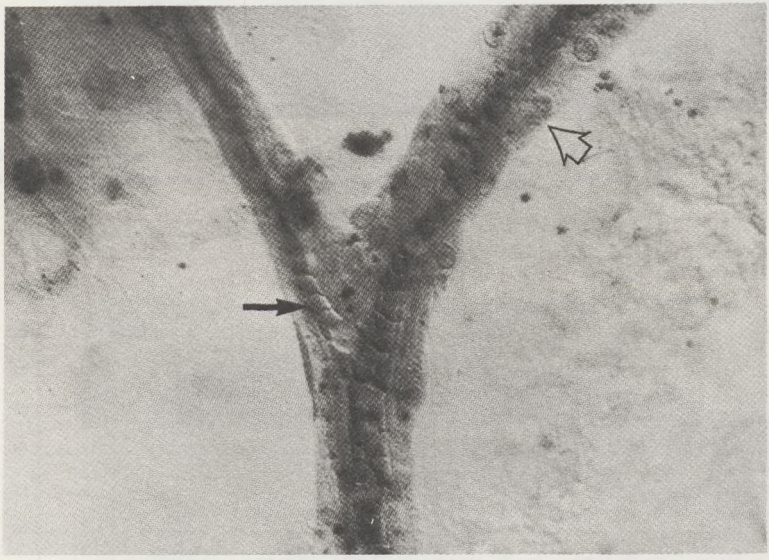


Fig. 7. The suspension vessel. Note: the many erythrocytes (*arrow*) "sludging" and the relative thick endothelial wall (*empty arrow*)

Histological Data

After death, the ossa temporalia were removed by the method of Ward and Lindsay (1964). The bones were fixed in 40% formalin and stained with O_5O_4 (Zetterquist). Microdissection was carried out, and surface preparations were made which were studied under the interference phase-contrast microscope (Hawkins and Johnsson 1975). Cytocochleograms were made of both ears. No anomalies were found in the tympanic membrane and the middle ear.

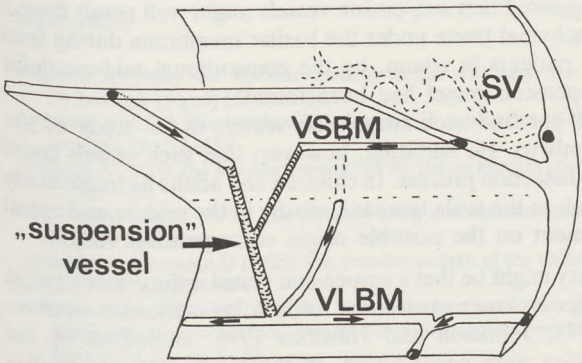


Fig. 8. A reconstruction of the blood flow pattern (*small arrows*) in the suspension vessel. SV = stria vascularis; VLBM = venule of the basilar membrane, VSBM = vessel of the basilar membrane (outer spiral vessel)

Hair Cells. All hair cells were counted and the percentage of remaining cells mapped out in a cytocochleogram (Fig. 3). As can be seen, the number of cells gradually decrease in the direction of the fenestra rotunda. In both ears, the losses among the outer hair cells (OHC 1, 2, 3) were more outspoken than among the inner hair cells (IHC). What actually struck the eye, was the extremely high loss of both OHC and IHC in the right ear at 24 mm from the apex (Fig. 4). This area virtually coincides with the 2,000 Hz area (Schuknecht 1974), which was the place where a dip in the audiogram had been found. Further, a very small area with extremely high OHC losses was found at 10 mm from the apex, but this loss did not show in the audiogram.

Vasculature of the Basilar Membrane. The left ear showed no conspicuous aberrations here. In the right ear, on the other hand, a blood vessel following an abnormal course was found in the area with the more outspoken hair-cell degeneration (21.1–24.1 mm from the apex) (Figs. 5 and 6).

This vessel sprang from the osseous lamina spiralis, ducked the basilar membrane and traversed the scala tympani toward the lateral wall, sprouting a tiny branch toward the basilar membrane meanwhile. The "suspension" vessel was crowded with erythrocytes. The wall of this vessel was obviously heavier than that of the other vessels in the organ of Corti (Fig. 7). Using the data of Axelsson, we reconstructed the direction of the blood flow in the suspension vessel. It seemed to form a link between the vessel of the basilar membrane (VSBM) and the venule of the basilar membrane (VLBM) (Fig. 8).

Stria Vascularis. In neither ear did we find any aberration by our technique. There was no atrophy, the vessels of the stria were normal and we did not find any cysts.

Discussion

A suspension vessel, like the one found in our research, is seldom mentioned in the literature. The nature and significance of this vessel are still obscure. Neither is the origin of the phenomenon known. As possible agents might be suggested: (1) a congenital defect, (2) an acquired defect (noise trauma, ototoxic medication or infection), (3) a phenomenon associated with aging.

To (1): Wolff (1935) suggested that suspension vessels might well result from a late resorption of mesenchymal tissue under the basilar membrane during fetal life. She described two patients in whom, by the conventional mid-modiolar slicing technique, a suspension vessel had been found.

Johnsson (1972) did not find such suspension vessels in his study of the cochlea of the human embryo. He admitted, however, that such vessels could have been missed in the dissection process. In children and adults he incidentally did find suspension vessels in the scala tympani, mostly in the middle and apical turns. He did not comment on the possible origin of suspension vessels.

To (2): Another possibility might be that a suspension vessel is the manifestation of a recovery process after an inner ear trauma. In histological studies of noise trauma (Schuknecht 1974; Johnsson and Hawkins 1976) anomalies of the vascular bed in the cochlea were never found. In the many ototoxity studies conducted so far, such a deviation in the vascular bed of the cochlea was never mentioned. In a study of a cochlea in a patient showing a serious hearing loss owing to treatment with gentamicin, we found a great number of anastomoses between the spiral vessels and the venules of the basilar membrane (Tange and Huizing 1980). No suspension vessels were found in this research.

To (3): As a third possible origin of the suspension vessel we mentioned old age. Johnsson (1971) and Johnsson and Hawkins (1972a, b), however, did not mention such vessels in the scala tympani in their extensive histological study of the inner ear of elderly people.

In conclusion it may be said that there is still no clarity about the mechanism generating suspension vessels in the scala tympani. In our case, we accidentally found a suspension vessel in a patient who complained of tinnitus in the right ear. Audiometrically, there was a high tone perceptive hearing loss with a relative dip at 2,000 Hz on the right side. In the right cochlea there was an extreme loss of hair cells in the 2,000 Hz region. In the same region, a vessel with an abnormal course was found. How it had developed, could not be ascertained in this study. By way of hypothesis it might be suggested that the suspension vessel was primarily generated by a congenital or acquired defect in the cochlea. A faulty hemodynamic function might have originated in the area serviced by the supplying arteriola. This might have caused a weak spot in the basilar membrane. As a result of aging, noise and infections, or ototoxic medications, hair-cell loss followed by nerve degeneration might then have occurred. In the present case one might wonder whether the tinnitus in the ear could have been result of the aberrant vessel in the scala tympani. Usually, however, tinnitus is assumed to be caused by hair-cell degeneration. In this study both phenomena were found. Although it seems rather speculative, tinnitus in the ears might indicate a suspension vessel in the scala tympani, which could mean that such ears are predisposed to inner ear traumata.

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Chapter 8

GENERAL DISCUSSION

Since Corti (1851) described the general microscopic details of the inner ear, many workers have studied the anatomy and pathology of the hearing organ. In the late 19th century the study of the human cochlea was carried out to a great extent by the aid of the dissection technique. Later, since the beginning of this century, these techniques have been almost exclusively replaced by the serial sectioning technique. In his superb book Schuknecht[1] gives a considerable amount of information on the morphology and pathology of the cochlea which has been obtained by this section technique. In certain respects, however, it is impossible to obtain exact and reliable information with this technique. This is particularly true of the quantitative estimates of the sensory haircell population, the innervation pattern and the surface structures of the stria vascularis and Reissner's membrane.
With the sectioning technique one cannot study these parts of the cochlea equally well[2]. It is for this reason that the microdissection technique with surface preparation was revived after a long period of oblivion.

At the same period of the revival of the microdissection technique, Hinshaw and Feldman[3] reported their findings of cochlear and vestibular toxicity of the drug streptomycin. Since that report, many ototoxic agents have appeared on the market. The most important ototoxic agents are the aminoglycoside antibiotics followed by some "loop" diuretics, certain analgesics and antipyretics. A relative newcomer to the family of ototoxic drugs is the antimitotic cis-diamminedichloroplatinum II=D.D.P. which is used in cancer treatment nowadays[4]. The histological changes in the cochlea that occur as a result of ototoxic drugs have been reported in the literature by many authors. In a variety of laboratory animals these workers have demonstrated the inner ear changes caused by these ototoxic drugs. Only a few comparable cases of human inner ear ototoxic pathology have been reported in the literature sofar[5,6,7]
In most animal studies the histological changes in the inner ear due to aminoglycoside antibiotics were evaluated by studying "representative" segments from every turn of the cochlea. Only a few workers used complete haircell-counting[8,9,10].

According to the general opinion the first signs of degeneration in ototoxicity are found in the basal turn. The inner most row of the outer hair-cells (OHC 1) is affected first; the damage then progresses towards the apex and the other outer hair-cells (OHC 2 and OHC 3). Subsequently, the inner ear cells (IHC) start to degenerate. This degeneration pattern also runs from base to apex. The last structures to show degeneration are the nerve fibres and the stria vascularis[11,12,13,14]

In our study we used the microdissection technique with surface preparations for the investigation of the degenerative changes in the cochlea due to two different ototoxic drugs; gentamicin and D.D.P. The microdissection technique has the advantage of enabling one to study the complete cochlea. Using only the new S.E.M. or T.E.M. techniques it would have been impossible to study the complete degeneration patterns of the hair cells as well as the stria vascularis and Reissner's membrane. Complete hair cell-counting (cytocochleograms) was used to establish the degeneration pattern of the whole cochlea in guinea pig.
We observed noticeable differences in damage caused by the two different ototoxic drugs.
In the gentamicin material the initial degeneration point was found to be localized in the basal turn at 6-8 mm from the round window. This part of the basilar membrane appears to be particularly "sensitive" to gentamicin. For D.D.P. on the other hand no typical "degeneration point"was found. D.D.P. starts its ototoxic action on the OHC's in all cochlear turns, whereas gentamicin has a preference for the basal turn. In the gentamicin as well as in the D.D.P. study, the OHC's were the first to degenerate, starting with OHC 1 and followed by OHC 3 and OHC 3. Subsequently, the IHC's started to degenerate.
We found differences between the two drugs concerning the pillar cells of the tunnel of Corti. In the gentamicin treated animals no noticeable loss of pillar cells was observed, whereas for D.D.P. a clear loss of outer pillar cells was found. This last phenomenon has also been seen by Micheals[15] in human inner ears.
No abnormalities of the stria vascularis were found in the gentamicin study, but it should be mentioned that T.E.M. was not used. In our D.D.P. material a noticeable degenerative change in the stria vascularis was found both in the surface preparations as well as in T.E.M. preparations. Severe changes were seen in the marginal cells of the stria vascularis. Even complete loss of marginal cells in the basal turn was observed. In Reissner's membrane the different effects of the two ototoxic drugs were noticed too. Intracellular vacuolisation was seen in the gentamicin guinea pigs while the D.D.P treated animals did not show abnormalities in the Reissner's membrane. Fig. 8 gives a survey of the

	Gentamicin	D.D.P.
Haircells	OHC>IHC Basal turn "Degeneration point"	OHC>IHC Every turn
Pillar cells	No changes	OPC loss
Stria Vascularis	No changes	Vacuolisation Protrusion MC MC loss
Reissner's Membrane	Intracellular Vacuolisation	No changes

Fig. 8. Differences in ototoxic expression of the two ototoxic drugs : Gentamicin and cis-Platinum (D.D.P.)
OHC = Outer haircell
IHC = Inner haircell
OPC = Outer pillarcell
MC = Marginal cell of stria vascularis

effects found in gentamicin intoxication as compared with D.D.P. intoxication.

This thesis was completed by examining two cases of degenerative changes in human cochlea by microdissection and surface preparations. One cochlea was intoxicated with gentamicin and showed all types of degenerative changes due to this drug. In the other inner ear an unusual blood vessel was found in the scala tympani. Around this aberrant vessel (suspension vessel) severe degenerative changes were found. There is still the question whether it would have been possible to find this vessel with other techniques than the microdissection technique. We believe that our technique has an important role in the research techniques for the inner ear. This statement finds support in the present investigations.

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Chapter 9

SUMMARY

This study gives the results of a series on inner ears carried out with the help of microdissections and surface preparations. The degeneration patterns of the cochlea of the guinea pig due to two different ototoxic drugs were investigated with this technique. Clear differences between the results of the ototoxic drugs (gentamicin and cis-platinum) in the inner ear were observed.

Gentamicin has a typical "starting point" of degeneration on the basilar membrane. From this "degeneration point" the loss of hair cells progressed towards the round window (fast) and the apex (slowly). Stria vascularis showed no signs of degeneration due to gentamicin. Reissner's membrane, on the other hand, showed cellular vacuolisation of the endolymphatic cells.

Cis-platinum (D.D.P.) showed no "degeneration point"; the loss of hair cells was found over the complete length of the basilar membrane, with a preference for the basilar turn.

Stria vascularis, on the other hand, showed severe degenerative changes due to D.D.P., whereas Reissner's membrane showed no change.

In using the microdissection technique we were in a position to discover these differences between the two ototoxic drugs.

This study was completed by two cases of microdissection of the human cochlea. In the first case, the postmortal findings gave a clear example of the degenerative changes in the human ear due to the ototoxic drug gentamicin.

In the other case, an unusual blood vessel was found in the scala tympani. Around that "suspension vessel" severe degenerative changes were observed in the organ of Corti.

This anomaly could be discovered thanks to the microdissection and surface preparations.

In our opinion the microdissection technique has an important place in inner ear research.

Chapter 10

SAMENVATTING

In deze studie worden de resultaten vermeld van een aantal studies, waarbij het binnenoor is onderzocht door middel van microdissecties en oppervlaktepreparaten. Het volledige degeneratiepatroon van de cochlea bij de cavia ten gevolge van twee verschillende ototoxische medicijnen werd met deze techniek onderzocht.

Er werden duidelijke verschillen gevonden in de ototoxische werking van deze twee medicijnen (gentamicine en cis-platinum).

Gentamicine heeft een duidelijk "startpunt" van de degeneratie van de haarcellen op het basilaire membraan. Vanuit dit "degeneratiepunt" treedt er een versneld verlies van haarcellen op in de richting van het ronde venster (snel) en in de richting van de apex van de cochlea (langzaam).

In de stria vascularis werden geen degeneratieve afwijkingen gevonden ten gevolge van gentamicine intoxicatie. Reissner's membraan daarentegen vertoonde intracellulaire vacuolisatie in de cellen aan de endolymphatische zijde.

Bij de studies waarbij cis-platinum (D.D.P.) als ototoxisch medicijn werd gebruikt kon geen duidelijk "degeneratiepunt" worden gevonden. Over de gehele lengte van het basillaire membraan werd verlies van haarcellen gevonden. Wel bestond er een geringe voorkeur voor haarcel-verlies in de basale winding van de cochlea. De stria vascularis daarentegen vertoonde forse degeneratieve veranderingen ten gevolge van D.D.P. In Reissner's membraan werden geen afwijkingen gevonden zoals deze bij de gentamicine studie werden beschreven.

Door gebruik te maken van de microdissectie techniek waren wij in staat deze verschillen in de ototoxische werking van de twee verschillende middelen vast te stellen.

De studie werd gecompleteerd met twee gevallen waarbij microdissectie werd verricht op menselijke cochlea's.

In het eerste geval werden de postmortale bevindingen beschreven van een patient met doofheid ten gevolge van gentamicine. De menselijke cochlea vertoonde een fraai beeld van de degeneratieve veranderingen in het binnenoor ten gevolge van gentamicine.

In het andere geval werd een afwijkend bloedvat gevonden in de scala tympani. Rond dit "zwevende" vat werden ernstige degeneratieve

veranderingen in het orgaan van Corti gevonden. Deze vaatafwijking kon dankzij de microdissectie techniek met oppervlaktepreparaten worden ontdekt. Naar onze mening heeft de microdissectie techniek met een oppervlaktepreparaten een belangrijke plaats in het onderzoek naar binnenoorafwijkingen.

Chapter 11

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CURRICULUM VITAE

The author was born 8-1-1950 in Oudenrijn-Utrecht. Raised and educated in Bilthoven and obtained his H.B.S.-B degree at de Werkplaats Kindergemeenschap Bilthoven in 1969. He studied medicine at the University of Utrecht and qualified in 1976. In 1976 he started to work as a clinical assistent in Otorhinololaryngology at the University Hospital "Dijkzigt", Rotterdam. He was registered as an E.N.T. specialist in 1980. Since two years he is working on the ENT department of the University of Amsterdam as a full-time staffmember. All the work of this thesis has been carried out at the Erasmus University in Rotterdam and was supported by grants from the Heinsius Houbolt Foundation.
The author is married and has a son and a daughter.

Stellingen behorende bij het proefschrift van R.A. Tange,
Degenerative ototoxic changes in the cochlea as seen in microdissections and surface preparations.

1. Bestudering van de gehele cochlea verdient de voorkeur boven de bestudering van slechts enkele fragmenten van de cochlea.

2. Voor het verkrijgen van een binnenoor voor histologisch onderzoek verdient de "bloc" methode (Ward/Lindsay, 1964) de voorkeur boven de "plug" methode. (Schuknecht 1974)

3. Er lijkt een duidelijk verschil te bestaan in de ototoxische werking van Gentamicine en cis-Platinum.

4. Microdissectie van het binnenoor zou in de opleiding tot Keel-, Neus- en Oorarts niet mogen ontbreken.

5. Ultrasone echografie van de bijholten levert nauwelijks meer diagnostische informatie op.

6. Bij de behandeling van Rhinosporidiosis dient een lange "follow up" periode in acht genomen te worden.
   Prins, L.C., Tange, R.A. and Dingemans, K.C. (1983)
   O.R.L. (accepted)

7. Intraveneus digitale angiografie is een aanwinst voor het vaatonderzoek in het hoofdhalsgebied.
   Tange, R.A., Overtoom, T.T.C., and Ludwig, J.W. (1983)
   (Arch. Otorhinolaryngol (accepted)

8. Het verdient aanbeveling een consequente literatuur verwijzing zoals het "Vancouver Style" systeem te hanteren.
   Lancet 1979; 1; 428-30.

9. Ultramicroscopische en histochemisch onderzoek zijn van wezenlijk belang voor de uiteindelijke diagnose primair adenocarcinoom van het middenoor.

10. De "anti-lawaai-campagne" verdient den krachtigen steun der geneeskundigen.
    Prof. Dr. H. Burger, 1936 Amsterdam

11. Naast de dag van de verpleging lijkt de dag van de arts niet onredelijk.

12. Een vluchtstrook zowel links als rechts kan de verkeersveiligheid bevorderen.

13. Microdissectie van het binnenoor is van een simpele eenvoud; men boort weg wat niet nodig is en de rest laat men zitten.

21 juni 1983 R.A. Tange