Scanning Microscopy

Volume 7 | Number 2 Article 16

3-5-1993

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Scanning Microscopy, Vol. 7, No. 2, 1993 (Pages 597-612)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/93$5.00+.00

DEGENERATION AND REORGANIZATION OF VESTIBULAR EPITHELIA AFTER LOCAL AMINOGLYCOSIDE APPLICATION IN THE MAMMALIAN INNER EAR

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(Received for publication January 1, 1993, and in revised form March 5, 1993)

Abstract

The development of degeneration of vestibular end organs and their possible reorganization have been observed over a 2 year period after local application of sisomicin in the inner ear using scanning electron microscopy (SEM). Degeneration of stereocilia took place as early as 5 days after the treatment in the utricule, the saccule and the cristae. At 10 days, almost the entire surface of these sensory epithelia presented a smooth aspect without specific structure. However, after 15 days, an epithelial reorganization developed with clear limits between cells. At 25 days, systematic kinociliary structures were observed at the apical surface of the cells. Five months after the treatment, the new kinocilium was still present and sometimes stereociliary-like structures appeared in the three types of vestibular organs. Two years after treatment, the kinocilium persisted and the embryonic-like ciliary tufts had disappeared. These SEM observations suggest that in mammals some vestibular epithelial regeneration is possible but stops at a stage which could correspond to an early developmental level.

Key Words: Scanning Electron Microscopy, degeneration, restructuring, vestibule, epithelia, guinea pig, morphology, aminoglycoside, sisomicin, topical application.

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Introduction

Neural epithelial regeneration in response to injury, such as chemical exposure, is a common process in the basal cell differentiation of the olfactory organ (Monti-Graziadei and Graziadei, 1979; Simmons *et al.*, 1981; Matulionis *et al.*, 1982; Costanzo and Graziadei, 1983; Morrison and Costanzo, 1989; Costanzo and Morrison, 1989). Similar undifferentiated stem cell populations exist in the inner ear and the lateral line organs of fish and amphibians (Corwin 1981, 1983, 1985; Corwin *et al.*, 1991; Jorgensen, 1981; Popper and Hoxter, 1984; Barber *et al.*, 1985; Tester and Kendall, 1969; Balak *et al.*, 1990). These cells provide the source for continuous generation of both supporting cells and hair cells throughout life and possible repopulation of the sensory organ following its alteration (for a review, see Corwin *et al.*, 1991).

Continued postembryonic production of hair cells is known to occur in the vestibular epithelia of anamniotes such as sharks (Corwin, 1981) and toads (Corwin, 1985) and in the lateral line organs of amphibians (Corwin, 1985; Corwin *et al.*, 1991). In amniote species, on the other hand, it seems that the adult total number of hair cells is determined at birth (Bredberg, 1968; Tilney *et al.*, 1986, 1987).

In mammals and birds, the vestibular sensory hair cells proliferate, migrate and differentiate during embryogenesis and/or early after birth (Weisleder and Rubel, 1992). As soon as cell proliferation terminates, it was classically thought that the ability to produce new cells is definitively lost for the duration of life. So, for many years it has been believed that the sensory structures of the adult vertebrate inner ear are incapable of regeneration and that sensory hair cells which have irreversible damage are replaced by neighbouring supporting cells (Bohne and Rabbit, 1983; Corwin *et al.*, 1991). Such damage can be observed in inner ear sensory organs during ototoxic treatment, acoustic overstimulation or mechanical disruption of the epithelia. In these conditions, the hair cell losses are apparently permanent and irreparable.

However, some authors have reported a continuous production of hair cells in the avian vestibular sen-

sory organs (Jorgensen and Mathiesen, 1988; Weisleder and Rubel, 1992), but not in the auditory basilar papilla. More recently, it has been observed that sensory hair cells regenerate after destruction due to various ototraumatic agents in the inner ear of birds. It was particularly demonstrated that cochlear hair cell stereociliary bundles recover a normal pattern after severe acoustic overstimulation (Cotanche, 1987; Ryals and Rubel, 1988; Ryals and Westbrook, 1990), and that hair cell regeneration occurs in the cochlea within a few weeks after they have been destroyed by ototoxic aminoglycoside antibiotics (Cruz *et al.*, 1987; Hashino *et al.*, 1992). Very recently, Weisleder and Rubel (1992) have presented preliminary accounts of experiments regarding regeneration of vestibular hair cells of birds after aminoglycoside intoxication. The overall consensus is that the avian vestibular system is able to regenerate hair cells, but this has not been demonstrated in mammals.

The purpose of the present study was to investigate at scanning electron microscopy (SEM) level the short and long term changes in the vestibular epithelia from the adult guinea pig after local injection of an ototoxic aminoglycoside antibiotic, Sisomicin, in the inner ear.

Materials and Methods

Inner ear destruction

Forty nine adult pigmented guinea pigs weighing 250-350 grams were first deeply anaesthetized by a mixture containing ketamine chlorhydrate (17 mg/kg) and xylazine chlorhydrate (4 mg/kg). Then, after shaving and cleaning the skin in the occipital area down the mastoid region, xylocaine was infiltrated and an incision made 1.5 cm behind the pinna. After resection of the muscular masses, the bulla was exposed. Using a dental burr, a 1 mm hole was drilled in the middle of the exposed bulla. Under the operating microscope, the round window was then searched through this hole in a postero-dorso-lateral approach. The membrane of the round window was then disrupted with a thin needle.

Twenty five milligrams of sisomicin sulfate were injected through the round window and allowed to diffuse in the whole inner ear and bulla. Then the hole in the bulla was plugged with a piece of a medical grade silicone rubber, one of the most inert subdermal implant materials (Silastic®, Dow Corning).

In another study we have determined that this unilateral and local application of sisomicin leads to a total and irreversible destruction of the afferent auditory neurons in the spiral ganglion and a severe but not total loss of afferent vestibular neurons in the Scarpa's ganglion (Dupont *et al.*, 1993).

Preparation of the specimens

After different survival periods of 5 (n = 12), 10 (n = 9), 15 (n = 8), 25 (n = 8), 150 days (n = 6) and 2 years (n = 6), the guinea pigs were deeply anaesthetized and rapidly perfused through the ascending aorta with Karnovsky's fixative containing 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3). Then the two bullae were removed and perfused again by intralabyrinthine flow through the round window with the same fixative and postfixed for 24 hours in the same solution. The cochleae were rinsed in cacodylate buffer and then postfixed with 1% OsO_4 for 1 hour. They were rinsed again, perfused with 70% ethanol and remained in this alcoholic solution until isolation of the inner ear sensory organs.

The organ of Corti and the vestibular organs were dissected in 70% ethanol. After dehydration in graded ethanols, the specimens were dried by the critical-point method (CO_2; Balzers CPD 010), mounted on stubs with double-coated sticky tape (Fullam) and covered with a thin layer of gold-palladium (7-8 nm thick) in an ion sputter coating unit (Balzers SCD 030). The specimens were observed in a scanning electron microscope (Philips 505) operated at 20-30 kV.

Figure 1. **a-b)** Degeneration of the organ of Corti 5 days after local injection of sisomicin. **a)** Organization of the normal cochlear epithelium in the guinea pig. Note the strict arrangement of the three rows of outer hair cells: OHC1, OHC2, OHC3; and one row of inner hair cells: IHC. Bar = 10 μm. **b)** 5 days after treatment the sensory cells have totally disappeared at the site of the organ of Corti (arrow) which is replaced by an undifferentiated epithelium still identified 2 years later. Bar = 0.1 mm. **c-f)** Macula of the saccule from a control guinea pig. **c-d)** Ciliary tufts are located over all the surface of the macula. Bars = 0.1 mm (in c) and 10 μm (in d). **e)** At high magnification, the ciliary tuft can be described as a straight type (arrow). This organization in straight rows of stereocilia and tall hair bundle leads us to imagine that we observed the apical surface of a type I vestibular hair cell (Lapeyre *et al.*, 1992). Note the presence of a very long kinocilium (curved arrow). Bar = 10 μm. **f)** Periphery of the macula consists of polygonal cells which exhibit only one kinocilium (arrow). Bar = 10 μm.

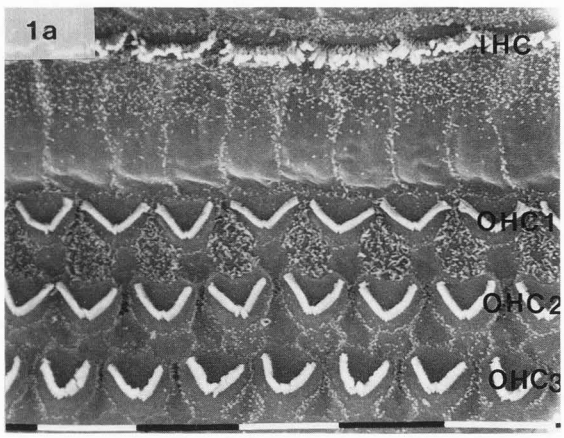
Results

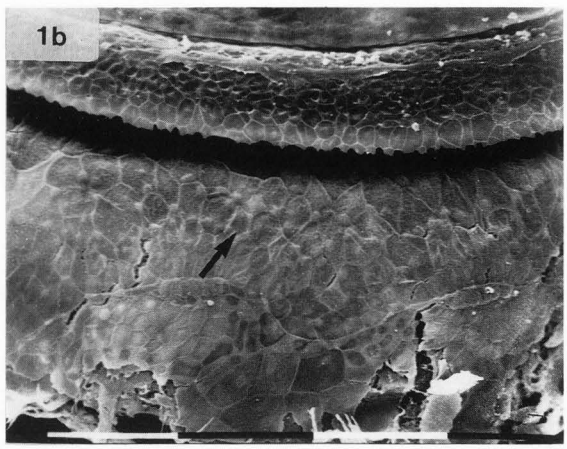
Degeneration of the organ of Corti:

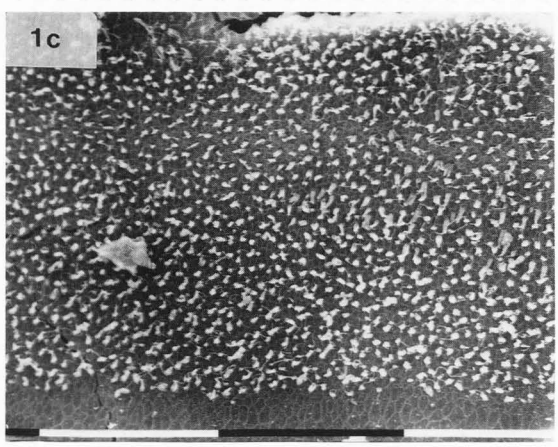
SEM observations showed a total and irreversible loss of sensory cells as early as 5 days after the local application of sisomicin. Even with longer survival times, up to 2 years, the normal organization of three rows of outer hair cells and one row of inner hair cells of the organ of Corti (Fig. 1a) was totally missing. The sensory epithelium was replaced by an undifferentiated tissue (Fig. 1b), however without further damage to other structures, such as the spiral limbus.

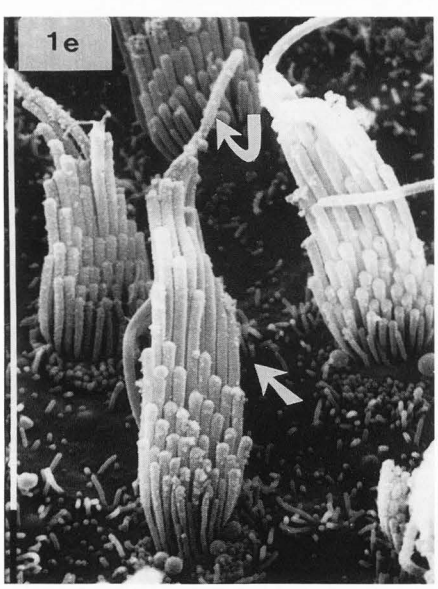
Short term degeneration of vestibular end organs

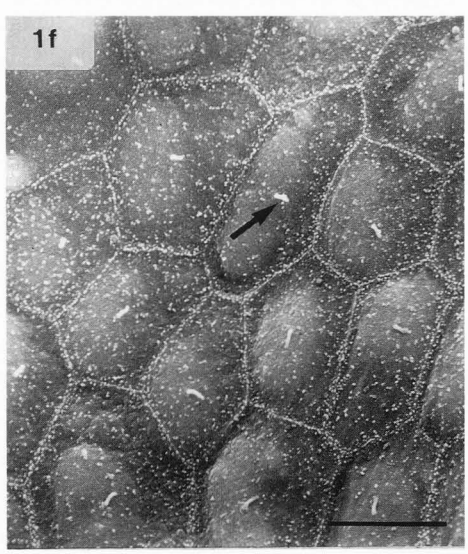
In order to evaluate the alterations of the neurosensory structures of the vestibule, we observed first the











well documented ultrastructural appearance of the vestibular organs from control guinea pigs. The surface of the saccular macula displays numerous ciliary tufts of the sensory cells (Fig. 1c and 1d). At a higher magnification, a very long kinocilium is systematically observed in the ciliary tuft (Fig. 1e). The border of the macula is made of a homogenous epithelial tissue mosaic with cells presenting one short kinocilium (Fig. 1f).

Five days after the local injection of sisomicin, the vestibular sensory epithelia exhibited numerous signs of alteration. At the surface of the utricular macula (Fig. 2a), the ciliary tufts were collapsed and the stereo-

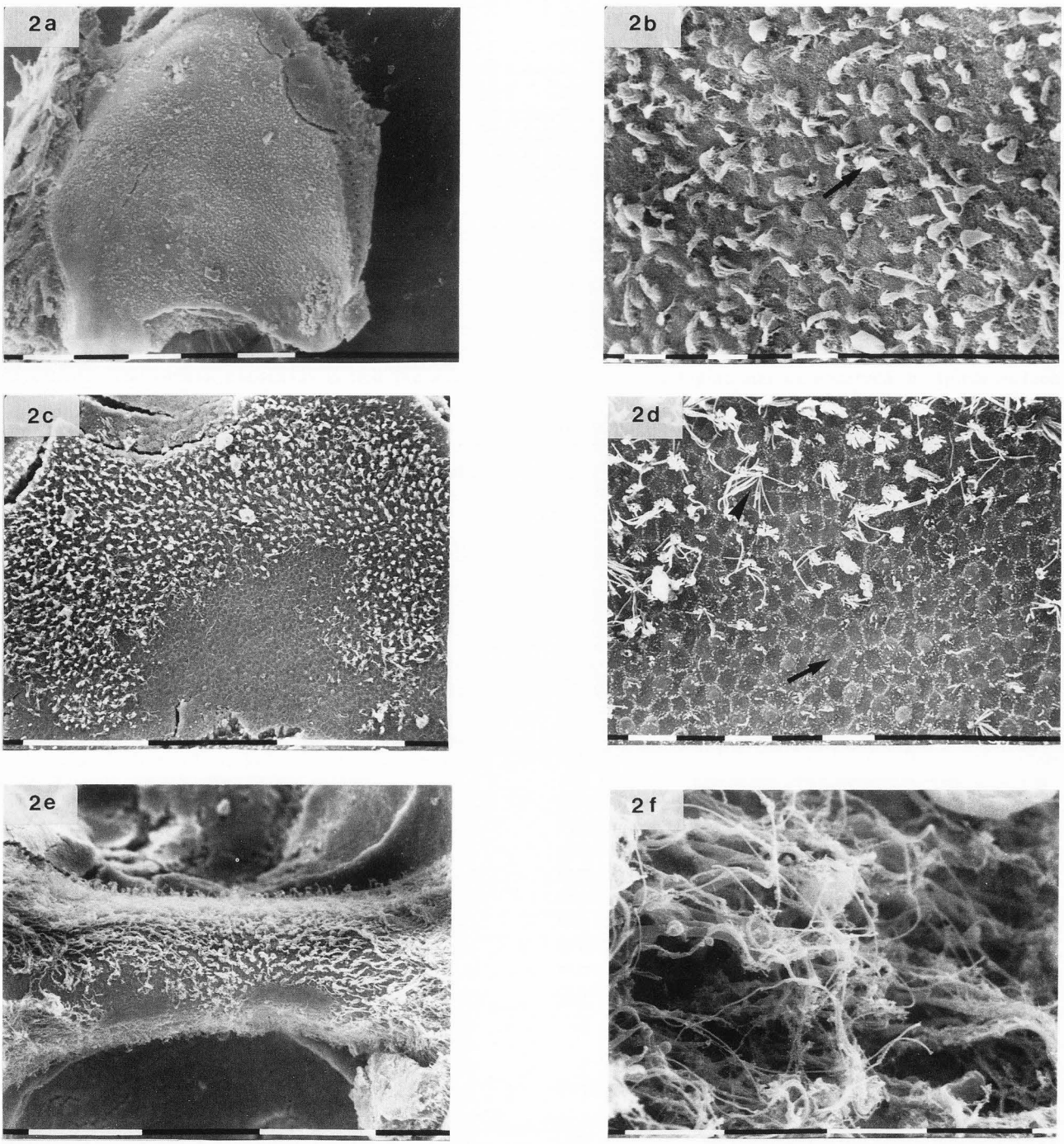
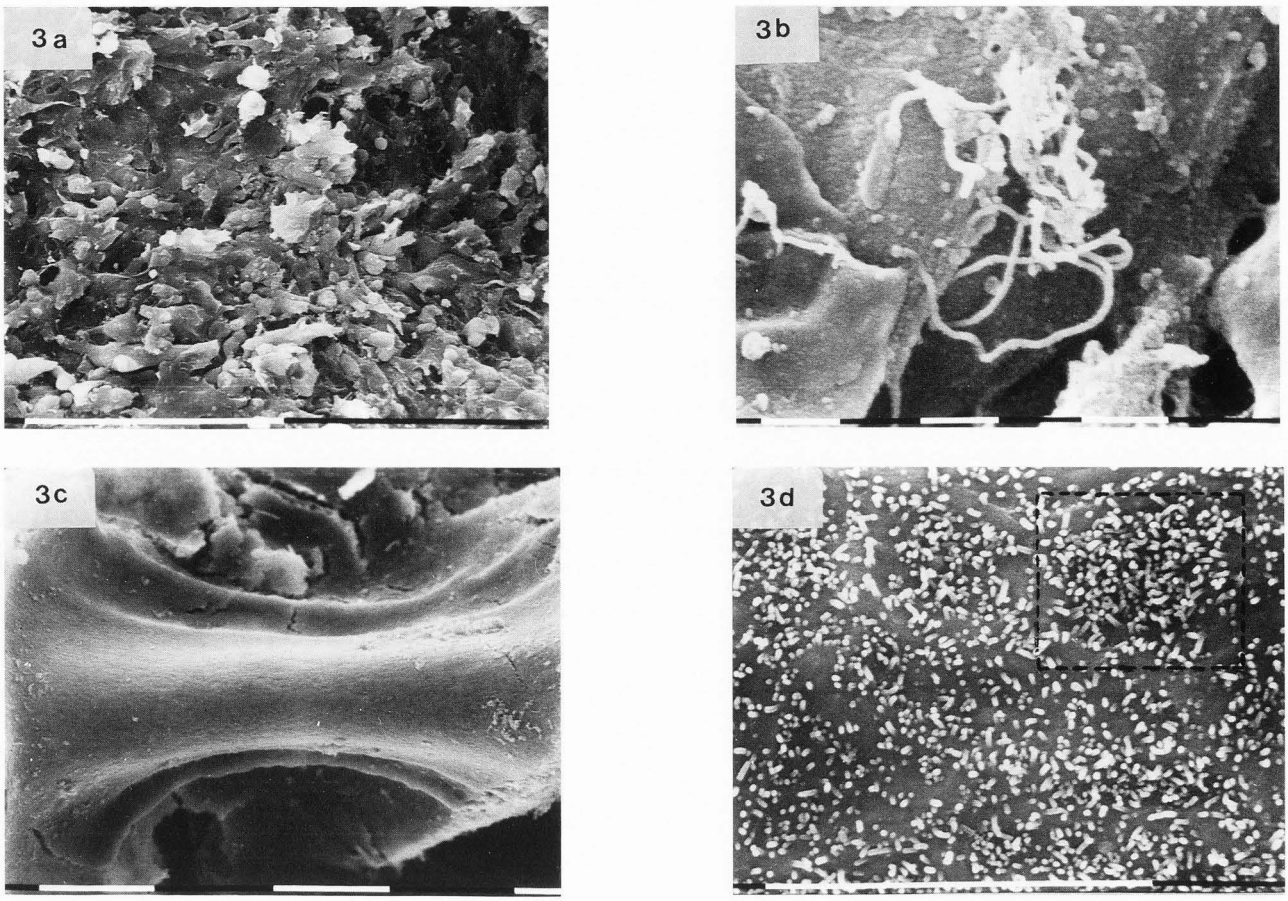


Figure 2. Vestibular sensory epithelia 5 days after local aminoglycoside application. Bars = 0.1 mm (in a, c and e); and 10 μm (in b, d and f). **a)** General view of the utricular macula. **b)** The ciliary tufts are severely damaged (arrow). The altered stereocilia are collapsed. **c)** At the surface of the saccular macula numerous ciliary tufts are observed but some areas are highly damaged. **d)** At higher magnification the remaining ciliary tufts are totally disorganized (arrowhead) and the region of the striola is devoid of ciliary structures (arrow). **e)** General view of one crista ampullaris which seems to be normal. **f)** At high magnification, stereocilia are collapsed and there is no real ciliary tuft organization over the crista crest.



cilia were completely fused (Fig. 2b) making it impossible to identify the shape of ciliary bundles. On the saccular macula, we observed large damaged areas where the ciliary structures had disappeared (Fig. 2c). The remaining ciliary tufts were totally disorganized and the stereocilia were broken off, most of them seemed to be shortened (Fig. 2d). At low magnification, the general aspect of the cristae were relatively preserved compared to controls (Fig. 2e), however at higher magnification, the long stereocilia had lost their original arrangement, showing severe damage such as collapse and a rough appearance (Fig. 2f).

After 10 days, the signs of degeneration increased. The macula of the saccule was totally destroyed and it was very difficult to observe any particular type of epithelium (Fig. 3a). There were no differentiated cells. Some remaining ciliary structures could be found in this highly damaged end organ but they did not have the appearance of stereociliary structures and were never organized in a tuft (Fig. 3b). Concerning the cristae ampullaris, the surface was totally stripped clean (Fig. 3c). No ciliary structures could be observed over the entire surface of the organ. At higher magnification, the epithelium seemed to be composed of undifferentiated cells, each containing a great number of microvilli (Fig. 3d). No cell outlines were observed, only some patches of more clustered microvilli were scattered over the surface of these vestibular organs (Fig. 3d).

Figure 3. Vestibular epithelia 10 days after local sisomicin application. Bars = 0.1 mm (in a and c), 1 μm (in b), and 10 μm (in d). **a)** General view of the saccular macula totally destroyed by the ototoxic treatment. **b)** At high magnification some ciliary debris was observed at the surface of this highly damaged epithelium. **c)** Note the smooth appearance of crista ampullaris at low magnification. **d)** At higher magnification no ciliary structures are observed, numerous microvilli cover the whole surface of the epithelium. Cell boundaries are not visible.

Short term restructuring of the vestibular end organs

Fifteen days after the local aminoglycoside application, the vestibular organs also exhibited important signs of disorganization. Two different pathological changes were observed at the surface of the utricular macula (Fig. 4a). In some areas, debris was scattered over the damaged epithelium. In other areas, the epithelium presented a more organized aspect (Fig. 4a). At higher magnification, this latter area appeared to be made of polygonal cells with well delimited borders of microvilli (Fig. 4b). A kinociliary structure could be

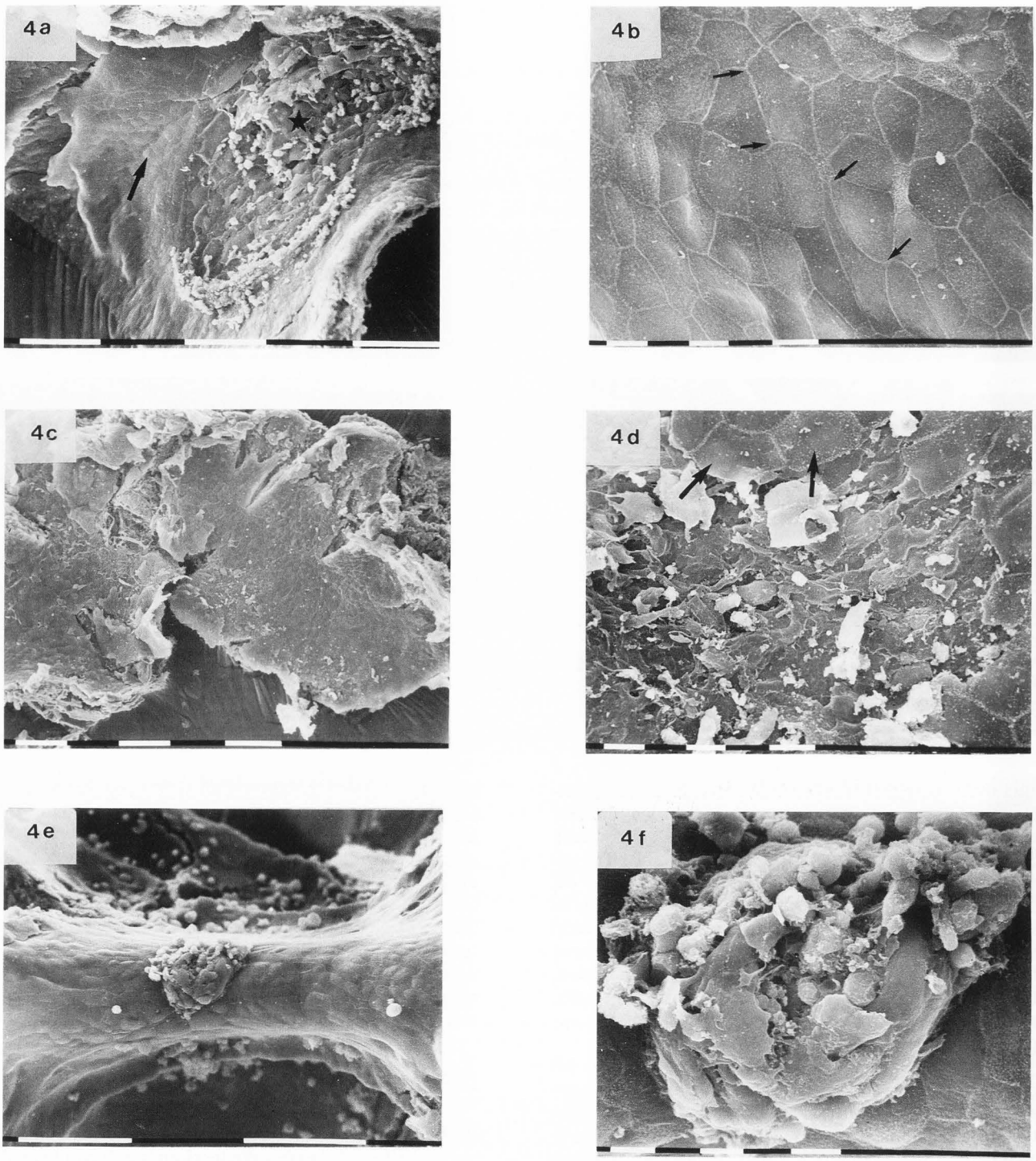
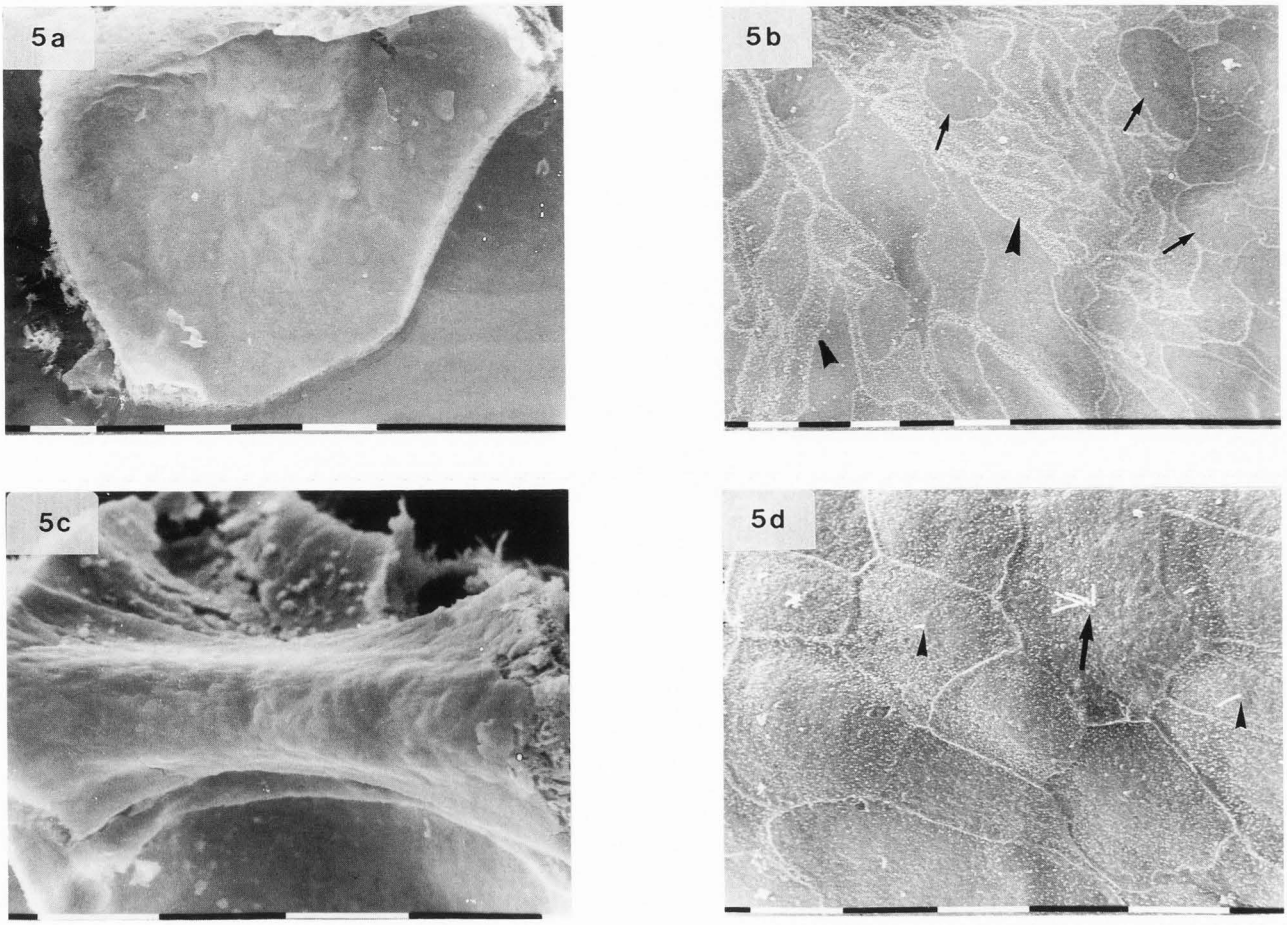


Figure 4. Vestibular end organ epithelia 15 days after the local aminoglycoside poisoning. Bars = 0.1 mm (in a, c and e) and 10 μm (in b, d and f). **a)** General view of the utricule displaying two pathological changes. One part of the epithelium is covered by much cellular debris (star), the other has a smooth appearance (arrow). **b)** High magnification of smooth part shows a rather well organized epithelium, made of polygonal cells delimited by borders of microvilli (arrows). **c)** Low magnification of the saccular macula. **d)** The saccular epithelium is also composed of polygonal cells (arrows) and exhibits numerous degenerated remains. **e)** General view of the crista ampullaris with a structured epithelium. **f)** Some cellular remains often circle the crest of the crista.



observed at the surface of some cells (Fig. 4b). Some areas of the saccular macula were also damaged, but the general appearance of the epithelium seemed more structured than at 10 days after treatment (Fig. 4c). At higher magnification we could observe the presence of polygonal cells constituting an organized epithelium, together with some debris in other severely altered parts (Fig. 4d). In the cristae the smooth aspect of the epithelium described 10 days after the destruction was no longer observed (Fig. 4e). This epithelium consisted of individual cells which have their limits well demarcated by the microvilli borders. Nevertheless, some debris was detected at the surface of the epithelium generally circling the crista crest (Fig. 4e, 4f).

Twenty-five days after the treatment, the macula of the utricule appeared totally smooth (Fig. 5a). At higher magnification, we could observe a well organized epithelium (Fig. 5b). Some cells had a great number of microvilli which covered their apical surface. Others cells with an apical surface smaller than in normal organs, presented a kinociliary structure. The same organization was observed in the cristae. Even if the surface of the epithelium had a smooth appearance, the tissue seemed more heterogeneous (Fig. 5c). Higher magnification observation revealed a clear structure of the epithelium with polygonal cells covered by microvilli which

Figure 5. Vestibular organs observed 25 days after the destruction. Bars = 0.1 mm (in a and c) and 10 μm (in b and d). **a**) The utricular macula has a smooth appearance at low magnification. **b**) At higher magnification, epithelium displays different cell types. Some cells have an apical surface covered by microvilli (arrowheads) others are polygonal and contain one kinociliary structure (arrows). **c**) The crista ampullaris has the same smooth appearance as the utricule. **d**) At higher magnification the crista crest consists of polygonal cells with a great number of microvilli and one frequently central kinociliary structure (arrowheads). Note that in some cases more than one kinocilium-like organelle is observed (arrow).

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made a border at the cellular limits. More interestingly, a kinocilium-like structure was systematically observed in these cells, often in a central position. In some instances more than one ciliary structure could be easily identified (Fig. 5d).

Changes of the vestibular organs at 5 months

Five months after the local application of sisomicin, the previously observed organization of the saccular and utricular maculae persisted. The epithelia seemed to be more differentiated than at 25 days (Fig. 6a-6c).

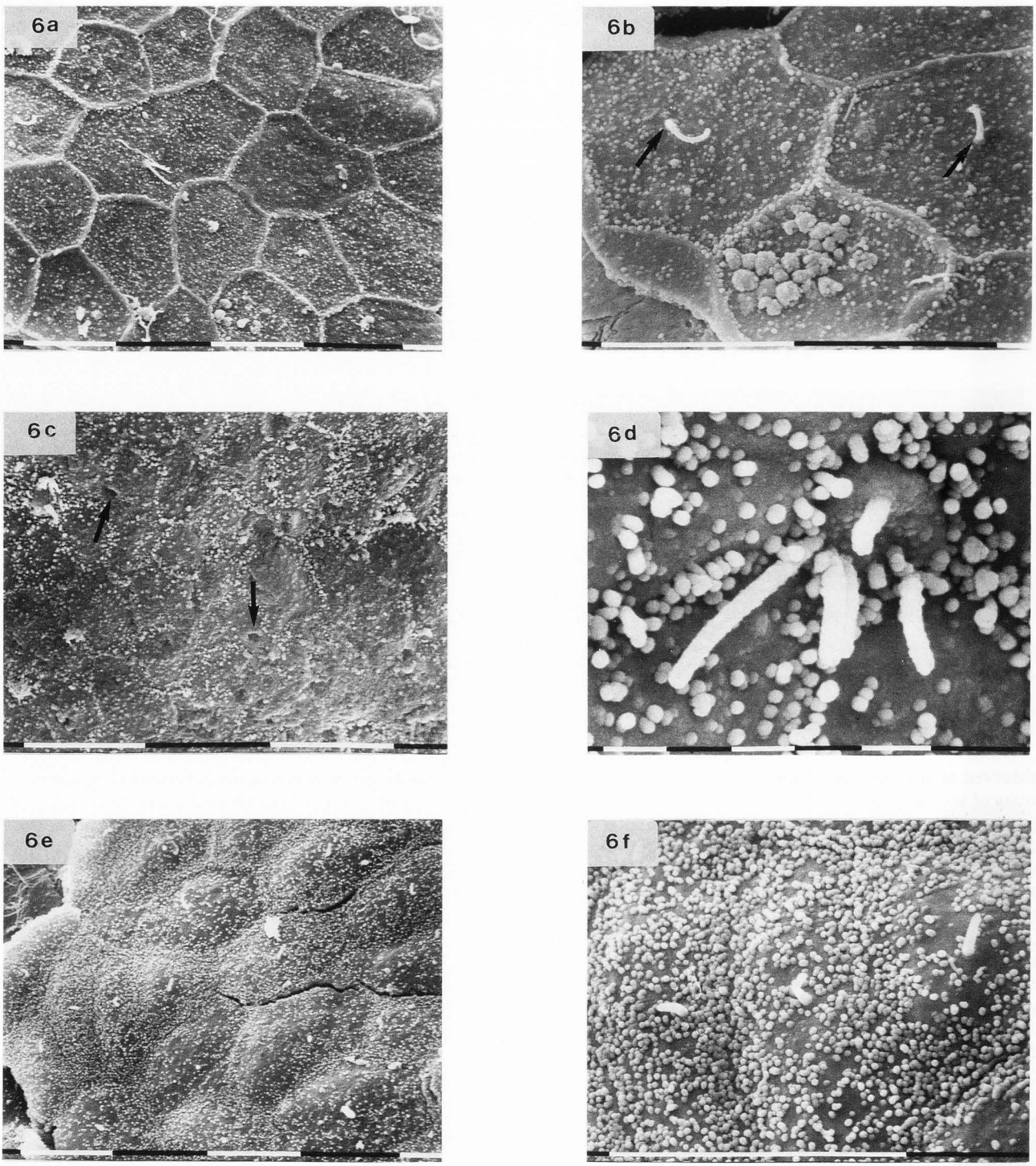

Figure 6. Vestibular epithelia 5 months after local ototoxic damage. Note the different degrees of reorganization of the epithelia in a, c, and e. Bars = 10.0 μm (in a, b, c, e, and f) and 1 μm (in d). **a)** On the utricular macula, polygonal cells are observed which reconstitute the epithelium. Note the clear limits between cells. **b)** At higher magnification, the kinociliary structures are still present (arrows). **c)** General view of an area from the saccular epithelium exhibiting some signs of alteration such as holes (arrows). **d)** On the same epithelium some cells have more than one ciliary structure: one long cilium and others shorter. **e)** Epithelium from the crista ampullaris contains numerous differentiated cells. **f)** These cells have a great number of microvilli and exhibit one central kinociliary structure.

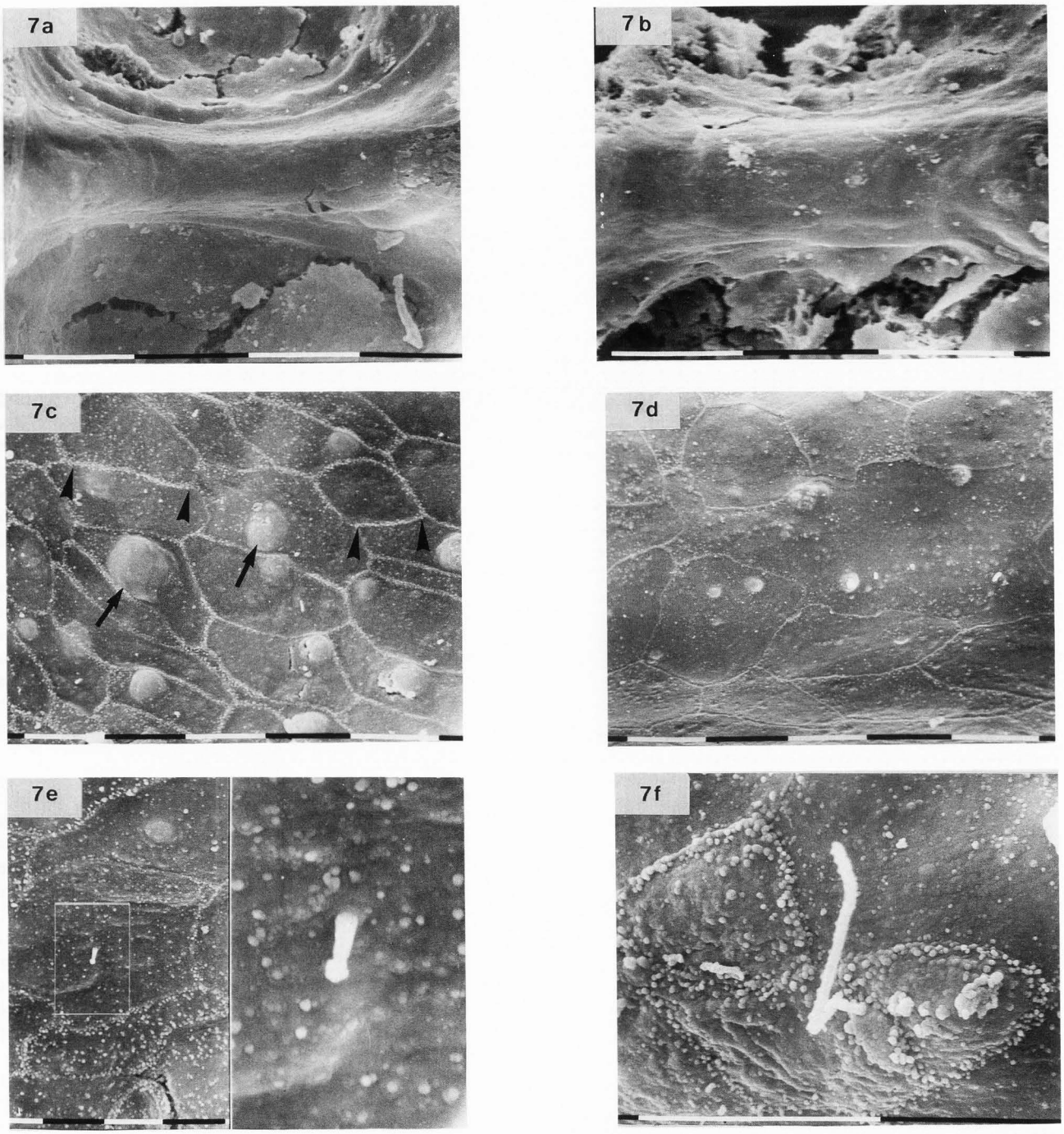

Figure 7. Labyrinthine end organs 2 years after local application of sisomicin. Bars = 0.1 mm (in a and b), 10 μm (in c, d, and f) and 5 μm (in e). **a**) Posterior crista ampullaris displays a smooth appearance where no ciliary tufts are observed at low magnification. **b**) At the surface of the horizontal crista ampullaris the same observation can be made. **c**) Higher magnification of the posterior ampulla epithelium shows that the constitutive cells are well delimited by borders of microvilli (arrowheads). Note the curved form of the apical surface of these cells (arrows). Kinociliary structures are still present at the surface of almost all cells. **d**) Higher magnification of the horizontal ampullar epithelium where limits between cells are clearly observed. The apical surface of the cells contains fewer microvilli and the general aspect of the epithelium is less reorganized than in the other crista. **e**) Over the posterior ampulla, kinociliary structures are observed differing from the microvilli. **f**) Some kinociliary structures observed at the surface of the horizontal crista ampullaris have sometimes a rather damaged form and seem to be broken.

Almost all the cells have a kinocilium-like organelle (Fig. 6b) and we could often observe more than one ciliary structure. In this latter case, one long cilium could be identified together with other shorter cilia (Fig. 6d) at the same apical surface. The same cellular reorganization observed on the surface of the otolithic organs was seen on the sensory surface of the cristae ampullaris (Fig. 6e). The cellular limits were less clear but the differentiated cells still exhibited one kinociliary structure and numerous microvilli (Fig. 6f).

Long term changes (2 years) of the vestibular end organs

Two years after cochleo-vestibular destruction by local injection of sisomicin, we could examine only the ampullar organs. Dissection of the otolithic end organs was not possible, due to a strong calcification of the membranous labyrinth, probably induced by local treatment with sisomicin. The saccular and utricular maculae were entirely included inside bone. We could observe the cristae which had a smooth but irregular appearance (Fig. 7a and 7b). At higher magnification we observed that the epithelium displayed some degenerative signs (Fig. 7c and 7d). The ciliary bundle-like structures observed at 5 months (Fig. 6d) were no longer visible although some remaining ciliary structures were still observed at the surface of the cells (Fig. 7e and 7f). Most of the cells exhibited only one cilium which resembled the kinocilium observed at 25 days and 5 months. Some of these kinociliary structures had an altered form as illustrated in Figure 7f. The irregular aspect of the crista surface could be due to the nuclei of the epithelial cells which seemed to be pushed out (Fig. 7c). Nevertheless, fixation artifacts inside these extremely clacified temporal bone could not be excluded.

Discussion

Different means of damage are classically used in studies of degeneration in the cochleo-vestibular structures. The audio-vestibular pathways can be sectioned surgically, the inner ear can be disrupted mechanically, or the application of a chemical drug can totally destroy the sensorineural structures of the labyrinth.

The goal of the present study was to investigate the anatomical changes in the sensory epithelia of the adult mammalian inner ear following local application of sisomicin which triggers morphofunctional changes in the central audio-vestibular system (Dupont *et al.*, 1992).

In the case of the cochlear sensory epithelium, we observed a total and irreversible destruction of sensory hair cells which was replaced by an undifferentiated tissue. This change could be observed a long time after destruction. In the three vestibular epithelia, there was also an immediate degeneration of the sensory structures, as seen at 5 days. The loss of ciliary tufts increased steadily until 10 days after the treatment. At 10 days, the maculae and the cristae seem to consist of undifferentiated stem cells covered by microvilli. Then a progressive reconstitution of these epithelia took place, although without the reappearance of sensory cells. As early as 25 days after destruction, we observed a partly restructured vestibular epithelia, which consisted of cells displaying a great number of microvilli. This particular cellular pattern was similar to that seen transiently on immature hair cells in the developing avian cochleae (Cotanche, 1987), and in regenerated chick hair cells 28 days after systemic gentamicin treatment (Duckert and Rubel, 1990). The kinociliary structures seen at the apical surface of the newly-formed cells look like those observed in epithelial cells at the periphery of the normal vestibular end organs. These cells are known to produce the sensory and supporting cells in an early stage of development, and they might be involved in a regenerative process (Corwin *et al.*, 1991). These kinociliary structures could be remains from damaged ciliary tufts. However, we observed that the sensory cilia were completely eliminated from the surface of all vestibular end organs by 10 days after the local application of sisomicin in all guinea pigs of this group. Moreover, it is not likely that such a local treatment could induce the destruction of sensory cilia, except one at the apical surface of the damaged cells.

Numerous studies have investigated the effect of aminoglycoside antibiotics on the vestibular sensory organs of mammals (Wersäll and Hawkins, 1962; Duvall and Wersäll, 1964; Harada, 1978; Fee, 1980). Unfortunately, these studies did not describe the ultrastructural aspect of the vestibular end organs following a lengthy survival time. The only study reporting some dynamic changes of vestibular receptors in adult mammals was that of Favre *et al.* (1989). Forty-eight hours after neurectomy of the superior branch of the vestibular nerve in the adult guinea pig, they observed that wallerian degeneration of nerve fibers was almost complete but a normal appearance of the sensory epithelia seemed to have been saved. However, 2 weeks or longer after the neurectomy, the sensory epithelium suffered profound changes. The sensory hair cells appeared "dedifferentiated" beginning with those locations on the epithelium which were the first to differentiate during ontogenesis. However the cells did not come to a completely undifferentiated state. Some of these cells had the appearance of neighbouring supporting cells.

After 5 months of recovery, we describe a more structured aspect of the vestibular end organs. However the state of organization in the maculae of the utricule and saccule, and of the cristae ampullaris, differed notably from one another. The reasons for these differences were unclear. It is possible that the extent of "reorganization" can be related to the amount of damage induced by variations in diffusion of sisomicin to the various parts of the labyrinth. The newly formed cells possessed one kinocilium and short stubby stereociliary-like structures which look much like large microvilli. This cell aspect is reminiscent of that seen at an early stage of hair cell differentiation (Swanson, 1988). Moreover the presence of a rather long kinocilium together with a

smaller than normal cell surface indicates that this new cell could be in an early stage of development (Cotanche, 1987; Hashino *et al.*, 1992). Concerning the appearance and organization of stereociliary tufts, it has already been established in the embryonic chick that stereocilia are derived from microvilli (Tilney *et al.*, 1986). In our study. we speculate that the observed stereocilia at 5 months develop following the same process.

In order to examine the outcome of such gradual changes, we studied the vestibular epithelia two years after the local application of sisomicin. In this case, it was not possible to observe the maculae of the utricule and saccule due to a high calcification of the labyrinth, which is a typical reaction following severe inner ear damage (Spoendlin, 1978). However, we could examine the cristae ampullaris which showed that the organization observed at 5 months was no longer present. The stereociliary-like structures disappeared but the kinocilium-like structures remained over an epithelium displaying numerous other signs of degeneration.

In the present study, we demonstrate that the vestibular epithelia of adult mammal are capable of anatomical reorganization after long term destruction. This observation of cells plasticity, with replacement originating from unidentified stem cells or cells that are ordinarily postmitotic, suggests that the possibility of self-repair after trauma should not be ruled out in mammalian inner ear simply because the cells are mitotically quiescent in normal postembryonic life. Our results extend the concept of neural epithelial regeneration to mammals which was developed in lower vertebrates and recently in birds where the cochlea has been shown to have anatomical regenerative capabilities (Swanson, 1988). In these species, after endogeneous injury due to aminoglycosides (Cruz *et al.*, 1987) or acoustic trauma (Cotanche, 1987; Girod *et al.*, 1989, 1991; Ryals and Rubel, 1988), a recovery of damaged cochlear sensory hair cells occurs with the production of new sensory cells (Corwin *et al.*, 1991).

In the present work, the most interesting structures in this partial recovery process are undoubtedly the kinocilia which could be best observed only by SEM. They have been described in other systems. In the olfactory sensory cells, cilia resembling the kinocilium of cochlear or vestibular hair cells have been shown to regrow after shortening caused by treatment with detergent (Adamek *et al.*, 1984). In similar conditions, our observations could reflect such a plastic phenomenon in the ciliary structures of vestibular sensory cells.

Furthermore, two years after the destruction we observed that the organization of labyrinthine epithelia still resembles that seen at a very early stage of the embryogenesis. The newly formed cells described at five months stop evolving, and this could suggest that they are physiologically immature or inappropriately innervated. Therefore, we are presently investigating the degeneration of the primary vestibular afferents in Scarpa's ganglion of the same animals. Initial results indicate that almost all neurons exhibit some signs of degeneration five months after the local application of sisomicin to the inner ear.

Only tritiated thymidine studies will indicate whether the newly formed cells derive from cell division. However, whatever their source, the stimulus for their production appears to be hair cell loss, as long as some differentiated epithelium remains in the damaged region (Swanson, 1988). If the stimulus for production of new cells can be exactly determined, it may eventually be possible to provide the technology to induce hair cell regeneration in the labyrinth of mammals and humans who have sustained vestibular pathologies due to ototoxic drug poisoning, aging, etc. At this point, different questions arise: Is recovery from ototoxic agents or other trauma related to the age at which the alteration occurs or is it related to the extent of the trauma? What would be necessary for a complete restructuring of the epithelia between five months and two years? Could treatment using trophic factors, such as nerve growth factor, epidermal growth factor or others, help in any way the definitive differentiation of these partly regenerated cells? Could such a treatment restore neural connections for the re-innervation of these self-repaired structures?

Acknowledgments

We wish to express our gratitude to Catherine Aurousseau for her technical assistance.

This study was supported in part by INSERM, "MRES" project number 34 and the "Conseil Régional d'Aquitaine" through a grant.

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Discussion with Reviewers

J.C. Saunders: This is an interesting paper whose results are new but only somewhat interesting. I say "somewhat" because it is not surprising that reorganization was absent in the organ of Corti of a mammal exposed to traumatic levels of aminoglycoside. Moreover, the fact that some cellular reorganization was apparent in the vestibular organs seems to be of a minor importance since there was essentially no evidence that the sensory aspects of the epithelium underwent any regeneration.

Authors: We think that some cellular reorganization, which is apparent in the vestibular organs, is not of minor importance but a very interesting observation in the guinea pig. Indeed, until now, regeneration of inner ear neuroepithelia was only observed in lower vertebrates. So we present here the first evidence that sensory epithelia of the vestibule are able to reorganize themselves after their total destruction in the adult mammals.

Furthermore, we are postulating, in the present paper, that there are some signs of "regeneration". We only think that the appearance of ciliary-like structures suggests a morphological reorganization. These are only SEM results, in order to interpret our observations we can only set some hypothesis. Of course, we are conscious that future experiments will be necessary to confirm these hypotheses. In particular, we will try to identify the exact origin of the newly formed cells: migration of cells from the periphery of the organs, cell division from the supporting cells or cellular repair mechanisms, etc. These aspects require the use of different techniques, such as, cellular tracing methods, cellular division tracers (triated thymidine or Bromodeoxyuridin) and transmission electron microscopy (TEM) observations. These investigations are partly in progress at present.

B.A. Bohne: Do you think that all cells of the maculae and cristae (sensory and supporting) break down and that cells at the periphery of the epithelia migrate in to replace the degenerated cells? I have the impression that the cells in Figure 5b were migrating toward the upper left of the field. What do you think?

Authors: To the question whether all cells of the maculae and cristae (sensory and supporting) breakdown, we cannot respond because we have not performed quantitative studies. Based on our qualitative observations, it seems that sensory cells are more sensitive to the local treatment than supporting cells. Nevertheless, it seems probable that supporting cells also breakdown, at least in part, in this type of trauma.

To the question if cells at the periphery of the epithelium migrate to replace the degenerated ones, we cannot respond on the basis of our studies. But it is one hypothesis, which could be tested by cellular markers and tracing methods applied in the periphery of the organs. It would be interesting to localize the labeled cells 15-25 days after the deafferentation in order to elucidate the problem of cellular migration.

Other hypotheses are cellular "dedifferentiation" from preexisting supporting cells, and also the possibility of cellular division, as proposed in the present paper. In these cases, it would be interesting to use methods such as triated thymidine incorporation within dividing cells.

J.C. Saunders: In the absence of TEM, it cannot be concluded that the protruding structures on the apical surface of the reorganized cells is a kinocilia. Similarly, it is not clear how the authors justify their conclusion that the "short stubby" protrusions on the reorganized cells are stereocilia.

Authors: We agree, as explained in the paper, SEM observations alone cannot allow us to conclude that protruding structures on the apical surface of the reorganized cells are kinocilia. In this perspective, only TEM studies of these reorganized neuroepithelia will enable improved understanding of the exact ultrastructural nature of these structures. The problem for stereociliary-like structures is the same as kinociliary-like structures. We feel that the observed ciliary-like structures "look much like large microvilli". We do not assume at this point of our study that they are indeed stereocilia, only TEM studies will provide a definite answer.

J.C. Saunders: There was no attempt to quantify many of the observations, and so it is not clear whether or not some of the more subtle observations are real events or impressions based on visual examination of the photomicrographs.

Authors: In our study, it was not necessary to quantify the reorganized cells because our observations until 5 months after deafferentation was true for the whole surface of the epithelia. Unfortunately, at low magnification, it is not possible to observe the kinociliary-like structures and low-magnifications are required to study the entire neuroepithelium. To quantify the newly formed cells requires that no overlap occurs in our observation, so as to avoid the double counting of some cells. This condition is extremely difficult in SEM studies; that is why quantitation was not considered in our study.

Nevertheless, as mentioned in the present manuscript, the whole surface of the vestibular neuroepithelia was reorganized as early as 15 days after the deafferentation. Concerning the kinociliary-like structures, they were systematically observed at the apical surface of the newly formed cells; but concerning the "pseudo" hair bundles of embryonic type, they were observed quite scattered over the epithelium 5 months after the destruction.

J.C. Saunders: The title of the paper indicates that the concern is with the vestibular epithelium. Why then do the authors report spiral ganglion damage and hair cell loss on the organ of Corti?
Authors: Our SEM study concerns the different neuroepithelia of the inner ear after local application of sisomicin. The inner ear includes two sensory apparatus: the cochlea and the vestibule. If the outcome of the organ of Corti has not been reported in the present paper, some could be puzzled by the lack of data concerning the auditory system. Moreover, it was very interesting to mention that the cochlear neuroepithelium does not present the same property of reorganization as the vestibule in the adult guinea pig, at least in our case of aminoglycoside intoxication. This shows that the regeneration of hair cells observed in the auditory epithelium of birds (for example) is no longer observed in adult mammals after severe ototoxic treatments with aminoglycoside antibiotics.

J.C. Saunders: Do hair cells on the normal vestibular epithelia pack with hexagonal array? If they do, than the reorganized epithelium is different in this respect.
Authors: The hair cells on the normal vestibular epithelia are indeed packed with hexagonal (at least polygonal) array. This can be easily observed in Figure 1. In this respect, the reorganized epithelium, observed as soon as 15 days after the treatment, does not differ from the normal organs. Indeed, the "newly formed" epithelium is made of polygonal cells until 2 years after the deafferentation.

J.C. Saunders: In connection with Fig. 5a, what do you really want to say by "totally smooth"? I think that the epithelium has been stripped of all its sensory hair cells and supporting cells.
Authors: This expression ("totally smooth") corresponds well to our observations and we think that it would be too speculative to interpret much more at present. Indeed we have no evidences to assume that the epithelium has been stripped of all its supporting cells; only TEM studies will allow us to conclude this. Furthermore, we think that it may be wrong since the work of Favre *et al.* (1989) demonstrates that supporting cells persist in the vestibular epithelia of adult mammals after deafferentation.

J.C. Saunders: The observations of the otolithic organs after two years is very interesting. However, more description would be of interest since many readers may have no idea what the term "strong calcification" means.
Authors: We are limited in the description of the otolithic organs, two years after deafferentation, especially in SEM studies. Further information can only be given after TEM observations. Concerning "strong calcification", Spoendlin (1975, 1978) well described this phenomenon after long term deafferentation. However, this process of calcification is a classical observation in the inner ear after long term exposure to aminoglycosides. This calcification affects not only the labyrinth but also the spiral ganglion (these date are being published elsewhere).

J.C. Saunders: The assumption that the nucleus is "pushed out" two years after deafferentation seems to be debatable. Indeed, it looks more like a bleb on the apical surface of the cell due to fixation artifact.
Authors: We agree that it is difficult to exclude the possibility of fixation artifact. The inner ears, two years after local application of sisomicin, are generally highly calcified. Only the non-otolithic organs of the vestibule could be dissected and the perfusion was more difficult. In these conditions, some fixation artifacts could occur. In order to confirm (if it is really protrusions of the nuclei), it is necessary to perform TEM observations. Nevertheless, the aspect observed in SEM resembles strangely the round form of a nucleus.

J.C. Saunders: Your conclusion that cells with a single kinocilia (if indeed it is a kinocilia) are hair cells seems to be not supported by any evidence. Moreover, the fact that the apical surface of these cells have a large population of microvilli does not mean much, since these are also found on immature supporting cells.
Authors: We do not conclude that hair cell with a single kinociliary-like structure is a mature hair cell. Nevertheless, if one cell displays a ciliary structure, it seems logical to call it "hair cell". In fact, we propose that these cells could be "embryonic-like sensory cells".

You assume that some cells, which have a large population of microvilli, are also found on immature supporting cells. It is true, but it is also the case for immature sensory cells! Furthermore, it has been demonstrated that in vestibular organs, when hair cells are damaged, they are replaced by supporting cells (Weisleder and Rubel, 1992). It has also been described that stereocilia derived from microvilli. Under these conditions, it could be considered that cells with large population of microvilli may be the future cells with stereociliary-like structures.

B.A. Bohne: What is the significance of the syncytium-like appearance of the sensory epithelium observed in Figure 3d and the variation in cell size in Figure 5b.
Authors: Ten days after the local application of sisomicin, we observed a very smooth aspect of the cristae. At higher magnification, we observed an undifferentiated-type epithelium without clear limits

between cells. We think that we probably have a very poorly differentiated tissue, with lots of nuclei and without clear limits between cells: a sort of cellular magma. Unfortunately, our observations are limited by the SEM method; only TEM ultrastructural studies could allow us to confirm the exact nature and the level of maturation of the epithelia. This type of tissue is well-known in the differentiation process such as the "trophoblastic syncytium" in the developing embryo. We can imagine that 10 days after the total destruction of vestibular hair cells, we could see the beginning of a transient step represented by a syncytium.

On the point of the significance of the variation in cell size observed at the surface of the restructuring vestibular neuroepithelia, it is very difficult to interpret the surface observations of these end organs. Nevertheless, we could hypothesize that the different sizes could correspond to different degrees of maturation. On the other hand, it could be that the apical surfaces are from different cellular types ("future supporting cells" and "future embryonic-type hair cells"). Moreover, this could be a representation of the phenomenon of migration of replacement cells from the periphery, which could have no defined morphology at this moment.

Concerning your impression that the cells were migrating in Figure 5b, it is a very interesting remark. If this could be confirmed, it will be a good indication in favor of the hypothesis cited above.

Reviewer III: There appears to be a problem in the methodology: you inject aminoglycoside directly into the cochlea, whereas it would be better to use the usual chronic gentamicin treatment (see e.g., Weisleder and Rubel, 1992).

Authors: Our initial objective was a total and rapid destruction of the sensory hair cells within the inner ear. Using aminoglycoside antibiotics two procedures can be used: the systemic injection and the local application. The major part of the studies about aminoglycosides ototoxicity use the general treatment which is rather long, if we expect a total destruction of sensory hair cells. Moreover, these aminosides systemically injected produce important decreases in the general biology of the treated animals, especially for sisomicin which is very neurotoxic. Second, the local application of sisomicin through the round window is well known for many years, since the studies of Spoendlin (1978).

Reviewer III: Does the pressure produced by the injection in the inner ear directly induce mechanical damage in the labyrinth?

Authors: The pressure applied in the inner ear has not been measured. We cannot exclude the fact that mechanical damage could occur, but it is not important in our demonstration. The exact mode of application of sisomicin is described in Dupont *et al.* (1993, in press).

Reviewer III: How can one be sure that sisomicin is distributed equally at the different receptors? How can one ensure that the same quantity of liquid is injected into each guinea pig?

Authors: Nobody can be sure of this, nevertheless the diffusion of the sisomicin into the cochlea and the vestibule is very rapid. As soon as 5 days after local application, we can reasonably assume that the sisomicin has diffused everywhere in the inner ear. In these conditions, the different receptors are exposed to the drug and will degenerate according to their own susceptibility.

We can ensure that the same quantity is injected in all guinea pigs: 25 mg of sisomicin diluted in 500 μl of excipient for each experimented animals. This injected volume corresponds to a solution filling almost all the space of the whole bulla.

Reviewer III: Since 25 mg appears to be quite a large quantity, have different quantities of sisomicin been used?

Authors: In a first series of experiments, we determined the dose of sisomicin necessary to produce a complete destruction of receptor cells of the inner ear. Twenty-five mg of sisomicin was determined as the threshold in local application to obtain a total and reproducible destruction of all the sensory cells 10 days after the treatment.

25 mg is a large quantity of drug in regard of the extremely low concentration of drug in the area of sensory cells during systemic injections. Nevertheless, the local application needs greater doses, as it has already been determined by Spoendlin (1978) who locally applied 500 mg of neomycin in order to destroy the receptors cells.

At lower doses of sisomicin than 25 mg, we did not ensure a total destruction of inner ear sensory cells.

Reviewer III: Why has sisomicin been chosen? Why did the authors not use the standard method of daily subcutaneous injections of aminoglycoside over a one week period?

Authors: As stated above, we used sisomicin to cause a total destruction of cochlear and vestibular sensory cells. Indeed, most of the aminoglycosides are more cochleotoxic than vestibulotoxic or the contrary; with sisomicin the toxicity in the cochlea and the vestibule is same. Moreover, we have undertaken a separate study (and will soon be publishing a paper) about the neurotoxicity of this antibiotic, this work has been performed on the same experimented animals. Finally, it is important to remember that sisomicin is one of the most (maybe the most) ototoxic aminoglycoside antibiotic, at least in the guinea pig.

In response to your second question, our laboratory has a great deal of experience in the ototoxicity of the aminoglycosides for thirty years, and we have never observed a complete destruction of all the sensory cells of the cochlea and the vestibule after systemic injection over a one week period, even after several weeks. In the case of amikacin, we have described an almost total destruction of the cochlear sensory cells, while the ves-

tibule is not so damaged. In the case of the dihydrostreptomycin, we have obtained an almost complete destruction of vestibular receptors while the cochlea was not damaged.

Under these conditions, systemic treatments of aminosides are not very appropriate to our experimental protocol. Even if sisomicin seems to destroy quite equally the cochlea and the vestibule, it is not possible to apply it by systemic treatment due to its high neurotoxicity which leads to a very high mortality. Local application of the drug seemed to be the best way to obtain a total cochlear and vestibular sensory hair cells destruction.

Reviewer III: A severe, but not complete, alteration of vestibular ganglion neurons was observed 5 months after the deafferentation, this would mean that some parts of receptors are innervated, but not others? What are the implications of these results?

Authors: A detailed paper about the neural degeneration of the primary cochleo-vestibular pathways after local application of sisomicin is in press (Dupont *et al.*, 1993). We can consider that some parts of vestibular receptors are innervated and not others. Under these conditions, this could imply that the remaining nerve endings may bring some substances, such as trophic factors or others, which could lead to a certain reorganization. Nevertheless, the observed reorganization of vestibular neuroepithelia cannot be much extended, because the vestibular nerve should continue to degenerate between 5 months and 2 years or more. In parallel, we have not observed such a reorganization of the organ of Corti; this could be explained by the fact that as soon as 25 days almost all the cochlear afferent neurons within the spiral ganglion have irreversibly disappeared. So, the destroyed underlying innervation of the cochlea could not exert a possible role in the way of regeneration. Even if recent reports in chicken conclude that innervation is not required to guide the differentiation of cochlear hair cells (Corwin and Cotanche, 1989 and Swanson *et al.*, 1990), it cannot be excluded that this innervation may initiate the reorganization of totally damaged neuroepithelia in the vestibule, at least in mammals!

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