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Hair-cell regeneration in organ cultures of the postnatal chicken inner ear

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The sensory epithelium of the avian inner ear retains into adulthood progenitor cells for inner-ear hair cells and other cell types in the epithelium. Hair cells are produced normally on an ongoing basis in the vestibular sensory epithelium, and hair-cell production is increased after insult in both auditory and vestibular sensory epithelia. The details of postnatal hair-cell production are not understood. In particular, molecular factors involved in the initiation and regulation of hair-cell genesis and differentiation are not known. Studies of this phenomena have been hampered by the lack of cell culture models. An organ culture system was developed which encourages generation and differentiation of hair cells in mature inner-ear sensory epithelia. Continuous labeling with tritiated thymidine showed genesis of both supporting cells and hair cells in normal vestibular epithelia grown in culture, and an increase in hair-cell and supporting-cell proliferation in damaged sensory epithelia grown in culture as compared to undamaged controls. This demonstrates, in vitro, both the division and differentiation of hair-cell progenitor cells in normal vestibular epithelia, and the maintenance of the hair-cell regeneration process in damaged inner-ear epithelia. This culture system should be useful for studies of hair-cell genesis and differentiation as well as studies of hair-cell and supporting-cell functioning in general.

Bird; Auditory; Vestibular; Hair cells; Hair-cell regeneration; Organ cultures

Introduction

In the vestibular sensory epithelium, the sensory receptor cells, the hair cells, are continuously generated from dividing progenitor cells in normal adult birds (Jørgensen and Mathiesen, 1988; Roberson et al., 1992). Continual inner-ear sensory-cell production is rare elsewhere in warm-blooded vertebrates. More extensive hair-cell generation is seen in the vestibular epithelium after experimental manipulations which lead to the death of significant numbers of hair cells (Weisleder and Rubel, 1992a, 1993). Similarly, new hair cells are generated in the auditory sensory epithelium after insult (Cruz et al., 1987; Cotanche, 1987; Corwin and Cotanche, 1988; Ryals and Rubel, 1988). The newly formed hair cells differentiate, and form appropriate, functionally active connections with the eighth nerve (Cotanche, 1987; Henry et al., 1988; McFadden and Saunders, 1989; Tucci and Rubel, 1990; Duckert and Rubel, 1990, 1993; Cotanche and Corwin, 1991; Cotanche et al., 1991; Marean et al., 1993).

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Regulatory factors for hair-cell generation and differentiation have not been definitively identified in the inner ear. Very little is known regarding the mitogenic effects of growth factors on the developing inner ear (Represa et al., 1990, 1991), and nothing is known regarding the factors which induce the generation and differentiation of hair cells in the mature or regenerating inner ear. It is anticipated that information obtained regarding hair-cell genesis and differentiation in the mature avian inner ear could eventually be applied to the mammalian inner ear and to other mature sensory systems to assist in the induction of sensory-cell production in these systems as well.

To identify the regulatory factors for hair-cell generation and differentiation, it would be useful to have an experimental system where the external milieu surrounding the inner-ear sensory epithelia can be easily and controllably manipulated in a well defined manner. Tissue culture techniques offer a number of advantages in studying microenvironment-mediated effects on the proliferation and differentiation of cells. These techniques eliminate some of the in vivo complexities and expose the cells to a culture medium which functions as a controlled microenvironment. Organotypic cultures (i.e., cultures that are obtained by transferring small pieces of tissue from the animal to the tissue culture vessel) offer several advantages over

other culture techniques. The major advantages are that they preserve the basic structural and connective organization of their tissue of origin. In organotypic cultures of inner-ear end organs, in situ topologic and connectional features are preserved in the sensory receptor epithelia. This facilitates the identification of the various types of sensory epithelial cells as well as the maintenance of normal intercellular relationships.

To identify the regulatory signals involved in hair-cell genesis and regeneration, we developed primary organotypic cultures of end organs from the postnatal chicken inner ear. Using these cultures as a tool, our ultimate aim is to identify growth factors and extracellular matrix components that are required to initiate and maintain cell proliferation and differentiation of progenitor cells in the inner-ear sensory epithelium. We describe the isolation and culture of inner-ear end organs and the morphological and proliferative features of the cultured sensory epithelium. The postnatal chicken inner ear was selected for the following reasons. The chicken inner-ear organs are easily accessible and have been well studied in vivo, both developmentally and in terms of their regenerative properties. Some preliminary information is available regarding the effects of certain growth factors on the developing chicken inner ear (Represa et al., 1990, 1991), and a body of literature exists regarding the anatomical and physiological properties of the regenerating, postnatal, chicken inner ear. Finally, the vestibular sensory epithelia in the chicken strongly resembles that in the mammal, and like the mammal, more than one type of hair cell is present in the avian cochlea.

The first aim of this paper is to provide an overview of the culture system we developed and the methods we currently use. The effects of medium type, serum type and concentration, and culturing paradigm on the cultured inner-ear sensory epithelia were assessed, and the optimal culturing conditions were determined. We describe the morphology of mature hair cells that were maintained in vitro for 1 to 7 days, and show the genesis of hair cells and supporting cells in the organotypic cultures by assaying DNA synthesis (tritiated-thymidine incorporation). This work demonstrates both the division and differentiation of hair-cell progenitor cells in a primary organ culture system.

The second aim of this paper is to prove that the process of hair-cell regeneration, namely, the increase in supporting cell and hair-cell generation following insult, is preserved in our culture system. We quantify and compare numbers of mitotically active cells (cells which incorporate tritiated-thymidine) in control and experimental cultures and demonstrate that proliferative activity stimulated by in vivo ototoxic insult is maintained in the organotypic cultures.

Preliminary accounts of portions of this data have appeared (Oesterle et al., 1992a, 1993a).

Materials and Methods

Animals

Eight-to-eighteen day-old postnatal chickens (White leghorn; *Gallus domesticus*; H & N International, Redmond, WA) served as the experimental subjects. The animals were killed by decapitation, and the saccule, utricle, and cochlea (containing the basilar papilla and the lagenar macula) were dissected free from the head using sterile technique. Entire inner-ear end organs were cultured. Both normal and drug-damaged chickens were used; drug-damaged subjects were used for the up-regulation portion of the study only. Drug-damaged chickens received daily intramuscular injections of 1200 mg/kg body weight streptomycin sulfate (Sigma, St. Louis, MO) during a consecutive 5 day period prior to explantation of inner-ear structures. All experiments were approved by the University of Washington Animal Care Committee under PHS grant DC00395 and NS07097.

Culture reagents

Two culture media were tested, Dulbecco's Modified Eagle's Medium (DMEM-F12; Gibco, Grand Island, NY) and Basal Medium Eagle (BME; Gibco). The DMEM-F12 medium was supplemented with 6 mg/ml glucose (Sigma), 1.1 mg/ml sodium bicarbonate (Sigma), 1.2 mg/ml Hepes buffer (Sigma), 100 μg/ml transferrin (Sigma), 25 μg/ml insulin (Sigma), 60 μM putrescine (Sigma), 30 nM selenium (Sigma), 30 nM progesterone (Sigma), penicillin (Sigma), and serum. The BME medium was supplemented with Earl's Balanced Salt Solution (Gibco; 2 parts BME to 1 part EBSS), 5 mg/ml glucose (Gibco), and serum. Five serum types, namely, fetal bovine serum (Gibco), fetal clone (Hyclone Laboratories, Logan, UT), calf serum (Gibco), chicken serum (Gibco), and horse serum (Gibco), were tested at concentrations ranging from 0.1 to 25%. The effects of serum-free media on the cultures were also studied.

Culture paradigms

Three culturing techniques were examined: (i) free-floating cultures, (ii) roller-tube cultures; and (iii) clotted-well cultures. For the free-floating cultures, isolated end organs were placed free floating into wells of 24-well tissue-culture plates (Costar, Cambridge, MA) filled with 1.5 ml of culture medium. The culture well plates were placed on a slowly moving nutator (Clay Adams, Parsippany, NJ) and incubated at 37°C under an atmosphere of 5% CO_2 in air.

With the roller-tube culture paradigm (Gahwiler, 1981), approximately 50 μl of chicken plasma (Cocalico

Biologicals, Reamstown, PA), that was freshly reconstituted from a lyophilized state in sterile water, was placed on a sterile glass coverslip. An individual, isolated end organ was set into the center of each drop. Excess plasma was removed with a sterile 0.5 cc insulin syringe and replaced with approximately 25 μl of thrombin (Sigma). The plasma and thrombin were mixed well to form a clot. Coverslips and organs were stored in a petri dish at ambient conditions for a sufficient period to allow the plasma to coagulate; usually 3 min. Once coagulated, cultures were placed into screwtop culture tubes containing 1.5 ml of growth medium, and these tubes were placed in a roller drum rotating at 10 revolutions/h. The volume of media was sufficient enough to coat the explant in a film of liquid periodically. The drum was stored in an incubator warmed to 37°C.

Finally, a combination of the free-floating and roller-tube culturing paradigms, what we have denominated the 'clotted-well' culturing technique, was also tested. Individual, isolated end organs were clotted onto round glass coverslips via a plasma clot in a manner similar to that described above. Once coagulated, coverslips were placed into wells of a 24-well tissue culture plate filled with 1.5 ml of medium (one coverslip per well). The culture well plates were placed on a nutator and incubated at 37°C under an atmosphere of 5% CO_2.

Cultures were grown for 1 to 7 days, and all procedures for cell culturing were carried out under sterile conditions. Culture media was not exchanged during the culture period. Each organ was cultured individually (e.g., one organ per well or one organ per coverslip).

Cell proliferation assay

The proliferation of cells was assessed by continuous labelling with tritiated-thymidine incorporation as described by Reh and Kljavin (1989). Briefly, a dose of 1 μCi/ml of 6.7 Ci/mmol tritiated thymidine (Amersham, Arlington Heights, IL) was added to the medium in each well or tube at the start of the culture period. The tritiated-thymidine was not replenished during the remainder of the culture period. As described in detail below, standard autoradiographic techniques were used to detect those cells incorporating tritiated thymidine.

Histological methods

Light microscopy and autoradiography

At the end of the culture period, cultures were immersion fixed with 3.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C for 24 to 48 h. The tissue was washed in buffer (0.1 M Na/K phosphate buffer, pH 7.4) before being post-fixed in 1% osmium tetroxide in 0.1 M Na/K phosphate buffer for one hour at room temperature. The organs were washed with buffer, dehydrated in a graded ethanol series and propylene oxide, and embedded in Spurr's epoxy resin (Polysciences, Warrington, PA). Each organ was cut transversely on a Sorvall MT2 microtome. Serial semi-thin (2 or 3 μm) sections were cut through the organ, and every third section was collected and mounted on acid-washed, chrome-alum subbed slides. The slides were coated with liquid photographic emulsion (Kodak NTB-2) diluted 1:1 with distilled water and stored at 4°C in light-tight boxes for 2–4 days. Slides were developed in Kodak D19 developer and fixed in Kodak Rapid Fixer before lightly counterstaining with 0.01% Toluidine Blue and coverslipping. Autoradiographic results were viewed by light microscopy using conventional optics or Nomarski differential-interference-contrast optics.

Electron microscopy

In addition to analysis at the light microscopic level, six organotypic cultures were also processed for transmission electron microscopy (TEM). Ultrathin sections (90 nm) were cut from the Spurr-embedded tissue on a Leica Ultracut microtome. After placement onto 50/200 mesh grids, sections were stained with alcoholic uranyl acetate and lead citrate and examined with a Philips EM 410 transmission electron microscope.

To delineate the pattern and extent of the streptomycin-induced lesion, vestibular end organs (utricles and saccules) were taken from 6 animals and processed for scanning electron microscopy (SEM). Three of these animals were drug-damaged, and the remaining 3 were age-matched control animals. The animals were decapitated, and the vestibular end organs were dissected free and immersion fixed with 3.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). After being held in fixative at 4°C for 24 to 48 h, the organs were washed with buffer, dehydrated in 70% ethanol at 4°C, and underwent final dissection to remove the otoconia. Final dehydration was accomplished with a graded series of ethyl alcohols and drying in a Tousimis (Rockville, MA) Critical Point Dryer using carbon dioxide. Specimens were mounted on aluminum stubs, coated to 400 Å with gold palladium in an Anatech (Alexandria, VA) Sputter Coater, and examined with a JEOL 840A electron microscope.

Data analysis

Identification of cell type in cultured tissue

The cell types constituting the sensory receptor epithelium, i.e., hair cells and supporting cells, were easily identified and distinguished from one another in the cultured tissue. Hair cells were identified by their ultrastructural features, the presence of a cuticular

plate and stereocilia, and location; cells located at the lumenal surface which do not extend to the basal lamina. Supporting cells were identified by the location of the their nuclei, the absence of a cuticular plate and stereocilia, and their staining properties with Toluidine blue. The cytoplasm of the supporting cells in the vestibular sensory epithelium is stained darker (a darker blue) than that of the vestibular hair cells, whereas, the reverse pattern is observed in the auditory sensory epithelium; the supporting-cell cytoplasm is stained lighter than that of the hair cells.

Cellular proliferation

Autoradiographic sections 45 μm apart (1 in 5) were analyzed using standard light microscopy for the presence of labeled nuclei in the sensory epithelium. Cells were considered labeled when five or more silver grains overlay the cell nucleus. In general, background was sufficiently low that silver grains were not present over the nuclei of unlabeled cells. That is, the distinction between labeled cells and unlabeled cells was unambiguous.

The sensory epithelium was artificially bisected into basal (BL) and lumenal (LL) layers (Fig. 1). The BL consisted of all cell nuclei within two-to-three nuclear diameters (approximately 18 μm) of the basal lamina. The LL consisted of the remaining lumenal 1/2 of the sensory epithelium thickness. In cultured vestibular epithelia (taken from normal animals), the BL contains supporting-cell nuclei and a small percentage of hair-cell nuclei. Hair-cell nuclei and a small percentage of supporting-cell nuclei are found in the LL.

To quantify the amount of cellular proliferation, a Dage 68 video camera was attached to a Zeiss Universal photomicroscope, and images of the sections were displayed with a MacIntosh IIcx computer. Using a 63 $\times$ oil immersion lens and Image 1.45 (Research Services Branch, NIMH, Bethesda, MD), the number of fields covering the length of the organ's sensory epithelium was noted. A random number generator chose four 95 μm by 95 μm fields of sensory epithelium that were analyzed in each section. The number of labeled and unlabeled cells within the LL and BL of each field was determined by counting nuclear profiles. Counts obtained from the four fields were averaged. This allowed determination of the labeled-nucleus linear density (number of labeled nuclei per millimeter of sensory epithelium) for the BL and LL as well as the total sensory epithelium (TE). Values for individual sections were averaged to yield a mean density value for each organ culture. In addition, the percentage of label in the BL, LL, or TE was calculated with the following equation: % linear label = (density / total number of cells (labeled and unlabeled) per mm) $\times$ 100.

Conceivably, it is quite possible that the total number of cells per mm of sensory epithelium is different in control and drug-damaged animals. This difference would affect estimates of average linear density. This potential bias, on the other hand, is eliminated from the % linear density calculations. Consequently, % linear density values are more accurate than estimates of average linear density, but average linear density values are included in this paper to aid comparisons of this study with previous published reports (e.g., Weisleder and Rubel, 1992a, 1993; Tsue et al., 1993).

Statistics

Significance values were determined by using the analysis of variance (ANOVA) and Duncan post-hoc test or the Student's *t*-test.

Methods specific to up-regulation study

Ototoxic damage was induced by injecting chicks once a day for 5 consecutive days with streptomycin sulfate, at a dose of 1200 milligrams per kilogram body weight. Vestibular end organs were isolated from drug-damaged and normal, age-matched control chicks one day after the final drug injection. The organs were cultured for 2 days in the presence of tritiated thymidine. The following culture paradigms were utilized: (i) free-floating culture technique with BME medium supplemented with 10% fetal bovine serum (FBS), (ii) clotted-well culture technique with BME medium supplemented with 25% horse serum (HS), (iii) free-floating culture technique with BME medium supplemented with 10% calf serum (CSS); and (iv) free-floating culture technique with DMEM-F12 supplemented with 2% CSS. A minimum of 4 drug-damaged and 4 control organs were cultured for each experimental paradigm. Cultured organs were fixed, dehydrated, embedded, sectioned and processed for autoradiography as described earlier. Processed tissue was viewed with standard light microscopic techniques. The amount of cellular proliferation was qualitatively assessed for the cultured lagenar and saccular maculae and was quantitatively assessed for the cultured utricular maculae. The average labeled-nucleus linear density (number of labeled cells per mm) and % linear label was determined for each experimental paradigm. Values calculated for cultures of drug-damaged organs were compared to those for concordant control cultures to determine whether the regenerative process was maintained in vitro.

Results

General observations

Both light and electron micrographs of in situ vestibular sensory epithelium in the chicken inner ear

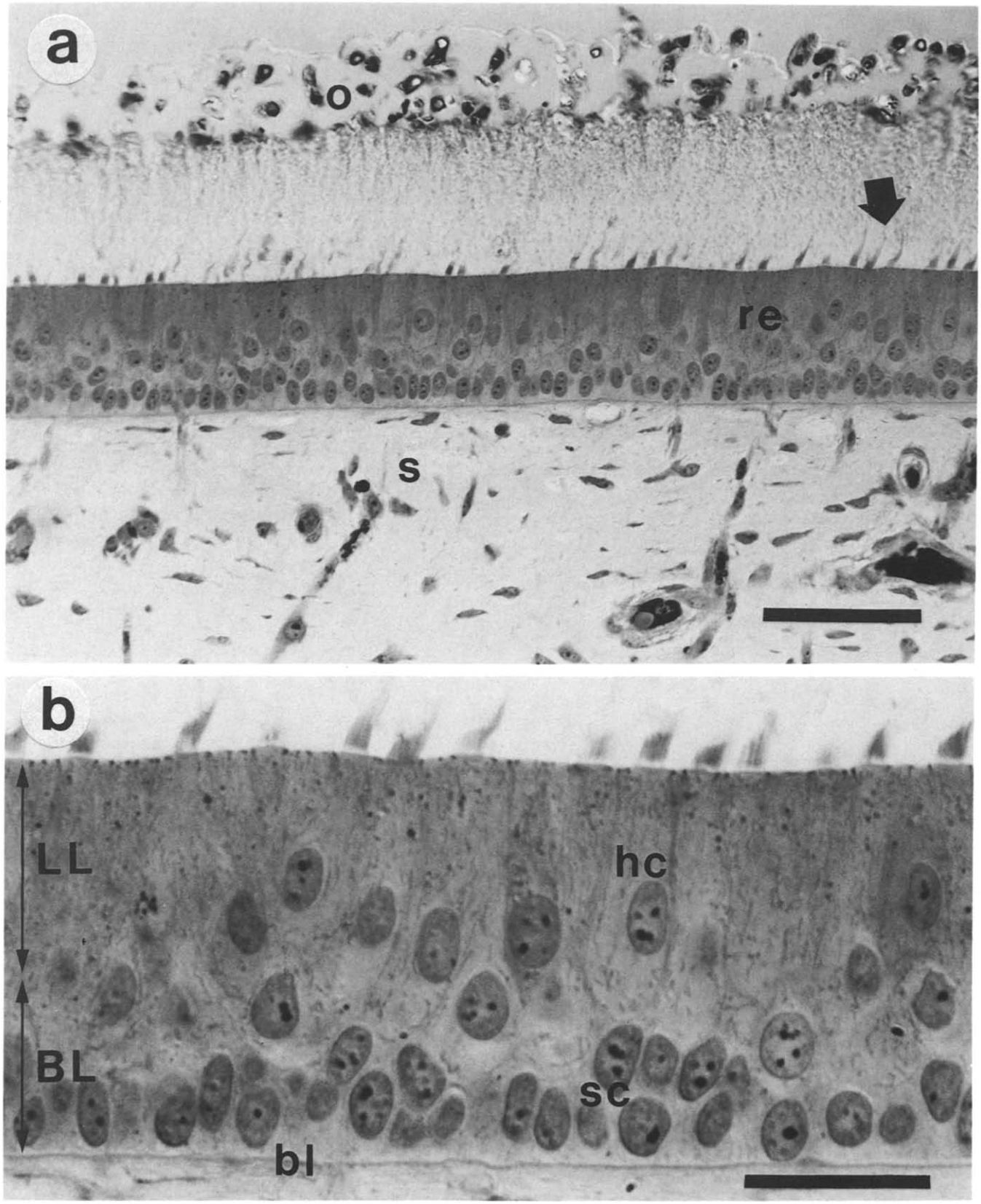


Fig. 1. Photomicrographs of a normal chicken utricle grown in culture for 2 days. a) Low magnification of the organ. b) Higher magnification of the region indicated by the arrow in (a). The cultured organ demonstrates morphology comparable to the in vivo state. In contrast to the in situ utricular maculae, large nerve calyxes do not envelope any hair-cell bodies in the cultured sensory epithelium. A free-floating culturing paradigm was used with the BME medium and 5% fetal bovine serum. Nuclear location within the sensory receptor epithelium is defined as being in the basal (BL) layer or lumenal layer (LL). s, stroma; o, otoconia; re, receptor epithelium; hc, hair cell; sc, supporting cell; bl, basal lamina. Unless noted otherwise, Nomarski differential-interference contrast optics was used to view the tissue shown in this paper at the light microscopic level. Scale bars (a) = 50 μm; (b) = 20 μm.

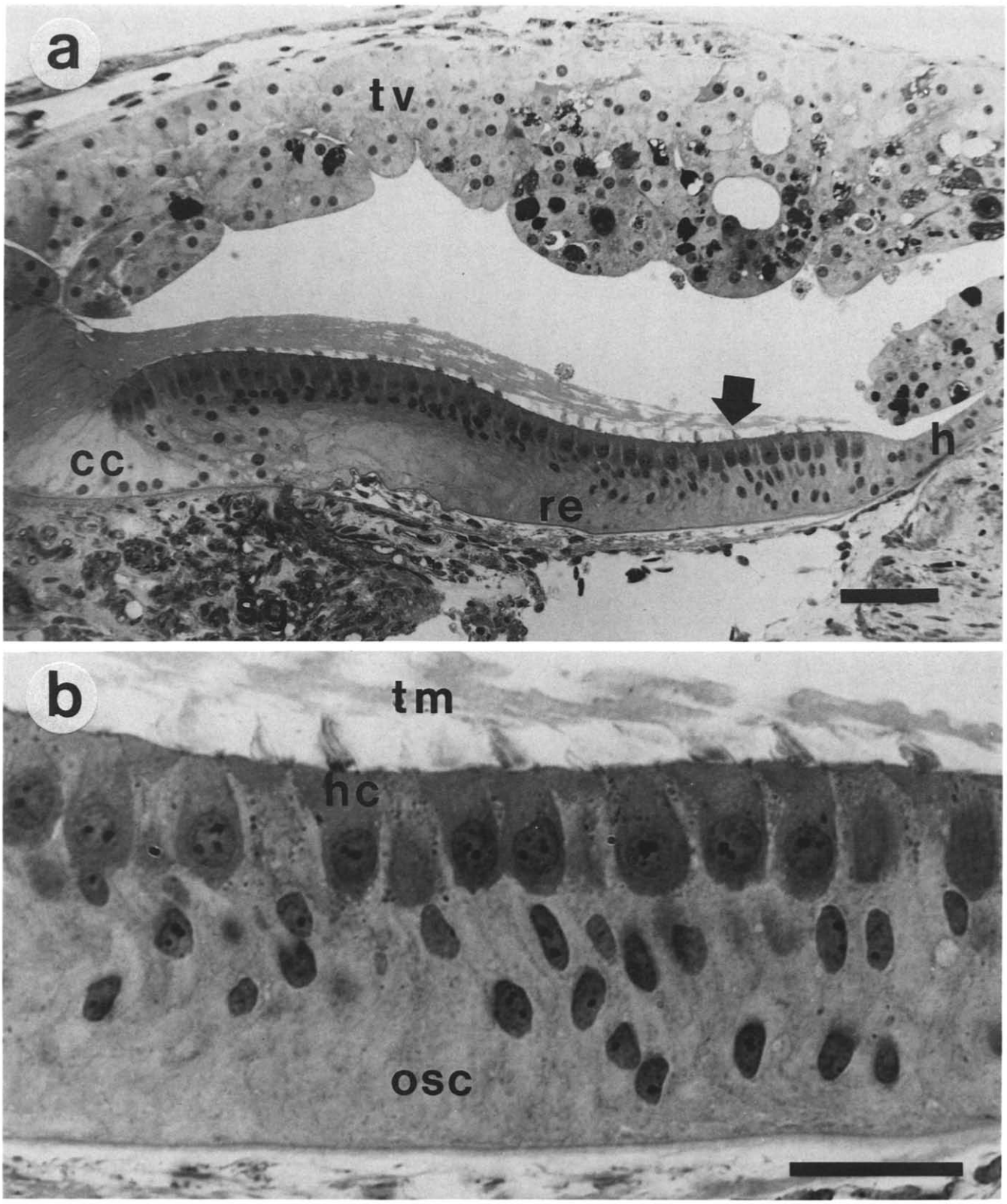


Fig. 2. Cross-sections from a cochlear duct that was grown in culture for 2 days. a) Low magnification of the organ. b) Higher magnification of the region indicated by the arrow in (a). Hair cells and supporting cells are well maintained in the cultured basilar papilla. In contrast, tegmentum vasculosum cells and spiral ganglion cells are poorly maintained. A roller culturing paradigm was used with the BME media and 5% calf serum. tv, tegmentum vasculosum; re, receptor epithelium; tm, tectorial membrane; hc, hair cell; osc, organ supporting cell; cc, clear cell; h, hyaline cell; sg, spiral ganglion. Scale bars (a) = 50 μm; (b) = 20 μm.

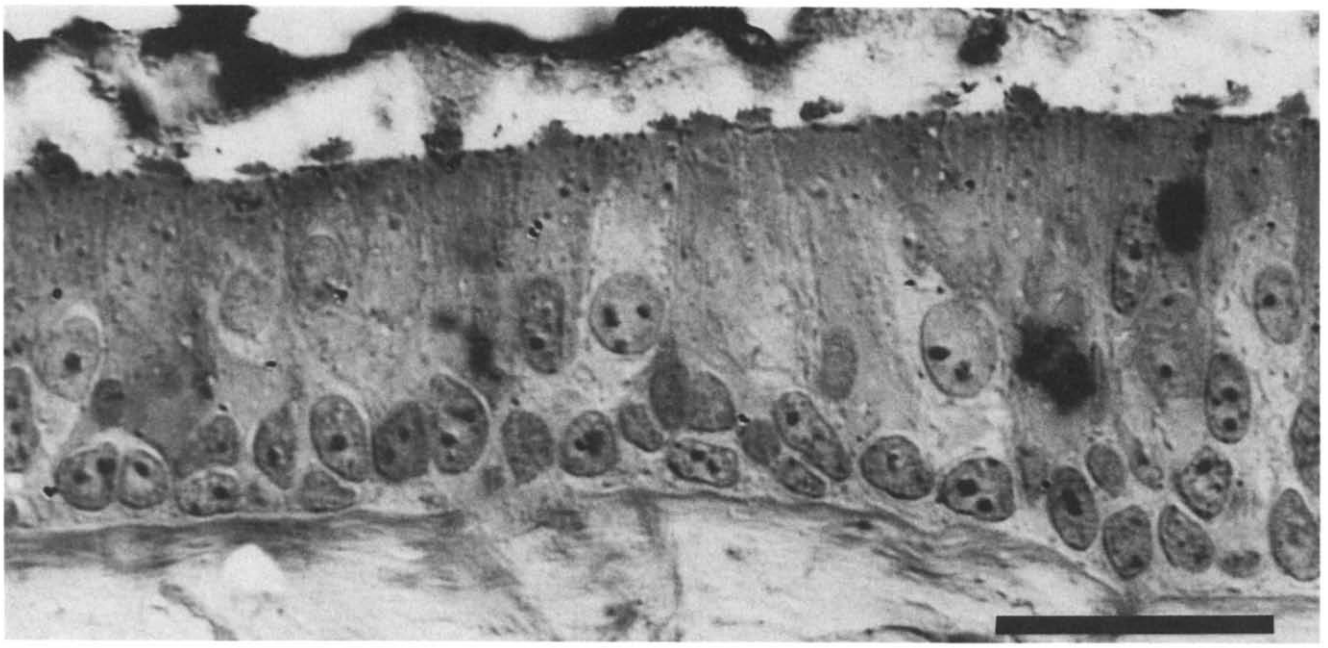


Fig. 3. Normal utricle grown in culture for 7 days. A clotted-well culturing paradigm was used with the BME medium and 5% fetal bovine serum. Scale bar = 20 μm.

have been published previously (Hirokawa, 1986; Weisleder, 1991; Weisleder and Rubel, 1993). Cultured inner-ear end organs demonstrate morphology comparable to the in vivo state. As illustrated by Figs. 1 and 2, the overall appearance of the in vivo end organ is retained in the organ cultures. A cross section from a utricle grown in culture for 2 days is shown in Fig. 1. Like the in situ utricle, otoconia lie above the otolithic membrane and sensory epithelium. The basal lamina separates the sensory epithelium from the underlying stroma. The characteristic cytoarchitecture and layered organization of the vestibular receptor epithelium is maintained in vitro, allowing easy identification of sensory and supporting cells. Although the distribution of nuclei in the cultured epithelia is more heterogeneous and less polarized into the classical basal support-cell layer and lumenal hair-cell layer than one sees in vivo, one to two rows of supporting-cell nuclei typically lie above the basal lamina in the lower half of the cultured sensory epithelium, and hair-cell nuclei are usually located in the upper half of the epithelium. Hair-cell nuclei have been observed occasionally just below the epithelium midline.

Vestibular hair cells are identified by their ultrastructural features (the presence of a cuticular plate, stereocilia, and kinocilia) and location (cells located at the lumenal surface which do not extend to the basal lamina). Fully differentiated vestibular hair cells taken from 8-to-18 day-old chickens and grown in culture for up to 7 days (Fig. 3) demonstrate morphology comparable to the in vivo state. Cultured vestibular hair cells are polarized having a central or basal nuclear region, an apical cuticular plate, and stereocilia and kinocilia extending from their lumenal ends (Fig. 4). Two types of hair cells, Type I and Type II hair cells, distinguished primarily by the morphology of their innervation are present in situ (Wersäll, 1956; Iurato, 1967; Hirokawa, 1986; Jørgensen and Cristensen, 1989). In culture, neural endings to the hair cells retract, consequently, innervation patterns can not be used to distinguish hair-cell type as is done in vivo. It is therefore difficult to ascertain whether both types of vestibular hair cells have been maintained in vitro. However, both club shaped and cylindrically shaped vestibular hair cells are present in the cultures.

A cross section from the chicken cochlear duct * grown in culture for 2 days is shown in Fig. 2 to illustrate that the overall appearance of the in situ auditory end organ is retained in the cultures. The basilar papilla, the auditory receptor epithelium, lies on the basilar membrane and is composed of hair cells and supporting cells. Clear cells and hyaline cells abut the superior and inferior edges of the basilar papilla, respectively. The basilar papilla is covered by a thick tectorial membrane which is attached to a wide band of nonsensory cells, the homogene cells, on the neural (superior) side of the duct.

* The avian cochlear duct contains the auditory sensory epithelium, the basilar papilla, which is the analog of the mammalian organ of Corti. The lagenar macula (vestibular sensory epithelium) is also housed in the apical end of the cochlear duct.

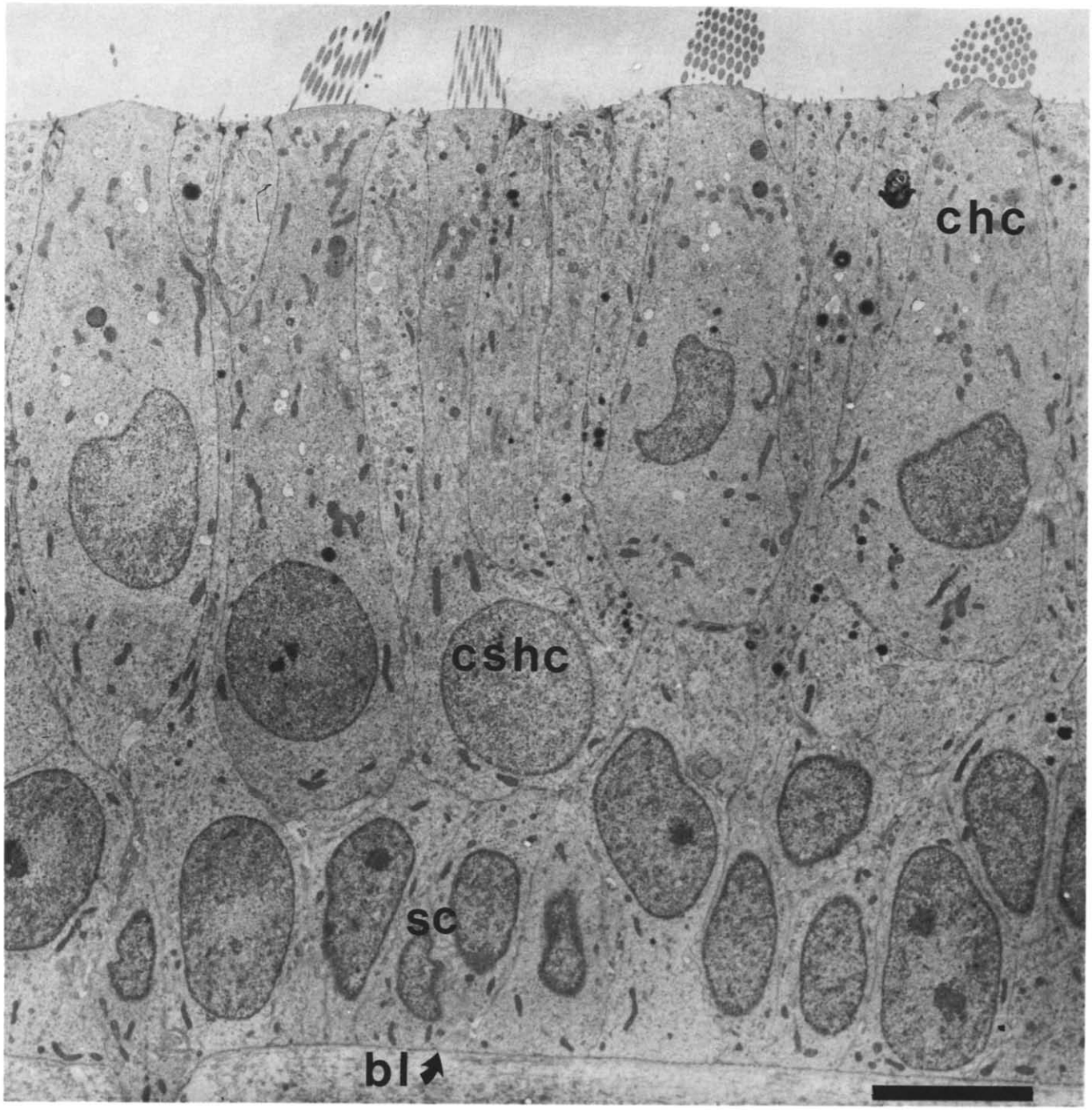


Fig. 4. Transmission electron micrograph of cultured vestibular sensory epithelium. This utricular macula was grown in culture for 2 days. Both club-shaped (cshc) and cylindrically shaped (chc) hair cells are present. Club-shaped hair cells are longer than cylindrically shaped hair cells. (For example, in this figure club-shaped hair cells are approximately 22 μm long whereas cylindrically shaped hair cells are roughly 18 μm long). In addition, the row of chc nuclei lies above the row of cshc nuclei in the epithelium. A free-floating culturing paradigm was used with the BME medium and 1% fetal bovine serum. sc, supporting cell; bl, basal lamina. Scale bar = 5 μm.

Like the vestibular sensory epithelium, the characteristic in situ cytoarchitecture of the auditory receptor epithelium is maintained in vitro. Specifically, hair-cell nuclei are located near the lumenal surface of the epithelium, above the nuclei of the organ supporting cells. Border cells (Oesterle et al., 1992b) are located at the inferior (abneural) edge of the basilar papilla and separate the sensory epithelium from the hyaline cells. Auditory hair cells are easily differentiated from supporting cells by their dark staining with toluidine blue, their lumenal location in the organ, and the presence of a cuticular plate and stereocilia. All types of basilar

papilla hair cells, tall, intermediate and short hair cells (Takasaka and Smith, 1971), are maintained in vitro, and they retain their polarized morphology. They have a central nuclear region, an apical cuticular plate, and stereocilia extend from their lumenal ends.

In general, the overall appearance of the cultured, inner-ear sensory epithelia strongly resembles that in situ. An important difference does exist, however, between the two conditions. Cultured hair cells, in contrast to in situ hair cells, are almost completely deinnervated, and cultured ganglion cells of the VIIIth nerve are drastically reduced in number. The deinnervation of hair cells in vitro is rapid. After 24 h in culture, nerve chalices surrounding Type I vestibular hair cells are undetectable at the light-microscope level. After 48 h in culture, qualitative TEM analysis of the vestibular and auditory sensory epithelia showed that afferent and efferent terminals on auditory and vestibular hair cells are rare, and those that are present show signs of degeneration. A small number of nerve fibers are present in the sensory epithelium proper (Fig. 5), and a few fibers still course to the hyaline-cell region adjacent to the basilar papilla (Fig. 6). Numerous spiral ganglion cells have died after 48 h in culture, and the ones that remain appear to be damaged. Pieces of the spiral ganglion are often retained with the isolated end organ, and in these cultures the condition of the spiral ganglion was assessed. After seven days of growth in culture, a few nerve fibers can be detected low in the organ, near the basilar membrane. Some fibers, especially those in the inferior (abneural) third of the organ, still course upwards towards the hair-cell region.

Cellular proliferation: General observations

Tritiated thymidine and autoradiographic techniques were used to identify proliferating cells and their progeny. Sections were examined for the presence of cells that had incorporated the nucleotide. In cultured vestibular sensory epithelia taken from normal postnatal chickens, occasional hair cells and supporting cells are labeled, indicating the replication of DNA in these cells during the culturing period. Labeled supporting cells are visible in all the cultures, whereas labeled vestibular hair cells with stereocilia are first detectable in 2 day-old cultures. To illustrate, a utricle taken from a normal postnatal chicken and grown for 24 h in the presence of tritiated thymidine is shown in Fig. 7. Two nuclei are labeled by the tritiated thymidine; a nucleus in the row of supporting-cell nuclei adjacent to the basal lamina is heavily labeled, and nearby, a nucleus in the lumenal one-half of the epithelium, in the region of the hair-cell nuclei, is also labeled. Several labeled vestibular hair cells with stereocilia are shown in Fig. 8. These utricular maculae

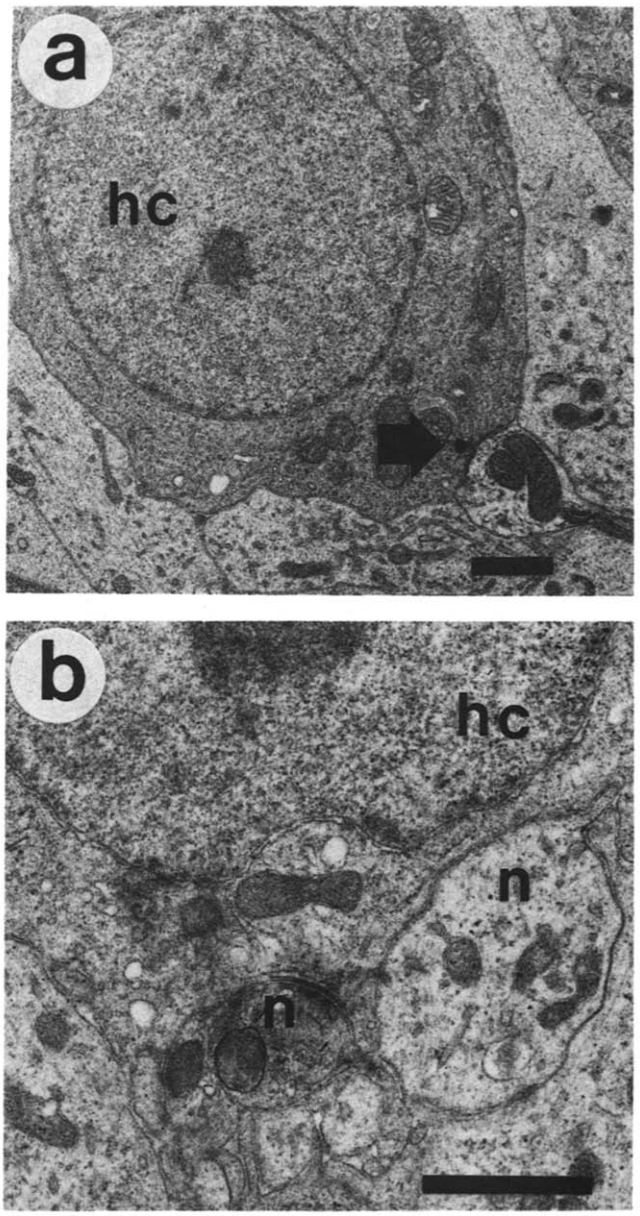


Fig. 5. Transmission electron micrographs of neural elements in inner-ear sensory epithelia grown in culture for 2 days. a) Neural element next to an auditory hair cell in cultured basilar papilla. A roller culturing paradigm was used with the BME medium, and 2.5% calf serum was used. b) Neural element next to a vestibular hair cell in the utricular macula. A free-floating culturing paradigm was used with the BME medium and 5% fetal bovine serum. Scale bars = 1 μm.

were taken from normal postnatal chickens and grown in culture for 2 days in the presence of tritiated thymidine. Importantly, the presence of labeled hair cells in the cultured sensory epithelium demonstrates the capacity of this primary culture system to support both the division and differentiation of hair-cell progenitors cells.

Labeled hair cells and supporting cells are present throughout the cultured vestibular sensory epithelium,

not just at the edges. A topographical predisposition to the peripheral or central zones of the macula was not observed in the vestibular end-organ types studied, the utricle, saccule, or lagena. Labeled cells occurred singly and in clusters throughout the sensory epithelium. Some cells outside the macula were also labeled by the tritiated thymidine. For example, label was observed in nonsensory cells adjacent to the receptor epithelium, in stromal cells, in capillary endothelial cells, in glial cells amongst the neural elements, and in fibroblast-like cells which form the outer boundary of the vestibular end organ.

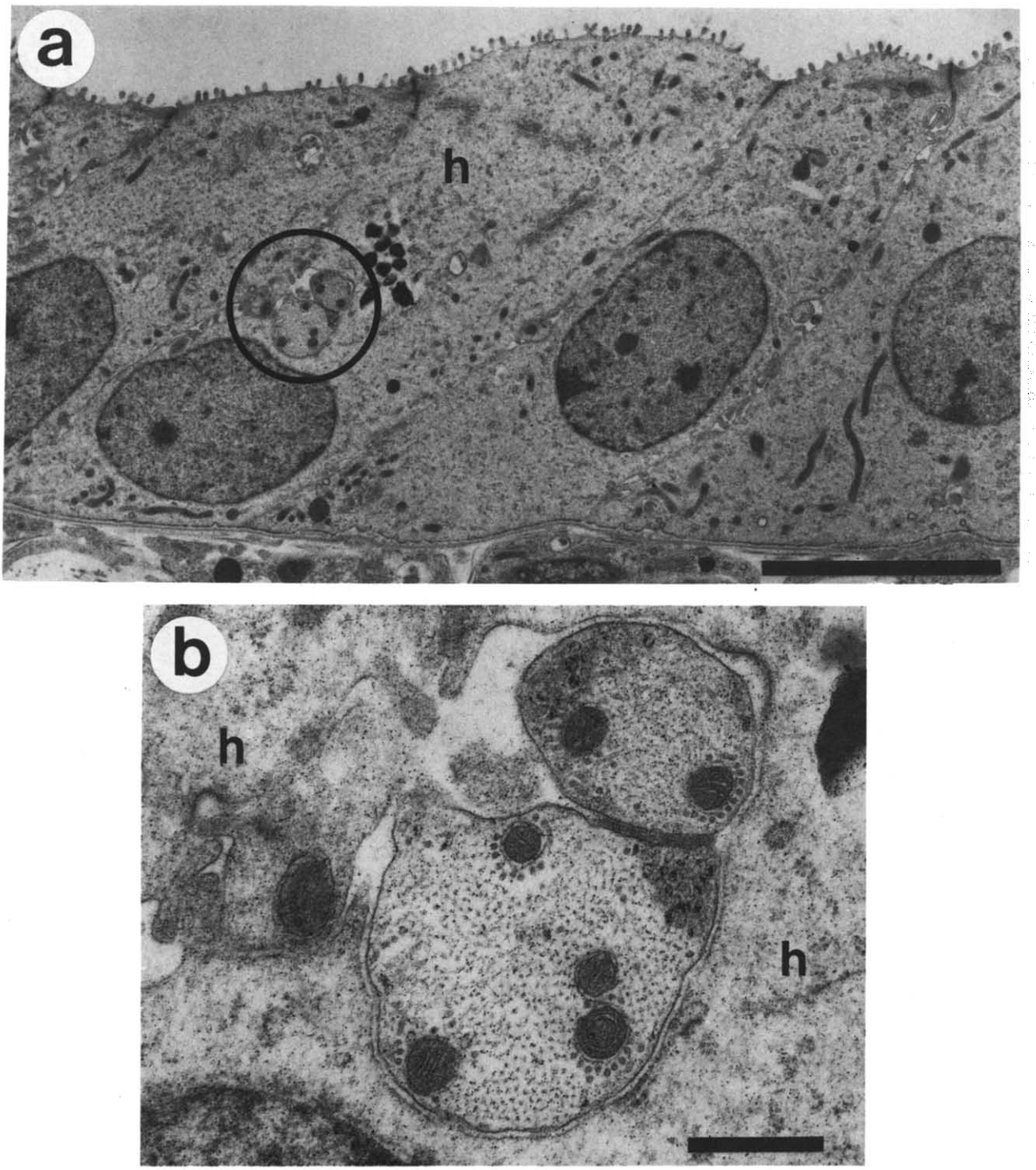


Fig. 6. Transmission electron micrographs of neural elements in inner-ear sensory epithelia grown in culture for 2 days. a) Low magnification of the hyaline-cell region in the cultured cochlear duct. b) Higher magnification of the neural elements shown in the circled region in (a). h, hyaline cell. A clotted-well culturing paradigm was used with the BME medium and 25% horse serum. Scale bars (a) = 5 μm; (b) = 0.5 μm.

Some auditory epithelial cells are labeled in cochlear ducts that were taken from normal postnatal chickens and grown in culture. In general, the number of labeled cells in the auditory sensory epithelia is markedly decreased relative to that seen in concordant vestibular sensory epithelia identically cultured. To illustrate, the number of labeled sensory epithelial cells per section was determined for the auditory and vestibular sensory epithelia housed in two identically cultured cochlear ducts. A free-floating culturing technique with a serum-free BME medium was used, and the cochlear ducts were grown in culture for 2 days. Regarding the auditory epithelia, 15 and 13 labeled cells were counted in 44 and 63 sections of two basilar papilla cultures, respectively, and the mean number of labeled cells per section of basilar papilla was 0.28 (range = 0 to 3). In contrast, 45 and 44 labeled cells were observed in 36 sections from each of two lagenar macula cultures, and the mean number of labeled cells per section of lagenar macula was 1.2 (range = 0 to 7).

Labeled basilar papilla supporting cells are first detectable in the 2 day cultures (Fig. 9), and labeled hair cells are seen occasionally with longer culture periods. The labeled cells are dispersed throughout the basilar papilla and do not appear to be preferentially localized to any particular region. In addition to labeled basilar-papilla cells, a number of cell types outside the sensory epithelium are also labeled. Tritiated-thymidine incorporation was found in the following cell types in the cochlear-duct cultures: clear cells, homogene cells, vacuole cells, hyaline cells, basilar membrane cells, tympanic border cells, capillary endothelial cells, cells of the superior and inferior cartilaginous plates, glial cells amongst spiral ganglion cells and nerve fibers, tegmentum vasculosum cells, and cells lining the scala tympani space.

Optimal culture conditions

To determine the optimal conditions for culturing postnatal inner-ear end organs, the effects of two types of medium, five types of serum (at a variety of concentrations), and three different culturing techniques were assessed. A minimum of two organs were examined for each experimental paradigm. The results were evaluated at the light microscope level by comparing the following items: (i) number of hair cells present, (ii) extent of intracellular vacuolization in hair cells and supporting cells, (iii) size and shape of hair cell and supporting-cell nuclei, (iv) amount of abnormal intracellular inclusions in hair and supporting cells, (v) size of the extracellular spaces in the sensory epithelium; and (vi) amount of cellular proliferation within the sensory epithelium. Decreased numbers of hair cells, numerous intracellular vacuoles, irregular and/or shrunken nuclear profiles, numerous darkly staining inclusions (with a Toluidine blue stain), large extracellular spaces, and large numbers of ^{3}H-thymidine labeled cells were considered abnormal. Some of the results are illustrated in Table I. Those six culture paradigms eliciting the best results were then also qualitatively assessed at the TEM level.

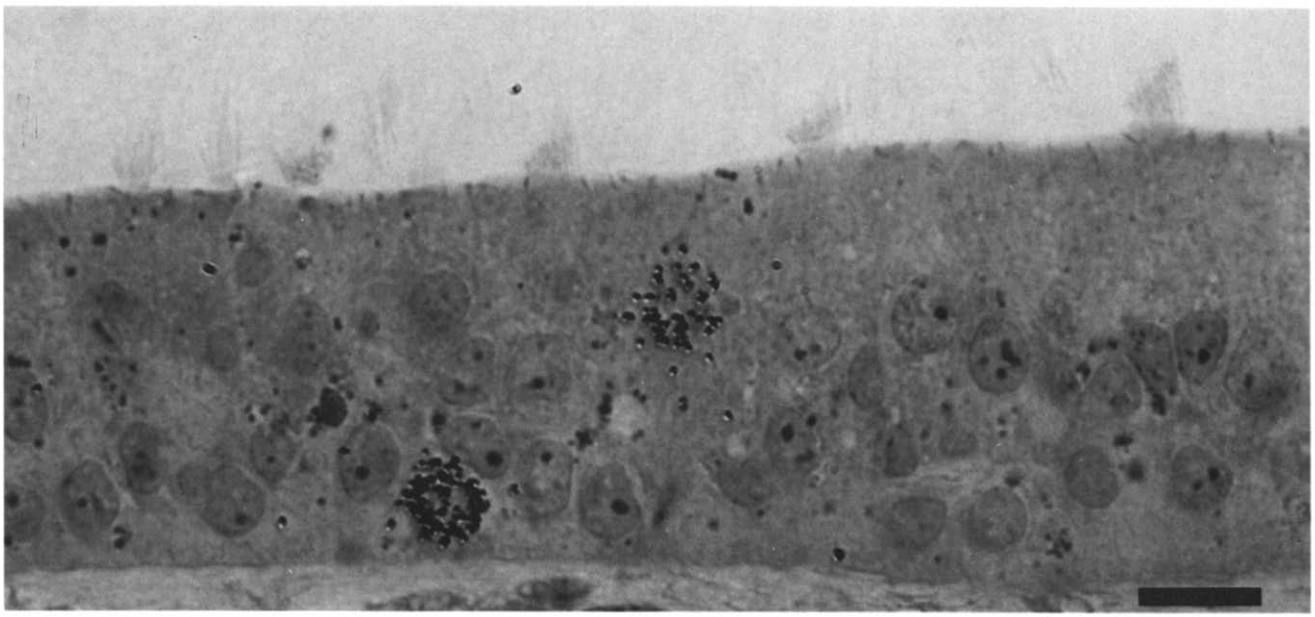


Fig. 7. Tritiated-thymidine autoradiographic labeling of proliferating cells in utricular sensory epithelium cultured for 24 h. The criteria for a labeled cell was 5 or more silver grains overlying the cell nucleus. A free-floating culturing paradigm was used with the DMEM-F12 medium and 2% calf serum. Scale bar = 10 μm.

Optimal conditions for culturing the vestibular sensory epithelium were found to be the following: a free-floating or clotted-well culturing paradigm utilizing the BME medium and supplemented with 5 or 10% fetal bovine serum. The best auditory sensory epithelium cultures were obtained when the free-floating or clotted-well culturing paradigm was utilized with the BME medium and 5% calf serum or 25% horse serum.

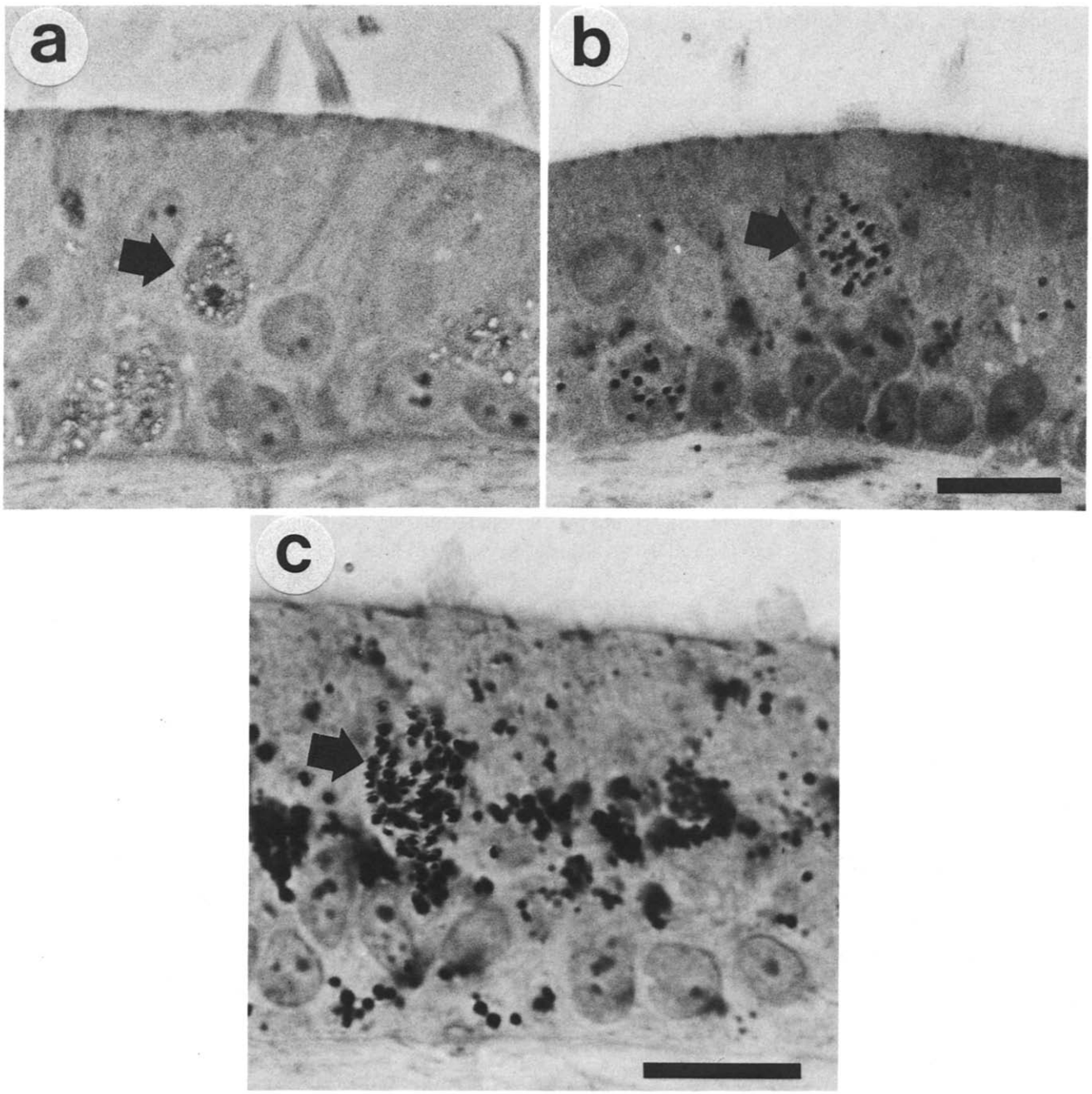


Fig. 8. Tritiated-thymidine labeled hair cells with immature (b and c) and more mature ciliary tufts (a) within utricular maculae grown in culture for 2 days in the presence of tritiated thymidine. The presence of labeled hair cells in the cultures demonstrates the ability of the culturing paradigm to support the production and differentiation of some new cells into hair cells. New hair cells and supporting cells are born in our cultures, in suboptimal (c) and optimal culturing conditions (a and b). The utricles in (a) and (b) were cultured with BME medium supplemented with 5% FBS, and a free-floating culturing paradigm was used. The utricle shown in (c) was cultured with suboptimal culturing conditions (free-floating culturing paradigm with BME medium and 25% horse serum) as evidenced by the large number of dark inclusions within the cell cytoplasms. Phase contrast and brightfield light microscopy were used in (a) and (b), respectively. Thus, in (a) grains appear as white dots over the nucleus. Scale bars = 10 μm. The magnification is identical in (a) and (b).

TABLE I

Some culturing condition results for the vestibular sensory epithelium

(All values in columns **i** to **vi** are averages [1])

Medium	Serum	Culture techniques	% Serum	i	ii	iii	iv	v	vi	Paradigm average
BME	FBS	free	0.1	4.5	5	4	4	5	5	4.6
BME	FBS	free	5	5	5	5	5	5	5	5.0
BME	FBS	free	20	4	4	5	4	5	5	4.5
DMEM-F12	FBS	free	0.1	4	2	3.5	4	3	5	3.6
DMEM-F12	FBS	free	5	4	2	3	5	3	5	3.7
DMEM-F12	FBS	free	20	3.5	2.5	3	4.5	4	5	3.8

[1] A minimum of 2 organs were examined for each experimental paradigm. For each organ culture, items **i** through **vi** were scored individually on a scale of 1 to 5, 1 being considered very poor and 5 very good. The individual values were averaged, and the mean value is included in the table. A mean value, the paradigm average, was determined for each experimental condition. i = number of hair cells present; ii = extent of intracellular vacuolization in hair cells and supporting cells; iii = size and shape of hair cell and supporting-cell nuclei; iv = amount of abnormal intracellular inclusions in hair and supporting cells; v = size of the extracellular spaces in the sensory epithelium; vi = amount of cellular proliferation within the sensory epithelium.

Below, the effects of the two types of medium, five types of serum, and three culturing techniques on the cultures will be discussed in more depth.

Culture medium

Two culture media, DMEM-F12 and BME, were tried, and the effects on the cultures are illustrated in Fig. 10. Shown here are sections taken from two utricles that were cultured identically except that different media were used. Note the greater number of intracellular vacuoles in both hair cells and supporting cells with the DMEM-F12 medium (Fig. 10a). Tritiated-thymidine-labeled hair cells and supporting cells were present in the cultures when either the DMEM-F12 or BME medium was used. Overall, the best results were obtained with the BME medium, hence the BME medium was used for the further development of a method to grow inner-ear organ cultures.

Serum type and concentration

Five types of sera, fetal bovine serum (FBS), fetal clone serum (FC), calf serum (CSS), chicken serum (CS), and horse serum (HS) were tested at concentra-

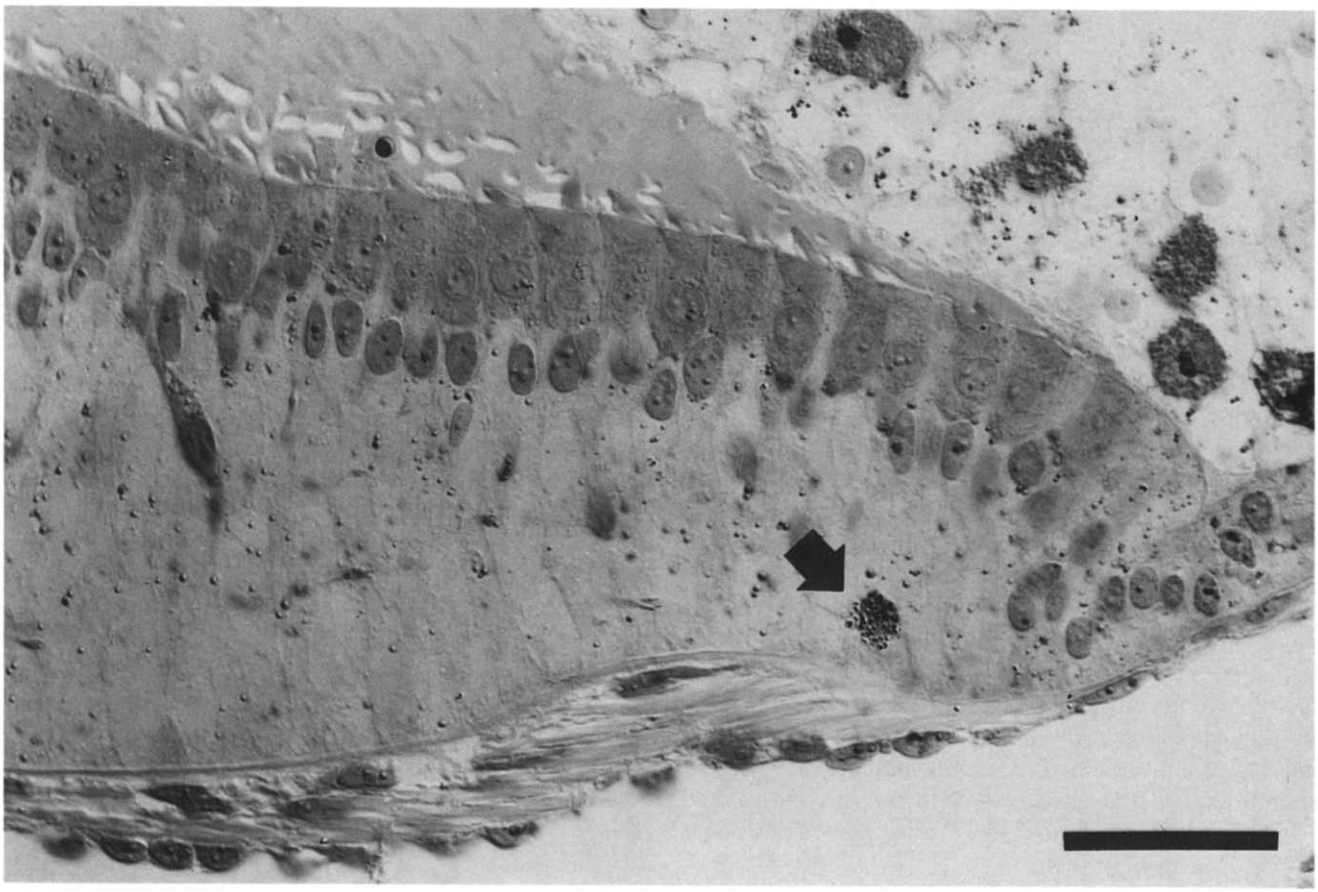


Fig. 9. Tritiated-thymidine labeled supporting cell (arrow) within a basilar papilla taken from a normal postnatal chicken and grown in culture for 2 days in the presence of tritiated thymidine. A clotted-well culturing paradigm was used with BME medium and 25% horse serum. Scale bar = 30 μm.

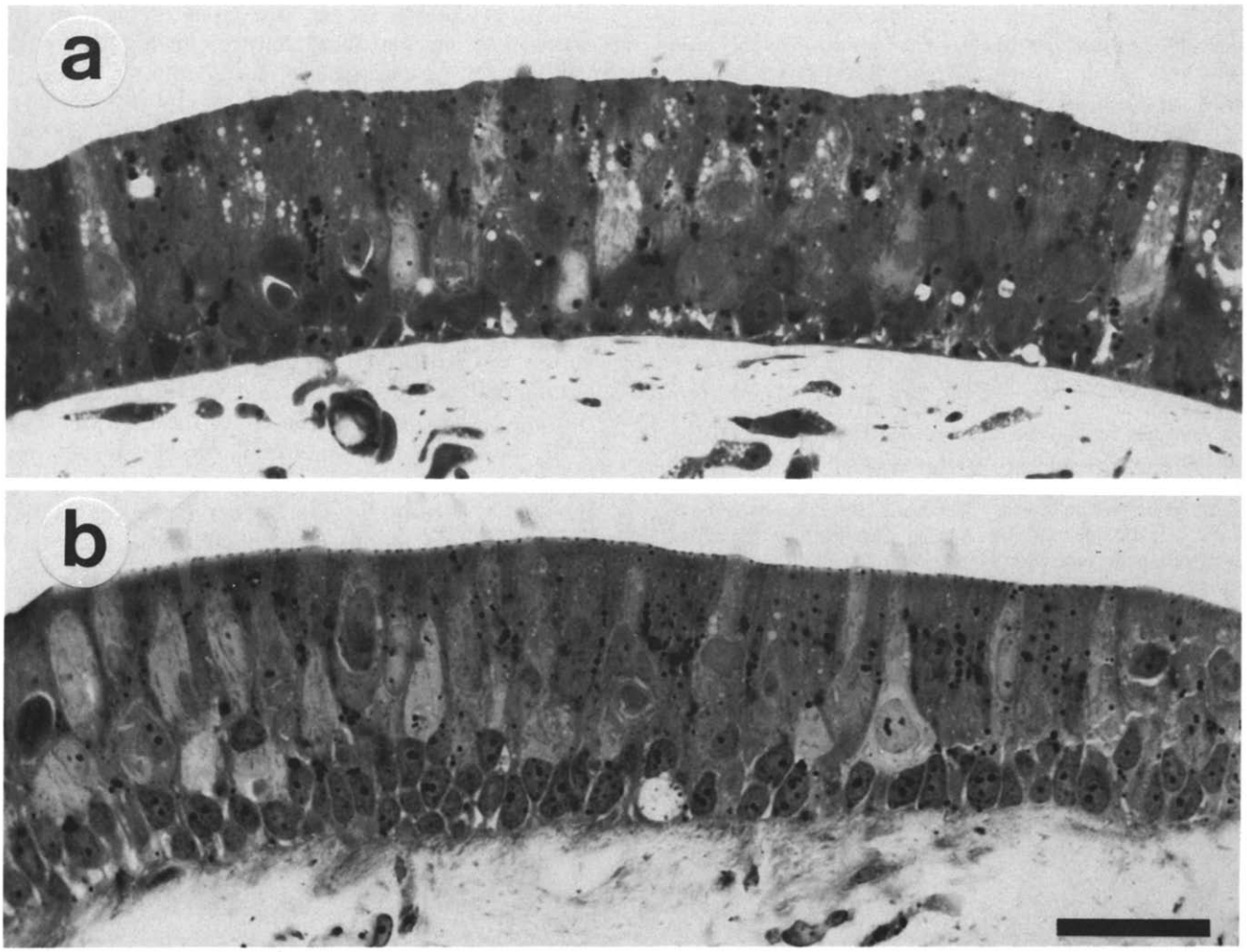
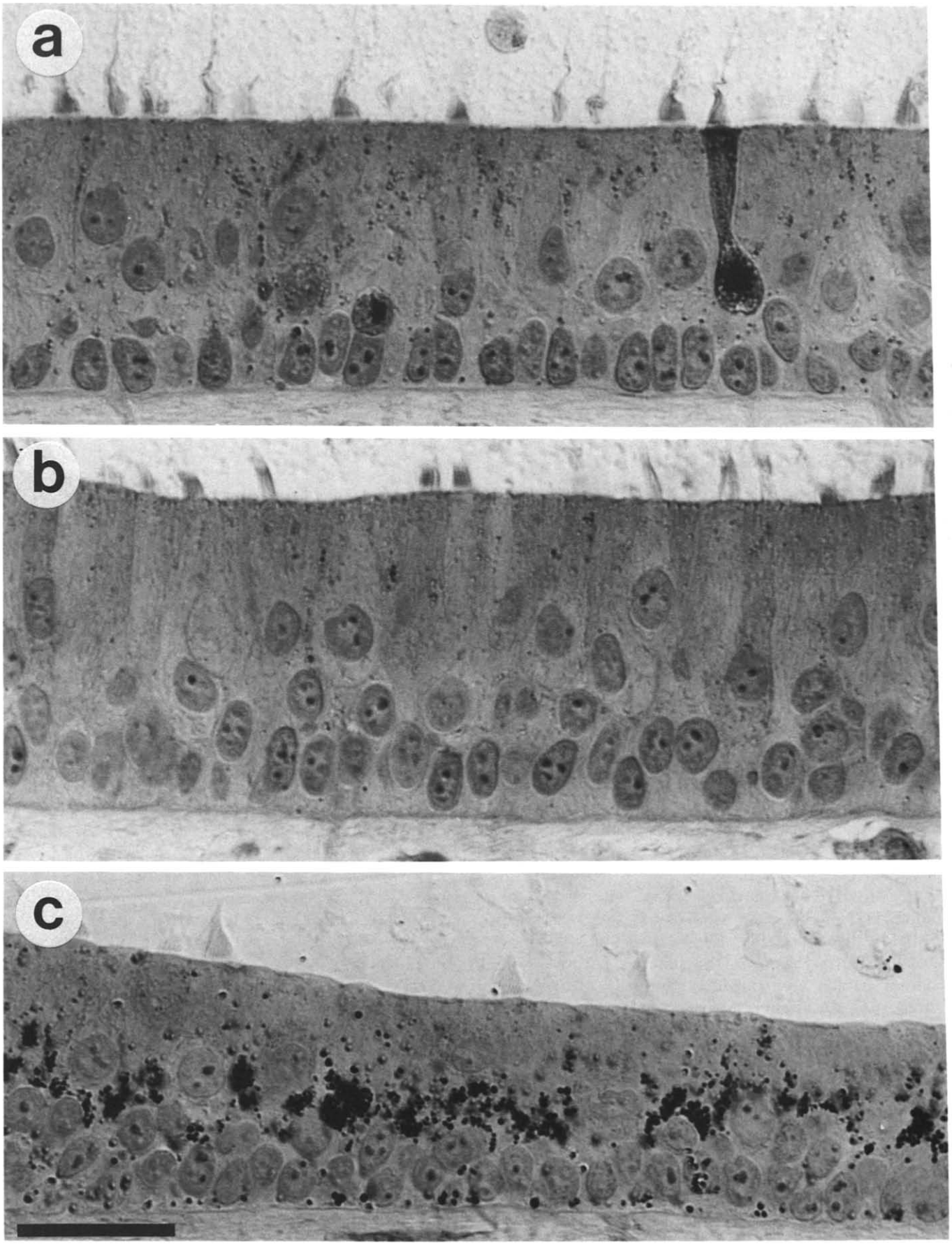


Fig. 10. Photomicrographs of normal postnatal chicken utricles cultured identically except that different media were used. a) Utricle cultured with DMEM-F12 medium, and b) Utricle cultured with BME medium. A free-floating culturing paradigm was used with 5% FBS. The organs were grown in culture for 2 days. Scale bar = 20 μm (**a** and **b**).

tions ranging from 0.1 to 25%, and the effects on the conditions of the primary cultures were assessed. Effects of serum concentration are illustrated in Fig. 11. As shown in Fig. 11c, abnormally large numbers of darkly stained inclusions are seen in the sensory epithelium when high serum concentrations, e.g., 20 and 25%, are used, and hair-cell numbers have declined substantially. Serum levels below 5%, on the other hand, can maintain near normal numbers of hair cells (e.g., Fig. 11a), but irregularly shaped supporting-cell nuclei become more numerous in the epithelium, and the number of intracellular vacuoles in supporting cells and hair cells increases. Greater numbers of darkly stained hair cells are also seen with the very low serum concentrations (Fig. 11a). The best results were obtained with FBS concentrations of 5 or 10%.

The effects of serum concentration on cellular proliferation within the sensory epithelium were also assessed. Specifically, two concentrations of FBS, 0.1 and 10%, were studied to determine whether serum concentration affected cellular proliferation. The results are shown in Fig. 12. This graph shows the mean labeled-nucleus linear density in the BL, LL, and TE for 12 normal utricles grown for two days (free-floating culture technique) in BME medium supplemented with 0.1 or 10% FBS. The overlapping values indicate that the amount of cellular proliferation did not vary significantly between these two conditions.

Fig. 11. Effects of various concentrations of FBS on cellular morphology in the vestibular sensory epithelium. Utricles were cultured identically for 2 days (free-floating culturing paradigm with the BME medium) except that different concentrations of FBS were used. a) 0.1% FBS, b) 5% FBS, and c) 25% FBS. The darkly stained cell in (a) is a dying or apoptotic hair cell. Scale bar = 20 μm (a–c).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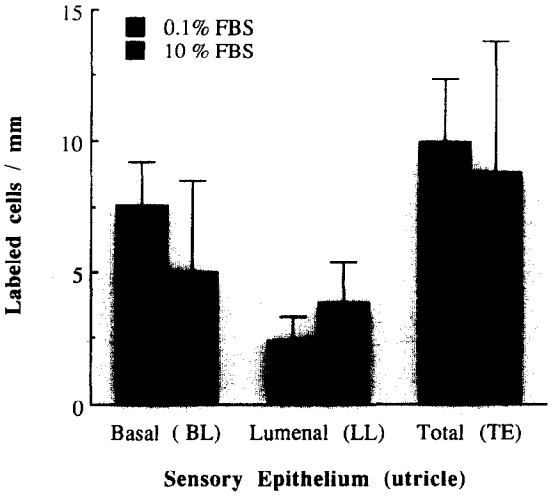


Fig. 12. Effect of FBS concentration on cellular proliferation in the utricular macula. Twelve normal utricles were grown in culture for 2 days; six were grown with 0.1% FBS and six were grown with 10% FBS. Mean tritiated-thymidine labeled nucleus linear density in the basal (BL) and lumenal (LL) sensory epithelial layers and the total sensory epithelium (TE) are shown. A free-floating culturing paradigm was used with the BME medium. Error bars represent SEM.

Serum-free cultures

Two utricles and two cochlear ducts were cultured in serum-free media, and the effects on the auditory and vestibular sensory epithelia were assessed. Auditory and vestibular hair cells are maintained in inner-ear sensory epithelium grown in culture with BME media only, without the addition of any serum supplement (Fig. 13).

Interestingly, tritiated-thymidine labeled supporting cells and hair cells are found in serum-free cultures of vestibular sensory epithelium (Fig. 13a). Cells outside the vestibular sensory epithelium are also labeled by tritiated thymidine. For example, some nonsensory cells immediately adjacent to the receptor epithelium, stromal cells, and glial cells amongst eighth-nerve fibers are labeled.

In serum-free cultures of normal auditory epithelium, organ supporting cells occasionally label with tritiated thymidine (Fig. 13b). Supporting cells above the habenular region, in the superior portion of the basilar papilla are labeled occasionally as are some supporting cells in the middle and inferior regions of the papilla. Labeled auditory hair cells, in contrast, were not observed. Other cell types in the cochlear duct labeled by tritiated thymidine in the serum-free cultures include the following: homogene cells, clear cells, basilar membrane cells, tympanic border cells, glial cells among the neural elements, cells in the tegmentum vaculosum, capillary endothelial cells and fibroblast-like cells lining the outer edge of the cochlear duct.

Culturing method

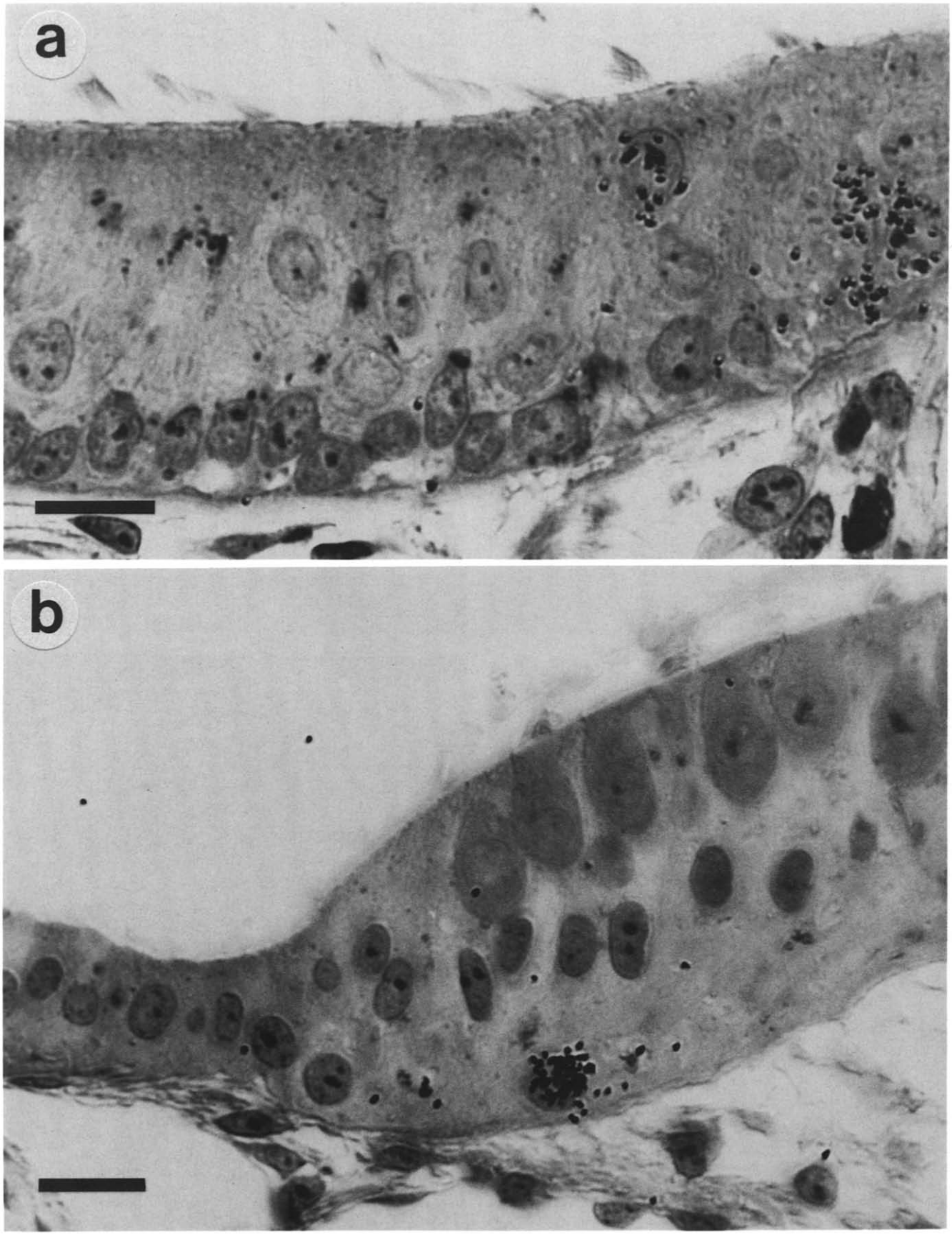
Three culturing techniques were tested, free floating, roller tube, and clotted-well culturing techniques. For short culture periods, 2 days or less, the free-floating paradigm consistently provided the best results, whereas clotted-well cultures were optimal for periods exceeding 2 days. Free-floating end organs, especially the larger organs such as the cochlear duct, tend to curl after two days in culture and become spherical in shape. Consequently, locational cues, such as the location of the high versus middle frequency region of the basilar papilla, become difficult to assess. In contrast, near normal end-organ shape and locational information is retained in clotted-well and roller-tube cultures. However, variability amongst identically cultured organs is increased for clotted-well and roller-tube cultures over that for free-floating cultures.

Up-regulation of proliferation following in vivo aminoglycoside ototoxicity

Fig. 14c–d shows the extent of the ototoxic lesion induced in vivo by the 5 day series of streptomycin injections: For purposes of comparison, a utricle from a normal, untreated animal is shown in panels (a) and (b). Utricles shown in Fig. 14 were not grown in culture. Instead, after decapitation, the inner ears were fixed, dissected free from the head and prepared for SEM. The surface of the normal in situ utricular macula is covered by stereocilia which belong to the underlying hair cells (Fig. 14a–b). After five consecutive days of streptomycin sulfate injections, at a dosage of 1200 mg/kg (IM), a large sickle-shaped region is present which is largely devoid of stereocilia (Fig. 14c). In the midst of this damaged region, regenerating hair cells can be observed (Fig. 14d). A similar pattern of damage was observed in streptomycin-damaged saccular maculae, but a larger percentage of the macula was damaged.

To determine whether the regenerative process could be maintained in vitro, vestibular end organs were dissected free from drug damaged and untreated, age-matched control chickens and grown in culture. Four culture paradigms were utilized: (i) free-floating

Fig. 13. Photomicrographs showing inner-ear sensory epithelia grown for 2 days in serum-free medium. a) Vestibular sensory epithelium. Note the presence of a tritiated-thymidine labeled cell in the LL of the utricular macula and several labeled supporting cells in the BL. b) Auditory sensory receptor epithelium and adjacent hyaline-cell region. Note the tritiated-thymidine labeled organ supporting cell. A free-floating culturing paradigm was used with the BME medium. Scale bars = 10 μm.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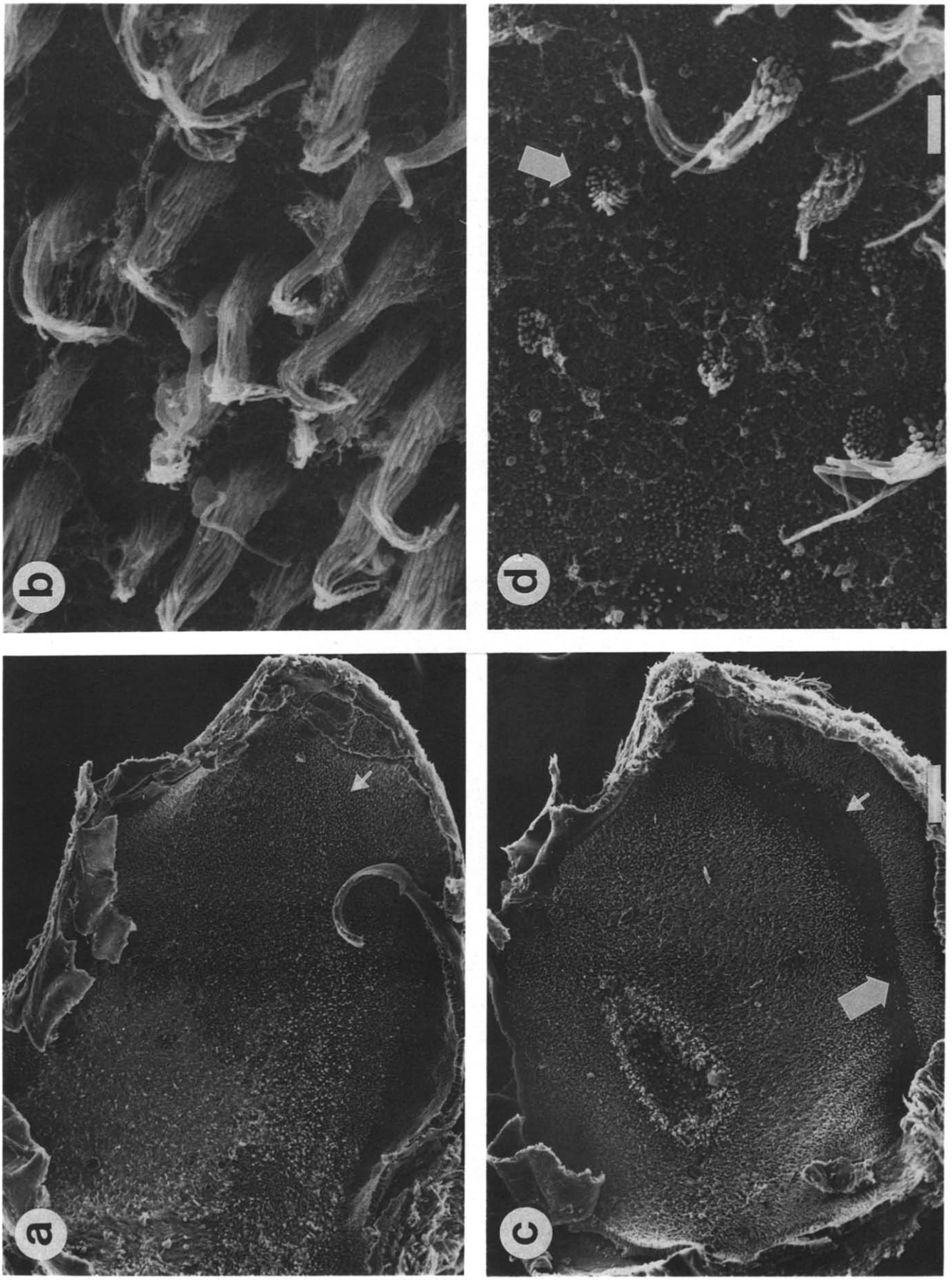
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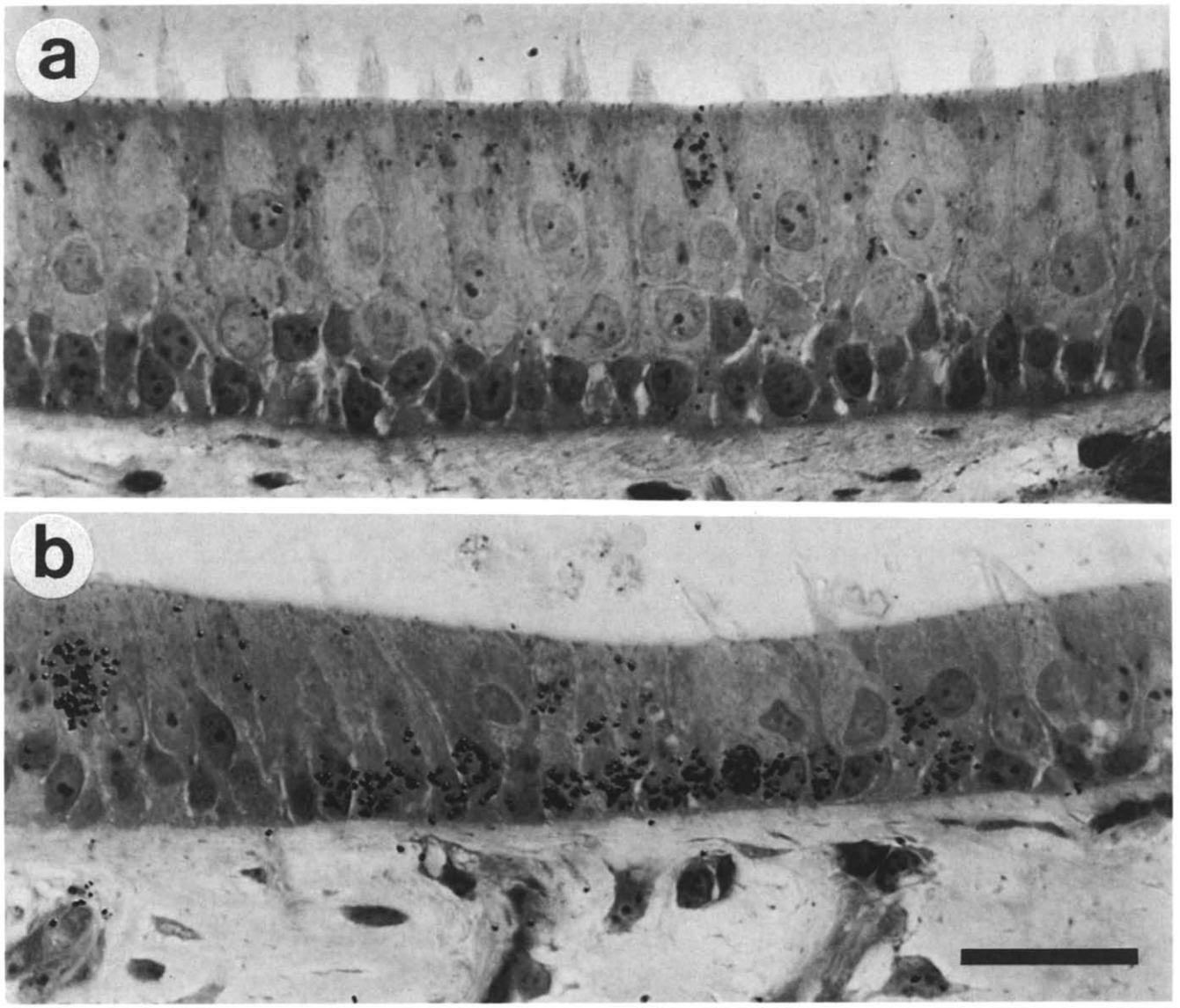

Fig. 15. Light micrographs of a) normal and b) streptomycin-damaged utricular sensory epithelium grown in culture for 2 days. To determine whether the regenerative process could be maintained in vitro, vestibular end organs were dissected free from streptomycin damaged and untreated, age-matched control chickens and grown in culture. Note the markedly greater number of tritiated-thymidine labeled cells in the explant of the drug-damaged tissue compared to the control tissue. A free-floating culturing paradigm was used with the BME medium and 10% fetal bovine serum. Scale bar = 20 μm (a and b).

culture technique with the BME medium supplemented with 10% FBS, (ii) clotted-well culture technique with the BME medium supplemented with 25% HS, (iii) free-floating culture technique with the BME medium supplemented with 10% CSS; and (iv) free-floating culture technique with the DMEM-F12 medium supplemented with 2% CSS. A minimum of 4 drug damaged and 4 normal utricles were cultured for each experimental paradigm.

Two cultured utricles are shown in Fig. 15. The

Fig. 14. Scanning electron micrographs of in situ utricles. a) Normal utricle from an untreated control animal. b) A higher magnification of the region of (a) indicated by the small arrow. c) Drug-damaged utricle showing the extent of the streptomycin lesion induced in vivo. After 5 days of streptomycin sulfate injections, a large sickle-shaped region is present which is largely devoid of stereocilia (large arrow). The position and shape of the damaged area suggests that this region is the striola. Recent papers on the fish (oscar: Yan et al., 1991; Lombarte et al., 1993) showed that aminoglycosides extensively damage the striola region. Drug-damaged hair cells are found throughout the entire sensory epithelium as well (Weisleder, 1991). The chicken was allowed to survive one day after the last drug injection. d) A higher magnification of the denuded region of (c) indicated by the small arrow. Note the regenerating hair cells (large arrow) in the damaged region. Bars indicate 100 μm in (a) and (c) and 2 μm in (b) and (d).

utricle shown in the top of the figure was taken from an untreated animal, and the utricle shown on the bottom was taken from a streptomycin-treated animal. They were cultured identically for two days in BME supplemented with 10% FBS. Note the orderly arrangement of the nuclear layers in the undamaged utricle in contrast to the disarray in the drug-damaged culture. Numerous hair cells are missing from the drug-damaged culture, and as a consequence, the sensory epithelium is thinned relative to that in the undamaged utricle.

Importantly, a markedly greater number of tritiated-thymidine labeled cells are seen in the explant of the drug-damaged tissue compared to the control tissue explant. As previously observed, labeled hair cells and supporting cells are rare in tissue from normal animals and only one cell is labeled in Fig. 15a. In contrast, many cells are labeled by tritiated thymidine in the drug-damaged utricle (Fig. 15b). In general, in the cultured utricular, lagenar and saccular maculae, a greater number of labeled cells are observed in the drug-damaged sensory epithelium than in the concordant control sensory epithelium.

The amount of cellular proliferation was quantitatively assessed for the cultured utricular maculae. The average labeled-nucleus linear density (number of labeled cells per mm) was determined for experimental paradigms (i), (ii), and (iii). The average density of labeled cells calculated for cultures of drug-damaged organs was compared to that for the concordant control cultures to determine whether the regenerative process was maintained in vitro.

Fig. 16 shows the findings for culturing paradigm (i), the free-floating culture technique with the BME medium supplemented with 10% FBS. Label density is greater in drug-damaged than control utricles. Specifically, the average density of labeled cells is roughly 3 and 5 times that for the control cultures in the basal and lumenal halves of the sensory epithelium, respectively. With respect to the total sensory epithelium, the average density of labeled cells is roughly 4 times that for the control cultures. Statistical comparisons of label density between damage and control cultures revealed a highly significant difference ($P < 0.01$).

A summary of the up-regulation results is presented in Table II. This table delineates the ratios (drug-damaged/ control organs) of mean labeled-nuclei linear density and % linear label in the BL, LL, and TE for culturing paradigms (i), (ii), and (iii), Cellular proliferation was increased in the drug-damaged sensory epithelium (relative to the controls) with culturing paradigms (i) and (ii). Findings from paradigm (iii), the free-floating culture technique with the BME medium supplemented with 10% calf serum, were equivocal.

In addition, the amount of hair-cell production was quantitatively assessed for experimental paradigm (i).

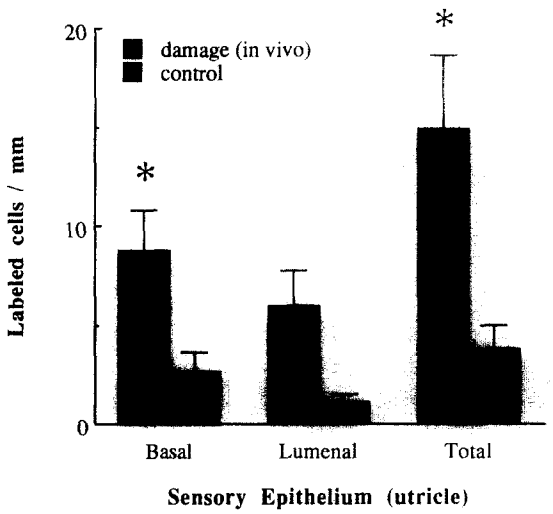

Fig. 16. Average linear density of labeled nuclei (average number of labeled cells per mm) in the basal and lumenal layers of the utricular sensory epithelium. Results for both normal and streptomycin-damaged utricles grown in culture are shown. Eight utricles taken from drug-damaged animals and 6 utricles taken from untreated controls were cultured identically for 2 days (free-floating culturing paradigm with BME medium and 10% FBS). The average number of labeled cells per mm was determined for each cultured utricle, and values for individual organs were averaged for each experimental paradigm. An asterisk denotes a value that is statistically significant from the control ($P < 0.05$). Error bars represent SEM.

The average number of tritiated-thymidine-labeled hair cells was determined for eight drug-damaged cultures (10 to 20 sections per organ) and compared to that for six control cultures (11 to 12 sections per organ) to determine whether the culturing conditions permitted increased hair-cell production in drug-damaged cultures. The criteria for the identification of a labeled hair cell was the following: 5 or more silver grains were

TABLE II

Up-regulation in cultured utricular maculae

(All values in the table are ratios, i.e., drug damaged/ control organs)

Culture paradigm [1]	labeled-nucleus linear density [2]			% linear label [3]		
	BL	LL	TE	BL	LL	TE
i	3.3 [a]	5.2	3.9 [a]	3.7 [a]	5.8	4.3 [a]
ii	2.4	3.7	3.0	1.9	2.5	2.1
iii	1.2	2.2	1.4	0.9	1.7	1.2

[1] i = free-floating culturing technique with BME medium and 10% FBS; ii = clotted-well culturing technique with BME medium and 25% HS; iii = free-floating culturing technique with BME medium and 10% CSS.

[2] labeled-nucleus linear density = mean number of labeled cells per mm sample area.

[3] % linear label = (number of labeled cells per mm/ total number of cell per mm) × 100.

[a] The value for the drug-damaged cultures is reliably greater than that from the control cultures ($P < 0.05$).

present over a cell nucleus located in the region of the hair-cell nuclei, the cell body lay in the lumenal half of the sensory epithelium, and stereocilia were present on the lumenal surface of the cell. Greater numbers of tritiated-thymidine-labeled hair cells are present in drug-damaged than control cultures. Specifically, the average number ($\pm$SD) of thymidine-labeled hair cells per section of drug-damaged tissue is 1.6 ($\pm$0.8), whereas the average number of labeled hair cells per section of control tissue is 0.4 ($\pm$0.3). Statistical comparisons of numbers of labeled hair cells between damaged and control cultures revealed a highly significant difference ($P < 0.01$).

Discussion

General summary of major findings

This study demonstrates the following: (i) That the sensory epithelium of the mature * avian inner ear can be maintained in vitro; (ii) That new hair cells and supporting cells are produced in vitro; (iii) That both division and differentiation of hair-cell progenitor cells occurs in vitro in the absence of sera; (iv) That the up-regulation of mitotic activity produced by damage in vivo is maintained when the organ is subsequently placed in vitro; and (v) That mitotic activity is initiated and maintained in vitro in the absence of neural electrical activity. Thus, a powerful tool has been developed that will now allow future determination of the regulatory signals involved in hair-cell proliferation and differentiation.

Organotypic cultures

Otocysts from chick embryos were first cultured over sixty years ago (Fell, 1928) and used by a number of investigators to study developing inner-ear sensory epithelia (e.g., Friedmann et al., 1977 and references therein, Ard et al. 1985; Sokolowski et al., 1993; Stone and Cotanche, 1991). Regarding the mature inner ear, only preliminary information has been published to date about culturing sensory epithelium from postnatal avian inner ears (Oesterle et al., 1992a; Warchol and Corwin, 1992). To the best of our knowledge, this is the first detailed report regarding explant cultures of the mature avian inner ear.

Importantly, this report is also a clear documentation of hair-cell genesis and differentiation in vitro in sensory epithelium taken from a mature, warm-blooded vertebrate inner ear. Warchol and coworkers have also recently demonstrated supporting-cell proliferation in organotypic cultures of utricles taken from mature guinea pig and human inner ears (Warchol et al., 1993) and basilar papillas taken from avian inner ears (Warchol and Corwin, 1992). The ability of their culture system to support the genesis and differentiation of hair cells has not been thoroughly investigated. The development of an in vitro model for hair-cell genesis and differentiation is of importance, for it provides a means to study the factors involved in the initiation and regulation of hair-cell regeneration.

Ongoing proliferation

Hair cells are produced throughout life in avian vestibular end organs (Jørgensen and Mathiesen, 1988; Roberson et al.,1992). New hair cells and supporting cells are added throughout the receptor epithelium (Roberson et al., 1992), and it has been suggested that the purpose of the addition is to replace cells that die during an ongoing normal process of cell death and replacement (Jørgensen, 1991; Weisleder and Rubel, 1993). Indirect evidence from drug-damaged and normal vestibular end organs indicates that supporting cells within the vestibular sensory epithelium are potential hair-cell precursor cells (Weisleder and Rubel, 1992b; Tsue et al., 1993). Findings of this study are harmonious with this idea in that after 24 h in culture (with continuous tritiated-thymidine incorporation), only labeled supporting cells were detected. The majority of the labeled supporting cells were located near the basal lamina, and a few were located in the lumenal half of the epithelium. Labeled hair cells were not found until later, after 2 days in culture. Tritiated-thymidine labeled supporting cells and hair cells are found throughout the vestibular sensory epithelium in explant cultures of normal inner-ear end organs, indicating that the ongoing proliferation that occurs in the normal, in situ vestibular sensory epithelium is maintained in our cultures.

Regarding the auditory sensory epithelium, there is a very low rate of ongoing postnatal proliferation of supporting cells in the normal in situ basilar papilla (Oesterle and Rubel, 1993b). Results from the in vitro basilar papilla are similar in that tritiated-thymidine labeled supporting cells are found occasionally in cultures of normal basilar papilla. The first labeled cells in

* Albeit, white leghorn chickens do not become sexually mature until 18 to 21 weeks of age (H & N International, personal communication) by the eighth postnatal day the inner-ear sensory epithelium has obtained its adult number of hair cells (Tilney et al., 1986; Katayama and Corwin, 1989; Weisleder and Rubel, 1993), nearly reached its adult number of supporting cells (Oesterle and Rubel, 1993), and nearly reached adult proportions (Ryals et al., 1984; Cotanche and Sulick, 1985; Tilney et al., 1986). Further, the endocochlear potential has reached its mature value (Cotanche et al., 1987), and compound action potentials have attained adult values (Rebillard and Rubel, 1981). Hence, we consider the inner-ear sensory epithelium of eight-to-eighteen day-old chickens to be essentially 'mature.'

the sensory epithelium were labeled organ supporting cells, and they were detected in cultures grown for 2 days. Labeled supporting cells are very rare in vivo but occur more frequently in the cultures. Conceivably, this increased mitotic activity is a reflection of the metabolic physiology of the epithelium being compromised by the artificial environment. Interestingly, several nonsensory cell types outside the organ, namely homogene cells, clear cells, and vacuole cells are labeled by tritiated thymidine in the cultures, whereas these cell types do not appear to proliferate in the normal in situ cochlear duct (Oesterle, personal observations).

Increased proliferation after insult

Weisleder and Rubel (1992a, 1993) recently reported that ototoxic damage to the vestibular organs in postnatal chicks increases proliferation of progenitor cells and differentiation of hair cells. Thus, the vestibular system in the young chicken is capable of regenerating its sensory epithelium in response to an ototoxic lesion. The purpose of this portion of the present study was to determine if the up-regulation of mitotic activity produced by damage in vivo is maintained when the organ is subsequently placed in vitro. In other words, can the culture system maintain or support the regenerative process? The increase in proliferative activity seen in drug-damaged organs relative to control cultures conclusively demonstrates that some culture conditions permit the regenerative process to occur in vitro. Further, the extent of up-regulation observed in vitro, a two-to-five-fold increase in proliferation at 48 h, agrees with the reports of Weisleder and Rubel (1993) and Tsue et al. (1993). Weisleder and Rubel found a four-fold increase in proliferation and Tsue et al (1993) report a two-to-three-fold increase in proliferation in vivo in chicken vestibular end organs 1 day after the completion of their streptomycin injections. Different tritiated-thymidine exposures between the studies prohibit any direct comparisons between these values, but qualitatively, the extent of the up-regulation observed in vitro agrees with the in vivo values.

Findings from this study indicate that hair-cell regeneration process may be locally maintained. The increased supporting cell and hair-cell production seen after insult is maintained locally. Signals for the maintenance of the increased proliferation could be generated from within the organ itself. Corwin et al. (1991) suggests that locally secreted growth factors play a role in regeneration of hair cells in the lateral line organ of salamanders.

Role of neural connections in ongoing proliferation and regeneration

Controversy still exists regarding the necessity of neural element presence for the following: (i) for hair-cell differentiation in the in situ developing inner ear, (ii) for maintenance of morphology in the mature inner ear; and (iii) for regeneration (reviewed in Corwin and Warchol, 1991 and Weisleder, 1991; Sans et al., 1993). It is not yet clear whether neural activity is necessary for regenerative processes in vivo. The presence of tritiated-thymidine labeled hair cells and supporting cells in explants of inner-ear end organs suggests that mitotic activity is initiated and maintained in vitro in this tissue in the absence of neural electrical activity. While neural electrical activity is not essential for ongoing proliferation or the maintenance of up-regulation in the mature avian inner ear, it may modulate this process (Weisleder, 1991). These results are concordant with those reported by Warchol and Corwin (1992) who presented preliminary evidence for increased proliferation in cultured chicken basilar papilla after laser damage.

Serum-free cultures

Our initial studies were designed to optimize the proliferation and differentiation of chicken hair cells in a serum-containing medium. Because serum-containing media contain variable levels of known as well as unknown factors that may influence mitotic activity and differentiation, we also examined results from media with very low serum concentrations or no serum supplement. Interestingly, new hair cells and supporting cells are found in organs taken from normal, undamaged animals that were grown in culture without serum (a serum-free medium). What are the factors that could be stimulating cell division and differentiation under these conditions? The finding that proliferative activity occurs in isolated inner-ear end organs cultured with serum-free media suggests that the signal(s) that trigger proliferative activity and hair-cell differentiation are local. These factors must be generated within the end organ itself and do not come from more remote regions of the body. We do not yet know what the signals are. Circulating growth factors, growth-factor release from phagocytic cells, and hair-cell extrusions could potentially trigger mitosis in otherwise quiescent supporting cells (Corwin et al., 1991; Stone and Cotanche, 1992). The development of the organotypic culture model will allow experimental testing of these and other putative trigger signals.

Hair-cell genesis and differentiation in culture

The presence of labeled hair cells and supporting cells in the cultured vestibular and auditory sensory epithelia indicates that our culture media and culturing conditions permit the formation and differentiation of new hair cells and supporting cells. Immature hair cells were seen after 2 days in culture. In vivo, immature

auditory hair cells (with stereocilia) have been observed roughly 72–96 h after the onset of damage (Girod et al., 1989; Janas et al., 1992; Stone and Cotanche, 1992), and new vestibular hair cells have been reported in normal vestibular sensory epithelium as early as 9 h after the administration of a tritiated-thymidine injection (Presson and Popper, 1990). The newly formed hair cells develop appropriately and take on a morphological appearance similar to that of newly generated hair cells observed in the posthatch chick.

Summary

In summary, this report is a description of a primary organ culture system which maintains sensory epithelium from postnatal avian inner ears in vitro. Hair-cell progenitor cells divide and differentiate in this culture system. In addition, proliferative activity stimulated by in vivo ototoxic insult is maintained in the organotypic cultures. The model developed here provides numbers of hair cells that would be adequate for biochemical or molecular studies. It is expected that this system will be useful for studies of hair-cell genesis and differentiation as well as studies of hair-cell and supporting-cell functioning in general.

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