musicians and musicians did not differ in body length ($P > 0.05$).
19. No formal test for perfect pitch was applied. Subjects were asked if they would be able to sing any tone without a reference tone and if they could name any tone that was given to them without a reference tone. In those musicians who described themselves as having perfect pitch ($n = 11$), this information was confirmed by review of their music school examination records for pitch discrimination task performance.
20. In distinguishing between musicians with or without perfect pitch, we were guided by a study of D. Sergeant [*J. Res. Mus. Educ.* **17**, 135 (1969)] investigating the possession of perfect pitch in a large sample of professional musicians. In this study almost all musicians who began training before the age of seven had perfect pitch, but almost none of those beginning after the age of 11. Similar results were also found by others suggesting that early exposure and disposition may underly this ability [A. Bachem, *J. Acoust. Soc. Am.* **27**, 751 (1955); C. L. Krumhansl, *Annu. Rev. Psychol.* **42**, 277 (1991); M. Klein, M. G. H. Coles, E. Donchin, *Science* **223**, 1306 (1984)].
21. For statistical analysis, δPT values (*16*) were subjected to a one-way analysis of variance (ANOVA) with three levels (musicians with or without perfect pitch, and controls). In this ANOVA two planned orthogonal (linear) contrasts were computed, comparing musicians versus controls and musicians with perfect pitch versus musicians without [R. E. Kirk, *Experimental Design: Procedures for the Behavioral Sciences* (Wadsworth, Belmont, CA, 1968), pp. 69–98]. The results were as follows: orthogonal contrast for musicians versus controls: [$F(1,57) = 5.12$, $P = 0.028$]; orthogonal contrast for musicians with perfect pitch versus musicians without: [$F(1,57) = 16.18$, $P < 0.001$]. Prior to the ANOVA, a Bartlett-Box-test for homogeneity of variances of δPT had revealed no deviation from the homogeneity assumption {[$F(2,4840) = 1.25$, $P = 0.32$]}.
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6 September 1994; accepted 28 November 1994

TECHNICAL COMMENTS

Mammalian Vestibular Hair Cell Regeneration

Birds and mammals are born with a full complement of inner ear hair cells, which were thought to be irreversibly lost when damaged (*1*). It is now well known that birds have the capacity to regenerate hair cells in their auditory and vestibular organs after damage by acoustic trauma or ototoxic drugs (*2*) and that these new cells can mediate functional recovery (*3*). Recent studies by A. Forge *et al.* (*4*) and by M. E. Warchol *et al.* (*5*) suggest that the vestibular epithelium of the mature mammalian inner ear may also have the ability to produce new hair cells by renewed mitotic activity in response to aminoglycoside injury in vivo (*4*) and in vitro (*5*). However, these reports do not provide convincing evidence that the DNA labeling, seen at a low frequency in vitro, is the source of the apparent recovery of hair cell apical surfaces observed in vivo.

Our study was undertaken to determine if cell division can be shown to give rise to new hair cells in normal mature mammalian vestibular epithelium or during the first 6 weeks after aminoglycoside ototoxicity. Three groups of young mature albino Hartley guinea pigs were used. The experimental animals in each group were treated with a single transtympanic injection of the ototoxic aminoglycoside, gentamicin, in the left ear (*6*). Animals in each control group were given an identical volume of 0.9% saline. The first group of animals was killed after 1 to 16 weeks and used for light microscopic evaluation of damage produced in the sensory epithelium of the utricle (*7*). The second group, killed after 1 to 16 weeks, was used for scanning electron microscopy (SEM) (*8*) in order to compare our results with those of Forge *et al.* (*4*). In animals of the third group, an osmotic pump filled with [^{3}H]thymidine was implanted under the skin of the back with its output leading to a cannula inserted into the perilymphatic space before treatment with aminoglycoside (*9*). These animals were killed after 1 to 16 weeks (*10*).

Hair cell damage and loss was evident in the light microscopic sections and SEM analyses of tissue from gentamicin-treated animals (Fig. 1). Experimental animals had fewer hair cells than controls, particularly in the striolar region. Other signs or damage observed by light microscopy of SEM included nuclear pyknosis, nuclear swelling, vaccuolization, cytoplasmic extrusion, and stereocilia fusion. The extent of damage was variable at all survival times. At 1 or 2 weeks after gentamicin treatment, hair cell injury was limited primarily to the striolar region in 10 of 16 animals examined by SEM. In three of the animals damage was observed over a larger area, extending from the striola toward the periphery of the organ. Complete destruction of the sensory hair cells was observed in the remaining three animals. Four weeks after gentamicin administration, one animal displayed hair cell damage extending out from the striolar region; in the other animal blebbing and fusion of stereocilia were seen over the entire surface of the sensory epithelium. In the animal killed 4 months after gentamicin, the surface of the utricle continued to show damaged stereocilia bundles throughout the entire sensory epithelium. The average length of the sensory epithelium and the linear support cell density remained constant between the control and experimental animals (Table 1) (*11*). However, the linear hair cell density was 51 to 85% lower in experimental animals than controls ($P < 0.001$).

Table 1. Results of treatment with gentamicin on guinea pig utricle: Length of sensory epithelium, hair cell density, and support cell density. Measurements are averages (± standard deviation).

Animal number	Treatment (weeks)	Sensory epithelium length (× 0.1 mm)	Hair cell density (per 0.1 mm)	Support cell density (per 0.1 mm)
94-01	1	7.2 (±2.0)	1.6 (±0.7)	11.6 (±4.6)
94-13	1	9.3 (±2.1)	3.4 (±0.8)	9.9 (±3.1)
93-42	1	7.2 (±1.6)	2.6 (±1.4)	10.4 (±1.5)
94-06	4	7.4 (±1.5)	1.3 (±1.5)	8.4 (±1.5)
94-05	6	8.7 (±2.0)	1.6 (±0.6)	7.1 (±1.6)
93-55	6	7.2 (±1.9)	4.3 (±0.8)	10.7 (±2.8)
93-57	0*	7.4 (±1.6)	8.7 (±1.7)	12.6 (±3.3)
93-38	6*	8.9 (±2.1)	6.6 (±1.9)	10.2 (±1.2)

*Control group received no gentamicin.

In all untreated animals, as well as in the gentamicin-treated animals, we identified small stereocilia bundles displaying the same morphological characteristics as those seen in embryonic chickens (*12*) and in drug-damaged guinea pig utricles (*4*). Stereocilia were short, uniformly sized, and clumped around a single, centrally located kinocilium. The apical surfaces of these immature-appearing stereocilia bundles were smaller than those of normal, mature hair cells. Because it was difficult to locate immature-appearing stereocilia bundles among the taller stereocilia bundles of more mature-appearing hair cells in the untreated animals, no attempt was made to quantify the number of immature-appearing stereocilia bundles. However, as reported by Forge *et al.*, there was a clear increase in the number of immature-appearing bundles in animals that survived 4 to 16 weeks after gentamicin treatment. From the limited sample of utricles on which we quantified the density of hair cells (Table 1), there did not appear to be a systematic change in hair cell density over the survival period examined.

Utricles from seven experimental and two control animals were continuously infused with [^{3}H]thymidine for 1 to 6 weeks. We examined every serial section (a total of 2293 autoradiographic sections) from these nine animals. No labeled hair cells were seen in any of the utricles from control or experimental animals. The only labeled nuclei present in the sensory epithelia were located in the support cell layer, adjacent to the basement membrane (Fig. 2). A total of 23 labeled support cells were found among four of the seven animals that had received both gentamicin and [^{3}H]thymidine (Table 2). Neither control animal had labeled nuclei in the sensory epithelia. At 1 to 4 weeks, only single-labeled support cells were found, indicating that mitosis had not occurred or that one daughter cell had degenerated. Three of five animals showed one or more labeled support cells. At 6 weeks we observed both single and pairs of labeled support cells in one animal and no labeled support cells in the other. Labeled nuclei in both the long- and short-term survivors were found exclusively in the striolar region of the sensory epithelium. It is unlikely that the lack of labeled hair cells and the paucity of labeled support cells is a result of limited availability of [^{3}H]thymidine during DNA replication. In all animals, sampling of the perilymph at the time of sacrifice yielded radioactivity sufficient to label dividing cells in tissue culture preparations of avian utricles (*13*). Also, all the utricles examined had many labeled stromal cells, pericytes, and Schwann cells in the tissues underlying and adjacent to the sensory epithelium (Table 2). Finally, when a similar procedure with identical delivery rate was used in the chick to label regenerating hair cells in drug-damaged cochleas, almost all regenerated hair cells were heavily labeled and stromal cell numbers were comparable to those seen in the present study (*14*).

Data presented in this comment have several implications. They support the idea that there may be a population of cells residing near the basement membrane in mammalian utricles that are capable of renewed cell division in vivo following damage to the sensory epithelium (*15*). While the number of labeled cells was small, in vitro studies suggest that the number of cells capable of division may be much larger if appropriate growth factors or cytokines are administered (*16*). Our observations also support the report by Forge *et al.* (*4*) of an increase in immature-appearing hair cells following gentamicin ototoxicity. On the other hand, we did not observe any labeled hair cell in control or experimental tissue, even when [^{3}H]thymidine was continuously present for up to 6 weeks. If immature-appearing hair cells in normal animals, or those shown by Forge *et al.* and this study to increase in number by 4 weeks after gentamicin treatment, were the result of support cell proliferation, they would have been labeled with [^{3}H]thymidine in this study. Thus, it appears that within the tem-

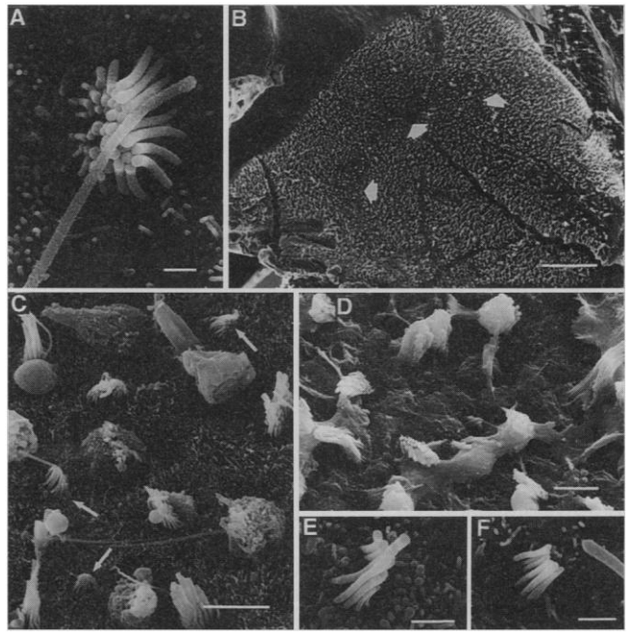

Fig. 1. SEM photomicrographs of representative utricles. (**A**) Immature-appearing sterocilia bundle from striolar region of control, untreated-mature guinea pig. (**B**) Low power photomicrograph from an animal 1 week after gentamicin treatment. Arrows point to striolar region with missing hair cells. (**C**) Same preparation as (B) at higher power, damaged hair cells and immature-appearing hair bundles (arrows). (**D**) Specimen from an animal 16 weeks after gentamicin treatment showing continued damaged surface. (**E** and **F**) Immature-appearing hair cells 4 weeks after gentamicin. Calibrations: 0.5 μm in A; 100 μm in B; 5 μm in C and D; 1 μm in E and F.

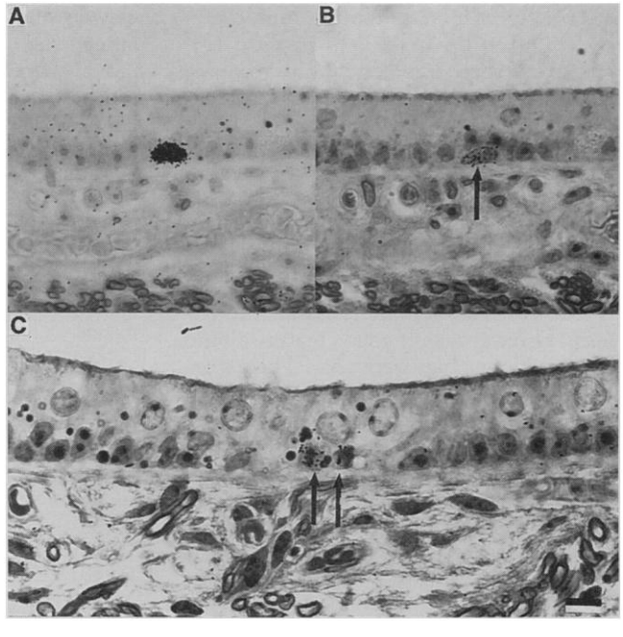

Fig. 2. Tissue sections of utricles showing [^{3}H]thymidine-labeled nuclei in support cell layer from animals 1 week (**A** and **B**) and 6 weeks (**C**) after gentamicin treatment. Adjacent sections show heavy labeling (A), and unusual size and shape of nucleus (B). A pair of labeled nuclei (C, arrows) suggests that mitosis had occurred during the labeling period. Relative lack of hair cell nuclei (A, B, and C) because of the gentamicin ototoxicity. Calibrations = 10 μm in all panels.

Table 2. Results of treatment by [^{3}H]thymidine infusion on guinea pig stromal cell nuclei. Counts are of heavily labeled nuclei in support cell layer of the sensory epithelium and total heavily labeled stromal cells up to 250.

Animal number	Treatment (weeks)	Nuclei	Stromal cells
93-42	1	Two support cell singlets	220
94-01	1	Eight support cell singlets	108
93-46	2	Stromal cells only	>250
93-45	2	Stromal cells only	>250
94-06	4	One support cell singlet	91
94-05	6	Stromal cells only	135
93-55	6	Three support cell doublets Six support cell singlets	>250
93-43	1*	Stromal cells only	140
93-38	6*	Stromal cells only	>250

*Control group received no gentamicin.

poral constraints of this investigation the immature-appearing hair bundles do not represent the apical surfaces of hair cells born after the induction of hair cell damage by gentamicin.

What might be the source of the immature-appearing hair bundles observed by Forge and colleagues and replicated here? They may have been derived from a process of cellular transformation, wherein another cell type in the sensory epithelium was converted to a hair cell. Alternatively, some hair cells might have sustained injury and loss of the stereocilia bundle after gentamicin and then repaired that structure by growth of a new sensory surface (*17*). Such reparative processes may normally occur at a low frequency, thereby explaining why the immature-appearing stereocilia bundles are evident in control tissue. Finally, we must acknowledge the possibility that immature-appearing hair cells seen in normal mature animals and at short survival times arise from cellular recovery or cellular transformation, while those emerging at long recovery periods are the result of renewed mitotic activity.

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6. Experimental methods and animal care procedures used in this investigation were approved by the University of Washington Animal Care Committee. Young albino guinea pigs (300 to 400 mg) with normal Preyer's reflexes were anesthetized with ketamine (40 mg/kg; Aveco, Fort Dodge, IA), and xylazine (5 mg/kg; Mobay, Shawnee, KS) and given a 4-mg dose of gentamicin (0.2 ml of 20 mg/ml solution; Solopak, Franklin, IL) injected into the left middle ear through the tympanic membrane. This procedure is used by A. Forge and colleagues, and the protocol was kindly provided by A. Forge.
7. Animals survived 1 (n = 4), 2 (n = 2), or 16 (n = 1) weeks after injection and then were euthanized by an overdose of sodium pentobarbital. The vestibule was gently perfused with 2% paraformaldehyde per 2% glutaraldehyde fixative for 10 min and immersed in cold fixative overnight. The utricle was osmicated, dissected free, embedded in Spurr's resin, and serially sectioned at 3 μm. Sections were stained with toluidine blue.
8. Animals were allowed to survive 1 (n = 10), 2 (n = 6), 4 (n = 2), and 16 (n = 1) weeks after gentamicin injection and then killed by an overdose of sodium pentobarbital. The utricles were harvested as described above (*7*), then dehydrated, critical-point-dried, sputter-coated with gold-palladium, and examined with the use of a JEOL JSM6300F Scanning Electron Microscope.
9. Seven experimental and two control animals were implanted with mini-osmotic pumps (Alzet, model 2002, Palo Alto, CA) containing 0.2 ml [^{3}H]thymidine (60 to 90 Ci/mol), which was infused at a rate of 0.5 μl/hour for 2 weeks. The mini-osmotic pump was surgically implanted under the redundant skin of the back. One bulla was then opened to visualize the ampulla of the posterior semicircular canal and the basal turn of the cochlea. A small fistula was drilled into the ampulla of the posterior semicircular canal, and a brain infusion cannula (Alzet, Palo Alto) was carefully inserted in the fistula. A polyethylene catheter was tunneled from the cannula under the skin of the neck to the pump. The bone-cannula junction was sealed with Vetbond (3M Animal Care Products, St. Paul, MN), the cannula was fixed in place with dental cement, and the skin was closed with nylon suture. The gentamicin (or saline) injection was then administered. Post-operative care consisted a perioperative dose of Ancef (10 mg/kg), once daily IM injections of Vistaril (Hydroxyzine, 1 mg/kg), and Compazine (Prochlorperazine, 1 mg/kg) until vertiginous symptoms subsided (usually approximately 4 days), and daily subcutaneous saline boluses until the animals were observed drinking water (also approximately 4 days). Daily weights and general condition were closely monitored. Any animals that developed symptoms of systemic infection or did not show recovery from vestibular symptoms within five days were euthanized by lethal injection. Fresh pumps containing [^{3}H]thymidine were exchanged at 2 and 4 weeks in those animals that were to be exposed to [^{3}H]thymidine for 4 or 6 weeks. At the end of the survival period, the animals were killed by overdose of sodium pentobarbital. Before fixation, the perilymph of the ear receiving [^{3}H]thymidine was sampled and analyzed by scintillation counts to insure that there was a continuous delivery of [^{3}H]thymidine to the targeted tissue. The vestibule was then perfused, harvested, fixed, and sectioned as described above. Every serial section was mounted, and all slides were processed for autoradiography.
10. Survival times: experimental animals, 1 (n = 2), 2 (n = 2), 4 (n = 1), and 6 (n = 2) weeks; control animals, 1 (n = 1) and 6 (n = 1) weeks. The slides were dipped in Kodak NTB-2 Nuclear Track emulsion (1:1 dilution) and exposed at 4°C for 3 to 6 days. The emulsion was developed in D-19 developer for 4 min, rinsed in distilled water, fixed in Kodak fixitive for 3.5 min at 13°C, and counterstained with 0.01% toluidine blue. A labeled nucleus was defined as one with six or more silver grains above it. Every section (190 to 400 per animal) was carefully examined at a magnification of 1250× in order to insure identification of every labeled cell in the sensory epithelium. Labeled cells in the stromal layer, below the epithelial basement membrane, were counted separately. Imaging software was used to generate mylar overlays in order to compare the labeled cells from adjacent sections to differentiate between same and adjacent cells.
11. Hair cells and support cells were counted in every tenth serial section through the utricular sensory epithelium of six group 3 experimental and two control animals at 1250×. One control animal was from group 3 and one was a normal, untreated, age-matched guinea pig. Sections were transverse to the sensory epithelium. In each section (20 to 40 in each animal), the total length of the sensory epithelium was measured, and the linear density of hair cells and support cells was calculated by dividing the number of hair cells (or support cells) in each section by the measured basement membrane length.
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Response: Rubel *et al.* outline results from in vivo studies of the utricle, one of the five vestibular organs that provide balance sensitivity in the mammalian ear. Their work appears to provide confirmation of the main results reported by Forge *et al.* and by Warchol *et al.* on 12 March 1993 pertaining to regeneration in the balance organs of the ear. The report by Forge *et al.* (*1*) was based on in vivo morphological investigations of the vestibular organs in juvenile guinea pigs. The report by Warchol *et al.* "Regenerative proliferation in inner ear sensory epithelia from adult guinea pigs and humans" (*2*), was based on radioactive and immunochemical labeling of proliferative cells in balance organ sensory epithelia maintained in culture after surgical removal from juvenile and adult guinea pigs and from adult humans. Rubel *et al.* have extended one of the cell labeling methods that was used by Warchol *et al.* in vitro into an in vivo situation similar to that used by Forge *et al.* Their comment provides new results and interpretations that were not predicted on the basis of the conclusions of the two preceding studies. Our examination of their data provides another interpretation. We suggest that data from a larger sample size, with specific monitoring of the integrity of the delivered nucleoside label and longer survival times, will be required for a definitive evaluation of the role of cell proliferation in the spontaneous, in vivo recovery of hair cells in mammalian balance organs.

Warchol *et al.* (*2*) reported that regenerative proliferation of supporting cells occurs in the utricles of mature mammals in response to death of sensory hair cells. Before that report, it had been assumed that cell proliferation did not occur in the sensory organs of the mammalian ear after embryonic development (*3*). The experiments examined utricles placed in organ culture for 3 to 28 days after surgical removal from guinea pigs and adult humans. Hair cells were killed by incubating the utricles for 24 hours in culture medium that contained an ototoxic antibiotic. Then the specimens were cultured for an additional 2 to 27 days in antibiotic-free medium that contained a tracer to label proliferating cells. Labeled supporting cells were found in all the specimens from guinea pigs in the experiments (*2*): in 18 utricles that had been treated with an ototoxic antibiotic to kill hair cells and in 4 control utricles. Labeled cells ranged from 3 to 26 in the 10 utricles that were cultured for 2 to 6 days after the antibiotic treatment, from 15 to 39 in the three utricles that were cultured for 27 days after the antibiotic treatment, and from 3 to 10 in the control cultures, which were not treated with the antibiotic. Two utricles from humans were cultured for 6 days after the antibiotic treatment, and their sensory epithelia contained 127 and 159 labeled cells at the end of that week. A third human utricle was cultured for 25 days after the antibiotic treatment, and contained even more labeled cells. In addition to finding cell proliferation evoked by hair cell loss, we observed that when antibiotic-lesioned utricles were kept in culture for 4 weeks, a subset of the labeled cells appeared to display some morphological features that are found in immature hair cells. The three utricles from guinea pigs that were cultured for 27 days after the antibiotic treatment contained a total of 78 labeled cell nuclei (15, 24, and 39). Of that number, 50 of the labeled nuclei (12, 13, and 25, respectively) were located in the lumenal stratum of the sensory epithelium, which is normally occupied by hair cell nuclei. Our report noted that labeled nuclei in the lumenal stratum were much more common in utricles that had been maintained in culture for at least 4 weeks, as compared with those that were cultured for 2 weeks or less.

The comment from Rubel *et al.* provides data on cell proliferation that was obtained from seven utricles after a single injection of gentamicin in vivo. One to 12 labeled supporting cells were observed in four utricles and no labeled supporting cells were observed in the remaining three, although they received the same treatment: injection of a bolus of that ototoxic antibiotic directly into the ear (*4*). The labeled nuclei were located in the lower stratum of the sensory epithelium, the stratum that is normally occupied by most, although not all, supporting cell nuclei. No labeled hair cells and no labeled nuclei in the lumenal stratum were observed in the four gentamicin-treated specimens that contained labeled cells. Rubel *et al.* conclude from their results that the immature-appearing bundles that are observed at recovery times of 6 weeks or less after a treatment with an ototoxic antibiotic in vivo are not arising through proliferation of cells in the sensory epithelium.

For several reasons, we believe that the results described by Rubel *et al.* should be interpreted with caution. Our in vitro study showed significant numbers of labeled nuclei in the lumenal stratum of the epithelium only in the group of specimens that survived for 4 weeks after the antibiotic treatment. The results suggest that 4 weeks may be required for the first stages of new cell differentiation, even under conditions which permit the antibiotic to be rapidly washed away from the sensory epithelium. In the study by Rubel *et al.*, only three of the animals that had been implanted with [^{3}H]thymidine pumps were allowed to survive for 4 weeks or longer after the ototoxic antibiotic was injected directly into the ear (*5*). One of those guinea pigs was killed after 4 weeks, the other two after 6 weeks. A single experimental utricle was obtained for autoradiography from each, so the pertinent negative findings reported by Rubel *et al.* rest on data obtained from a total of three specimens.

The long-term survival group in our in vitro study of guinea pig utricles (27 days) also consisted of three specimens, but each of those utricles contained at least 12 labeled cell nuclei in the lumenal stratum of the epithelium, for a total of 50 positively labeled nuclei in that location. In the present context, a sample group of three specimens that displays 50 unambiguously positive results seems more meaningful than a sample group of three specimens that show a negative result, specifically the absence of labeled cell nuclei in the lumenal stratum.

The labeling studies by Warchol *et al.* (*2*) and those by Rubel *et al.* were carried out under entirely different experimental conditions. In our experiments, the utricles were cultured for 27 days after hair cell lesions were dissected from ears that had not been subjected to an ototoxic antibiotic in vivo. They were denervated as they were dissected from the ear. The roof of each utricle was removed, the otoconial masses were removed, the otolithic membranes were removed from above the apical surface of the hair cell epithelia, and the organs were placed in tissue culture dishes containing a controlled concentration of either neomycin or gentamicin. After 24 hours, they were rinsed twice with fresh antibiotic-free medium in order to remove the ototoxic drug, and then they were transferred to coverglasses in fresh labeling medium and embedded in Matrigel (Collaborative Biomedical Products/Becton Dickinson, Mountain View, California). Matrigel is a basal lamina product secreted by the Engelbreth-Holm-Swarm tumor cell line (*6*). In addition to extracellular matrix components of basal lamina, Matrigel has been shown to contain the growth factors bFGF, EGF, IGF-1, PDGF, and TGF-β. We suspect that one or more of those factors, such as TGF-β, which has been shown to induce differentiation of epithelial cells in other organ systems (*7*), may have contributed to cell differentiation in our specimens.

It has been established that the nuclei of the proliferating cells that give rise to hair cells and supporting cells in the ear undergo an elevator-like "interkinetic" movement during the cell cycle (*8*, *9*). This takes nuclei from a more basal location at the start of the cell cycle (during S and G_2 phases) to an apical position where mitosis occurs (M). Yet, we did not observe many labeled nuclei in the lumenal stratum in cultures that were maintained for 14 days or less. They appeared in appreciable numbers only in the

group of epithelia that were cultured for 4 weeks. Our data showed labeled cells in mitosis by 2 days after the end of the antibiotic treatment, the earliest stage that we examined [figure 1A of our report (2) showed such a cell]. Thus, cells of the mammalian vestibular hair cell epithelium can cycle from the S through the M phase in 48 hours. On the basis of those data, we concluded that the labeled cell nuclei that appeared in the lumenal stratum in the epithelia from the 4-week culture group were not simply undergoing interkinetic movement associated with progression to mitosis. Furthermore, some of the cells with labeled nuclei in the lumenal stratum were in isolated groups consisting of a labeled lumenal nucleus located near comparably labeled nuclei in the basal strat- um, which suggests that those labeled cells might have originated from the same cell divisions.

There was little labeling in the sensory epithelia of the guinea pigs in the experiments conducted by Rubel *et al.*, but there are potential explanations for this negative result. For example, variability is a part of in vivo ototoxicity studies, and as described by Rubel *et al.*, the direct injection of gentamicin into the ear resulted in lesions that showed considerable variation in their severity. More significantly, we do not find the measure of total radioactivity in the perilymph of the ear persuasive as an argument that the nucleoside tracer was continuously delivered in a stable form that could label proliferating cells. The potential for breakdown of the labeling compound is a serious concern in any experiment where radionuclides must be held for weeks at high specific activity, in the elevated temperatures of a mammal's body, and with the potential for exposure to biological breakdown. Infusion of a solution that contained only the breakdown products of [^{3}H]thymidine (products which would not label proliferating cells) would give the same reading in terms of radioactivity referred to in the comment by Rubel *et al.* as would infusion of a solution that contained the actual tracer before breakdown. The measure does not distinguish between the actual tracer and its breakdown products. Thin-layer chromatography has been used even in some short-term studies of proliferation in the ears of endotherms and would have provided a valid measure of the stability of the [^{3}H]thymidine and its capacity to label any cells after it resided for 2 weeks at a specific activity of 60 to 90 Ci/mol in a mini-osmotic pump within a guinea pig (8).

The data in table 2 of the comment by Rubel *et al.* suggest that the concern outlined above has more than theoretical significance. The rate of stromal cell proliferation should not be influenced by variation in the severity of damage to the sensory hair cells of the overlying epithelium. Stromal cells should continue to proliferate throughout the experiment, so the cumulative incidence of labeling in stromal cells provides an internal check on the delivery of the [^{3}H]thymidine. The number of stromal cells that were labeled should have been a function of the length of time that stable [^{3}H]thymidine was delivered to the ear. The table shows that the incidences of labeled stromal cells in utricles from the three guinea pigs that were given the tracer for 1 week were 108, 140, and 220. If [^{3}H]thymidine had been delivered to the tissues in a form that would label proliferating cells continuously for 4 and 6 weeks in the long-term labeling groups, then at least 400 to 800 and 600 to 1200 stromal cells should have been labeled over the respective periods (even when the potential for multiple generations of labeled cells is not factored in). In fact, the observed incidence of labeled stromal cells in the one guinea pig that was implanted with labeling pumps for 4 weeks after the gentamicin injection into the ear was only 91, and the incidence of those labeled cells in one of the two guinea pigs that were implanted with labeling pumps for 6 weeks was only 135. Those results strongly suggest that at least two of the three relevant utricles examined by Rubel *et al.* might not have been exposed to [^{3}H]thymidine in a form that was capable of labeling proliferating cells for the duration of the survival of the guinea pigs after hair cells were lesioned. Interestingly, the utricle of the other guinea pig that received labeling pumps for 6 weeks after the antibiotic administration contained ">250" labeled stromal cells, and its sensory epithelium contained the largest number of labeled cells in the entire sample of specimens, 12 in total. That specimen was also the only one in which labeled supporting cells occurred in "doublets."

The second part of the issue raised by Rubel *et al.* relates to scanning electron microscope observations of immature-appearing hair bundles. Forge *et al.* (1) noted immature-appearing hair bundles throughout utricles from control guinea pigs. Such bundles are particularly visible at the outer edges of the epithelium, at a location analogous to the site where newly produced (labeled) hair cells are known to be added to epithelia in other species during postembryonic life (10, 11). That immature-appearing hair bundles could be observed even in the epithelia of control guinea pigs necessitated a quantitative approach to the data reported by Forge *et al.*, which confirmed that substantially larger numbers of immature-appearing bundles appeared by 4 weeks after a 10-day course of subcutaneously administered gentamicin. Rubel *et al.* report the presence of immature-appearing hair bundles in all specimens that were processed for scanning electron microscopy, including controls. They also report an increase in the number of immature-appearing bundles in animals that survived for 4 to 16 weeks after gentamicin treatment.

On the basis of the negative labeling data presented in their comment, Rubel *et al.* conclude that mechanisms of hair cell regeneration that do not depend on cell proliferation are giving rise to immature-appearing hair bundles in the balance organs of mammalian ears. In view of the technical concerns that we have outlined above and in note (5), we feel that the limited negative data presented should be interpreted with caution.

We do not reject proposals that nonproliferative mechanisms for hair cell regeneration may occur. In advancing such a proposal Rubel *et al.* join other investigators who have provided evidence and theoretical explanations concerning the potential existence of modes of hair cell replacement that do not depend on renewed cell proliferation (12). The potential for healing of the hair bundles of damaged hair cells and the potential for formation of immature-appearing hair bundles without cell proliferation have long been concerns in studies of hair cell formation in the postembryonic ear (1, 13). The possible occurrence of cell phenotype conversion in the sensory epithelia of the ear was a major topic of discussion at a recent meeting in this field (14).

Although studies of hair cell regeneration in mammalian balance organs are at an early stage, three facts are clear. First, morphological recovery of hair bundles occurs in the vestibular organs of mammals over a course of weeks after damage to hair cells caused by ototoxic antibiotics. This result was initially reported by Forge *et al.* (1) and appears to have been confirmed by Rubel *et al.* Second, supporting cells, which are known to serve as the progenitors of hair cells in auditory, vestibular, and lateral line epithelia of fish, amphibians, and birds (11, 15), begin to proliferate after hair cells have been killed in the vestibular organs from mature mammals, including humans. This result was initially reported in the in vitro studies by Warchol *et al.* (2), has been confirmed by another in vitro investigation (16), by the in vivo results of Goldstein *et al.* (4), and by the in vivo results of Rubel *et al.* Third, the amount of supporting cell proliferation that occurs in the vestibular organs from mature mammals can be enhanced by the application of specific mitogenic growth factors in vitro (16, 17).

Hair cell regeneration and postembry-

onic hair cell addition have been studied extensively in the inner ear sensory epithelia and lateral line organs of fish and amphibians and in the cochlea and vestibular organs of birds. In those cases, regeneration of hair cells and other forms of postembryonic production of hair cells can occur through the differentiation of progeny produced through proliferation (*11*, *15*). This has been demonstrated by positive labeling of new hair cells. It seems likely, therefore, that trauma-evoked cell proliferation and hair bundle recovery within the sensory epithelia of vestibular organs are linked, so that regenerative proliferation may be one mechanism by which the balance organs of the mammalian ear can recover from injury. In theory, additional mechanisms may contribute to regeneration of hair cells in all vertebrates. Independent of whether hair cell regeneration in mammalian balance epithelia occurs through a nonproliferative mechanism or through a process that is dependent on new cell production, the process of trauma-evoked supporting cell proliferation is certain to play a vital role in self-repair of damage to the hair cell epithelium. The challenge now is to discover what factors will enhance the production, the differentiation, and the survival of the sensory cells of the inner ear.

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4. In the data contained in the comment from Rubel *et al.*, the fraction of the sensory epithelia that contained labeled cells and the levels of labeling within the four sensory epithelia that contained labeled cells are low as compared with the incidence of BrdU labeling reported from in vivo experiments involving subcutaneous injection of antibiotic for 7 days [B. J. Goldstein, M. Y. Huang, J. T. Corwin, *Assoc. Res. Otolaryngol.* **17** (Abstr. 524) (1994)]. In that study, 17 ± 12 cells in the sensory epithelia of the treated utricles (range: 7 to 38) were labeled by BrdU that was delivered systemically from mini-osmotic pumps over the course of 7 days. Thus, labeling ranged from one to more than five cells per day, on average.
5. Cautious interpretation of the small sample of negative results also seems reasonable in view of the potential for long lasting influences of the ototoxic gentamicin after delivery of a large bolus directly into the ear. The impracticality of assessing the degree of damage and the degree of hair bundle recovery occurring in ears that received the drug treatment followed by implantation of osmotic pumps to deliver label is another limitation of the protocol.
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Response: The work by Rubel *et al.* appears to confirm both of the essential findings of the reports by Forge *et al.* (*1*) and Warchol *et al.*, which are (i) that developing hair cells appear after drug-induced damage to the mature vestibular sensory epithelia of guinea pigs and (ii) that cell proliferation is stimulated in those epithelia after damage. These reports independently demonstrated a previously unsuspected capacity for recovery after trauma-induced damage in the mammalian inner ear. The results of the study by Warchol *et al.* (*2*) are significant because it had not been previously shown that in mammals, unlike all other vertebrates, postembryonic cell proliferation could occur in the sensory epithelia of their inner ears. That study was performed in vitro using organotypic cultures and the results were subsequently substantiated in other in vitro studies (*3*). The results of Rubel *et al.* demonstrate that proliferation is also stimulated in vivo. This has apparently been confirmed in another study performed in vivo by Goldstein *et al.* (*4*).

A point at issue, however, seems to be whether, or perhaps to what extent, the appearance of immature hair cells is related to the stimulation of proliferation. In the morphological studies we initially reported (*1*), a small number of immature hair cells, identified from their hair bundle morphology as seen by scanning electron microscopy, were present in normal utricles, an observation Rubel *et al.* appear to confirm. One week after treatment of animals with gentamicin, hair cells had been lost and replaced by expansion of supporting cells. However, by 4 weeks after treatment, immature hair cells, identified both by scanning electron microscopy and from thin sections, had reappeared and were present at significantly greater numbers than in undamaged tissue (*1*). The results of Rubel *et al.* appear to support this finding. Warchol *et al.* (*2*), using organotypic cultures, found that cells labeled with markers for cell proliferation were present at 1 week after exposure of the cultures to aminoglycoside, but nearly all the labeled nuclei were present at the level normally occupied by supporting cell nuclei, and no labeled hair cells were apparent. This, too, seems to be supported by the in vivo results of Rubel *et al.* as well as by the other in vivo study of Goldstein *et al.* (*4*). After 4 weeks in culture, labeled nuclei were present at a position in the epithelium, which is normally occupied by

hair cell nuclei. The morphological features of those labeled cells were similar to immature hair cells. The most parsimonious explanation of these findings is that the immature hair cells that appear after treatment in vivo develop from cells that have undergone proliferation, as had been suggested in the studies of organotypic cultures. The number of labeled cells that could be "putatively" identified as hair cells in the organotypic cultures was rather less than the number of cells with characteristics of immature hair cells that were identified from the in vivo morphological study, but this could be a result of differing capacities for proliferation and subsequent differentiation in vitro and in vivo. Alternatively, the apparent discrepancy could result because, in addition to proliferation, there are, as Rubel *et al.* suggest, other processes that lead to development of hair cells after damage, including self-repair of nonlethally damaged cells, or the direct conversion of some nonsensory cell type in the epithelium into a hair cell. Self-repair through a process that involves redevelopment of their apical structures has never previously been recognized in the many, varied studies of hair cell injury in vivo. If such does occur, it seems unlikely that it is confined to the mammalian vestibular system, and this would have implications for studies of those sensory epithelia where recovery of hair cell numbers through proliferation has been reported. However, phenotypic conversion of supporting cells directly into hair cells has been suggested previously (*5*) as a supplementary mechanism for hair cell production, although at present the evidence is indirect and inconclusive.

Rubel *et al.* however, appear to contend that in vivo most of the immature hair cells arise from some process other than from the proliferating nonsensory cells. None of the labeled cells in their study could be identified as hair cells. That they did not find any labeled hair cells is an unexpected and potentially significant result.

However, the drug treatment protocol used by Rubel *et al.* appears to have induced extensive damage over prolonged periods, with marked interanimal variability in the amount of damage caused. The variability between animals in the pattern of damage could be reflected in a variability in the number and timing of appearance of immature hair cells. Some of the data do appear to show that the pattern of damage influences the pattern and extent of proliferation. In one of the two thymidine-exposed, drug-treated animals examined at 6 weeks after treatment, there are no labeled cells. The density of mature, remaining hair cells in the utricle in this animal is low, suggesting damage was extensive. The other animal examined at this time is the only sample showing pairs of labeled nuclei, and it is suggested that progression to cell division had occurred by this time. This animal is also the one that shows the highest density of hair cells after treatment, which suggests that it suffered the least damage. Furthermore, we have found significantly greater numbers of hair cells at 12 weeks than at 4 weeks after treatment, as assessed from SEM (*6*) and from thin sections (*7*). If this is correct, then it may be that the development of hair cells after injury to the epithelium continues over a period more prolonged than that examined by Rubel *et al.* It would be of interest to determine the developmental fate of those daughter cells that Rubel *et al.* found had been produced 6 weeks after drug exposure.

It is difficult from the results presented by Rubel *et al.* to draw firm conclusions as to the extent to which proliferative regeneration is involved in the appearance of immature hair cells in the drug-damaged mammalian urtricles. Nevertheless, the results of their study indicate that proliferation might not be the only process involved. This raises important questions that need to be considered in future work.

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Regeneration and Mammalian Auditory Hair Cells

Until recently, it was assumed that deafness in mammals resulting from the loss of auditory receptors (by administration of ototoxic drugs or by physical injury) was permanent, although such is not the case for cold-blooded vertebrates and birds (*1*). P. Lefebvre *et al.* report that retinoic acid (RA) stimulated the regeneration of hair cells on the cochleas of neonatal rats maintained in vitro after ototoxic poisoning (*2*). Finding a molecule or molecules that could initiate regeneration of hair cells would have a great impact on the treatment of deafness.

To test whether RA can stimulate hair cell regeneration, we performed tests with RA and neomycin separately and then compared the effects of RA and neomycin together (Fig. 1). Corti explants from 3-day-old rats were maintained for 10 to 13 days in vitro with fetal bovine serum (FBS) and N1 (*3*). Normal cochlear organization with one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) was observed by phalloidin staining (*4*). The apical surface of hair cells with their cuticular plates and stereocilia bundles were normal. However, some qualitative differences were observed compared to in vivo control cochleas. For example, stereocilia of IHCs were more elongated and stereocilia of OHCs sometimes still had an immature shape, irrespective of in vitro conditions. The mean number density of hair cells in vitro compared to 3 days after birth (DAB) in vivo control cochleas is slightly lower, because an increase in the intercellular spaces between hair cells. No dying hair cells were observed. Postnatal cochlea explants may have the potential to produce supernumerary hair cells in vitro, (*5*), but previous observations (*6*) have shown that explants from postnatal cochleas, even from the apical part, do not reliably produce supernumerary hair cells. Moreover, our control explants from three DAB, maintained 10 to 13 days in vitro, have less hair cells whatever the origin of the explant in the cochlea (Fig. 2).

We investigated whether RA is able to stimulate production of supernumerary hair cells in postnatal explants (*7*). Explants exposed to RA alone for 7 days (*8*) did not show an increase in the number of hair cells and no extra rows of hair cells were observed (Fig. 1).

Is antibiotic treatment, as used by Lefebvre *et al.* (*2*), able to destroy virtually all hair cells? After treatment (*9*), the apical surface of the former sensory epithelium is replaced by polygonal cells. Few hair cells were visible in the basal turn (Figs. 1 and 2A). However, explants from the last half of the apical turn showed remaining hair cells. Over half