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Macrophage and Microglia-Like Cells in the Avian Inner Ear

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ABSTRACT

Recent studies suggest that macrophages may influence early stages of the process of hair cell regeneration in lateral line neuromasts; numbers of macrophages were observed to increase prior to increases in hair cell progenitor proliferation, and macrophages have the potential to secrete mitogenic growth factors. We examined whether increases in the number of leukocytes present in the in vivo avian inner ear precede the proliferation of hair cell precursors following aminoglycoside insult. Bromodeoxyuridine (BrdU) immunohistochemistry was used to identify proliferating cells in chicken auditory and vestibular sensory receptor epithelia. LT40, an antibody to the avian homologue of common leukocyte antigen CD45, was used to label leukocytes within the receptor epithelia. Macrophages and, surprisingly, microglia-like cells are present in normal auditory and vestibular sensory epithelia. After hair cell loss caused by treatment with aminoglycosides, numbers of macrophage and microglia-like cells increase in the sensory epithelium. The increase in macrophage and microglia-like cell numbers precedes a significant increase in sensory epithelial cell proliferation. The results suggest that macrophage and microglia-like cells may play a role in releasing early signals for cell cycle progression in damaged inner ear sensory epithelium. J. Comp. Neurol. 398:241–256, 1998.

Indexing terms: auditory; vestibular; hair cell regeneration; cochlea; leukocytes proliferation

Acoustic overstimulation and aminoglycoside antibiotic toxicity are among the leading causes of sensorineural hearing impairment. In mammals, these insults result in sensory hair cell loss and formation of scar tissue. Avian, fish, and amphibian inner ears, in contrast, are capable of self-repair postembryonically (Cotanche, 1987; Cruz et al., 1987; Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Baird et al., 1993; Lombarte et al., 1993; Weisleder and Rubel, 1993). Regeneration of new hair cells and associated neural elements occurs in both the auditory and vestibular sensory epithelia with resultant functional recovery (Hashino and Sokabe, 1989; Linzenbold et al., 1993; Marean et al., 1993; Saunders et al., 1995; Carey et al., 1996). Sensory epithelial supporting cells are precursors of the new hair cells (Corwin and Cotanche, 1988; Girod et al., 1989; Balak et al., 1990; Presson and Popper, 1990; Raphael, 1992; Baird et al., 1993; Hashino and Salvi, 1993; Jones and Corwin, 1993, 1996; Weisleder and Rubel, 1993; Stone and Cotanche, 1994; Tsue et al., 1994b; Warchol and Corwin, 1996).

How might precursor cells be triggered to enter the active cell cycle? It has been hypothesized that precursor cell proliferation may be regulated by local diffusible factors, wherein damaged hair cells, or scavenging cells such as macrophages, cause the release of a mitogenic substance into the extracellular environment. In vitro studies with serum-free media (Oesterle et al., 1993; Warchol and Corwin, 1993; Warchol, 1995), laser-ablated hair cells (Warchol and Corwin, 1996), and coculture experiments (Tsue et al., 1994a) suggest that a local release of growth factors may be involved in the induction of precursor cell proliferation. Growth factors such as insulin-like growth factor-1 (IGF-1) and transforming growth factor alpha (TGFα) have been shown to stimulate cell proliferation in mature inner ear sensory epithelium (Lambert, 1994; Yamashita and Oesterle, 1994, 1995; Kuntz and Oesterle, 1996; Oesterle et al., 1997; Zheng et al., 1997).

Resident tissue macrophages respond rapidly to injury in lateral line organs of axolotl salamanders (Jones and Corwin, 1993, 1996) and cultured avian auditory sensory epithelium, the basilar papilla (Warchol, 1997). Macro-

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phages may play an important role in initiating hair cell regeneration by releasing a variety of mitogenic factors including interleukin-1, basic fibroblast growth factor (bFGF), heparin-binding epidermal growth factor (HB-EGF), TGFα, transforming growth factor beta (TGFβ), IGF-1, platelet-derived growth factor (PDGF), and tumor necrosis factor alpha (TNFα; Baird et al., 1985; Shimokado et al., 1985; Assoian et al., 1987; Nathan, 1987; Madtes et al., 1988; Rappolee et al., 1988; Rom et al., 1988; Higashiyama et al., 1991).

We undertook this study to determine whether: (1) macrophages are present in vivo in chicken auditory and vestibular sensory epithelia; and (2) whether macrophage numbers increase prior to increases in sensory epithelial cell proliferation following aminoglycoside treatment. To our surprise, microglia-like cells were detected in the in vivo avian inner ear sensory epithelia along with macrophages. Macrophage and microglia-like cells increased in number prior to any significant proliferative increase in precursor cells, making these cells potentially important in triggering sensory epithelial cell proliferation. A preliminary report of portions of these data has appeared (Bhave et al., 1996).

MATERIALS AND METHODS

Animals

White Leghorn chickens (*Gallus domesticus*) from H & N International (Redmond, WA) were housed in a thermoregulated brooder in the animal care facility and were provided with free access to food and water throughout the experiments. All experimental protocols were reviewed and approved by the University of Washington Animal Care Committee.

Experimental treatment plan

Three-day-old (P3) chicks were treated with a single dose of aminoglycoside antibiotic (Bhave et al., 1995; Janas et al., 1995) and were grouped by survival times following the aminoglycoside treatment. Hair cell damage caused by the antibiotic treatment was visualized by scanning electron microscopy (SEM). 5-bromo-2′-deoxyuridine (BrdU), a thymidine analogue, was used to label the proliferating sensory epithelial (SE) cells in control and experimental birds (Gratzner, 1982). BrdU is incorporated in the DNA of cells passing through the S-phase of the cell cycle, thereby labeling dividing cells and their progeny. Auditory and vestibular tissue were prepared as wholemounts and processed for immunohistochemistry. Antibody LT40 (gift of Dr. Michael Ratcliffe, Montreal, Quebec, Canada), an antibody to the avian homologue of common leukocyte antigen CD45 (Paramithiotis et al., 1991), was used to label leukocytes. CD45 is a membranous antigen present on cells derived from white blood cell precursors. CD45 is immunohistochemically detectable in cells from monocytic lineage, including macrophages and microglia (Penfold et al., 1991; Provis et al., 1995; Johnson et al., 1996). An anti-BrdU antibody was used to detect proliferating cells that had incorporated BrdU (Bhave et al., 1995).

Ototoxic drug treatment and BrdU injections

Basilar papilla. The basilar papilla, the auditory sensory receptor epithelium in the chicken, is analogous to the mammalian organ of Corti. Chicks received a single subcutaneous injection of gentamicin sulfate (250 mg/kg; Lyphomed, Deerfield, IL) to kill basilar papilla hair cells. Birds were then divided into 1 day (n = 14), 2 day (n = 12), 3 day (n = 10), 5 day (n = 6), 7 day (n = 5), and 15 day (n = 8) postinjection survival groups. Age-matched control chicks (posthatch day 4 [n = 2], 5 [n = 2], 6 [n = 3], 8 [n = 3], 10 [n = 1], and 18 [n = 1]) received no drug. Experimental and control animals received a total of three intraperitoneal injections of BrdU (100 mg/kg; Sigma, St. Louis, MO) delivered at 24 hours, 20 hours, and 16 hours prior to killing.

Utricle. Chicks received a single subcutaneous injection of streptomycin sulfate (1,200 mg/kg; Sigma) to kill utricular hair cells. Birds were then grouped into 1 day (n = 6), 2 day (n = 6), 4 day (n = 6), 10 day (n = 6), and 15 day (n = 6) postinjection survival groups. Age-matched control chicks (posthatch day 4 [n = 2], 5 [n = 1], 7 [n = 1], 13 [n = 1], and 18 [n = 1]) did not receive any drug. Experimental and control animals received a total of two intraperitoneal injections of BrdU (100 mg/kg) delivered at 24 hours and 22 hours prior to killing.

Tissue preparation for immunohistochemistry

Basilar papilla. Chicks were decapitated, and the animals' right cochlear ducts were perfused by intralabyrinthine perfusion with 1% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 2 minutes. After two 5-minute PBS rinses, cochlear ducts were dissected out of the bony cochlea and fixed again (immersion fixation) in 1% paraformaldehyde for 20 minutes, washed, and processed for LT40 immunohistochemistry as wholemounts. Temporal bones from the left ear of the same chicks were handled in an identical manner, except that the initial 1% paraformaldehyde perfusion was 10 minutes in duration. Left cochlear ducts were immersion-fixed in 4% paraformaldehyde/PBS (pH 7.4) for 20 minutes and processed for BrdU immunohistochemistry.

Utricle. Utricles from both ears were harvested. The utricle from the right ear was immersion-fixed in 1% paraformaldehyde for 30 minutes for LT40 immunohistochemistry, whereas the utricle from the left ear was immersion-fixed in 4% paraformaldehyde for 30 minutes for BrdU immunohistochemistry. Following PBS rinses, otoconia were gently removed from the lumenal surface of the utricular macula, and the organs were then processed for immunohistochemistry.

LT40 (anti-CD45) immunohistochemistry

Endogenous peroxidase activity in the wholemounts was blocked by incubating the tissue in 0.5% H_2O_2 in methanol for 30 minutes. To reduce nonspecific staining, tissue was incubated in 10% normal horse serum in 0.1% Triton X-100/1% bovine serum albumin (BSA) for 30 minutes. Sequential incubations were carried out with LT40 antibody (1:20 in 0.1% Triton X-100/1% BSA) for 3 hours, biotinylated anti-mouse IgM antibody (1:200 in 0.1% Triton X-100/1% BSA; Vector Labs, Burlingame, CA) for 30 minutes, and avidin-biotin with horseradish peroxidase (1:50 in 0.1% Triton X-100/1% BSA; Vector Labs) for 45 minutes. All incubations were carried out at room temperature, and the tissue was washed with PBS, pH 7.4, between incubations. For the development of the peroxidase reaction, wholemounts were placed in 0.05 M Tris buffer, pH 7.6, for 5 minutes, followed by incubation in

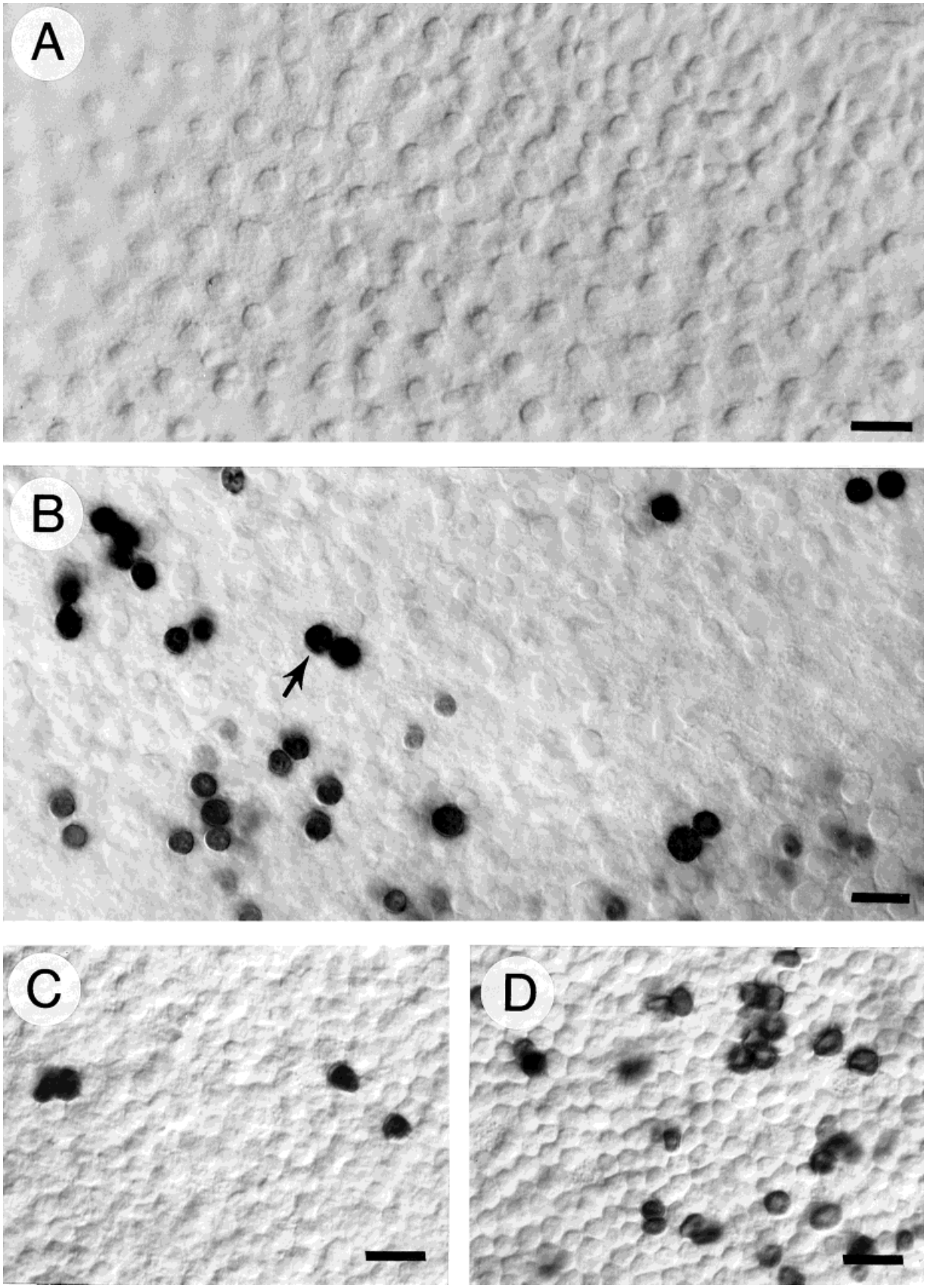

Fig. 1. Proliferating sensory epithelial (SE) cells in normal and drug-damaged chicken SE. Photomicrographs of bromodeoxyuridine (BrdU)-immunohistochemical staining in chicken basilar papilla and utricular macula. Plane of focus is at the level of the supporting cell nuclei. **A:** Normal control basilar papilla demonstrating no immunostaining within the supporting cell layer. **B:** Basilar papilla 3 days postgentamicin injection demonstrating a significantly increased number of BrdU-positive cells in the damaged proximal region. Arrow points to closely associated "daughter" nuclei found in areas with the highest proliferative activity. **C:** Normal control utricular macula. Some BrdU-stained nuclei are present among the supporting cell nuclei. There is an ongoing production of SE cells that occurs normally in the undamaged utricular macula. **D:** Utricular macula 2 days poststreptomycin injection, demonstrating a significantly increased number of BrdU-positive cells in the damaged region. Scale bars = 10 µm.

diaminobenzidine (DAB; 0.25 mg/ml; Sigma) in 0.05 M Tris buffer for 5 minutes, and DAB/0.01% H_2O_2 for 5 minutes. Following the immunohistochemistry, the tissue was washed in Tris buffer, mounted onto slides in 90% glycerol in PBS with 1% sodium azide, and coverslipped. Immunostaining was evaluated by light microscopy with Nomarski differential interference contrast optics.

BrdU immunohistochemistry

BrdU immunohistochemistry (Gratzner, 1982) was performed using methods similar to those described in Raphael (1992), Hashino and Salvi (1993), and Stone and Cotanche (1994). Basilar papillas and utricles were placed in 1 N HCl diluted in 0.1% Triton X-100 in PBS for 30 minutes at 37°C. After rinses in PBS, endogenous peroxidase was blocked with 0.5% H_2O_2 in methanol for 30 minutes. Organs were incubated in 10% normal horse serum in 0.1% Triton X-100/1% BSA for 30 minutes and then incubated with anti-BrdU antibody (1:40 in 0.1% Triton X-100/1% BSA; Becton-Dickenson, San Jose, CA) for 1 hour, biotinylated horse anti-mouse IgG (1:200 in 0.1% Triton X-100/1% BSA; Vector Labs) for 30 minutes, and avidin-biotin with horseradish peroxidase (1:50 in 0.1% Triton X-100/1% BSA) for 45 minutes. With the exception of the HCl treatment, all incubations were carried out at room temperature, and the tissue was washed with PBS between incubations. Wholemounts were then placed in 0.05 M Tris buffer for 5 minutes, followed by DAB (0.25 mg/ml 0.05 M Tris) for 5 minutes and DAB/0.01% H_2O_2 for 5 minutes. Tissue was rinsed thoroughly with Tris buffer, mounted onto slides in 90% glycerol in PBS with 1% sodium azide, coverslipped, and analyzed by using light microscopy with Nomarski optics.

Immunohistochemistry controls

To simulate the diffusion conditions encountered in wholemount specimens, thick tissue sections (~50-µm-thick) were prepared for use as positive controls. Sections of chicken gut, taken from the same animals from which the inner ear end organs had been harvested, served as positive control tissue for the BrdU immunohistochemistry. Tissues used as LT40-positive controls were chicken spleen, retina, and brain parenchyma. All positive controls were processed following the identical procedures used for the inner ear tissues. For negative controls, basilar papillas and utricles from experimental and control animals were treated in the same manner, but with the omission of the primary antibody.

Scanning electron microscopy (SEM)

To delineate the pattern and extent of aminoglycoside-induced hair cell loss, basilar papillas taken from control (n = 2) and 3-day postinjection (gentamicin sulfate) survival groups (n = 2), and utricles from control (n = 3) and 4-day postinjection (streptomycin sulphate) survival groups (n = 3), were processed for SEM.

Basilar papilla. Animals were decapitated, and the cochlear ducts were perfused by intralabyrinthine perfusion with 2.5% gluteraldehyde, 1% paraformaldehyde, 0.1 M sucrose, and 5 mM $MgSO_4$ in PBS (pH 7.4). Temporal bones containing the cochlear ducts were harvested and immersion-fixed in the same fixative overnight at 4°C. After postfixation in 1% osmium tetroxide in PBS for 1 hour and PBS rinses, the tegmentum vasculosum and tectorial membrane were dissected away to expose the lumenal surface of the basilar papilla. Tissue was then dehydrated with a graded series of ethyl alcohols and dried in a Tousimis (Rockville, MA) critical point dryer with carbon dioxide. Specimens were mounted on aluminum studs, sputter-coated with gold/palladium alloy (Anatech, Alexandria, VA), and examined with a JEOL JSM 6300F scanning electron microscope.

Utricles. Utricles were isolated and immersion-fixed in 2.5% gluteraldehyde, 1% paraformaldehyde, 0.1 M sucrose, and 5 mM $MgSO_4$ in PBS (pH 7.4) overnight. Following PBS rinses, otoconia were gently removed, and the utricular macula was exposed. Utricles were postfixed in 1% osmium tetroxide for 1 hour and rinsed with PBS. Dehydration was accomplished with a graded series of ethyl alcohol and dried in the critical point dryer. Specimens were mounted on aluminum studs, sputter-coated with pure gold, and examined with the JEOL microscope.

Data analysis

Wholemounts were examined using a 40× objective on a Leica Axiplan microscope and Nomarski optics. BrdU-positive nuclei were easily detected in the inner ear SE by their dark brown nuclear staining (e.g., Fig. 1B). Macrophage-type cells were identified by their large round shape, presence of a large unstained single nucleus, and their dark-brown-stained LT40-positive cytoplasm (Fig. 2B, inset). For purposes of further discussion, these macrophage-type cells will be referred to herein simply as macrophages. Microglia-type cells were identified by their characteristic star- or flower-shaped pattern formed by branched processes that radiate from a small cell body and their LT40 cytoplasmic immunostaining (Fig. 3A,B, insets). Hair cells and supporting cells were unlabeled with the anti-CD45 LT40 antibody.

The number of BrdU-labeled SE cells or LT-40-labeled macrophages and microglia-like cells in the SE were counted for each organ using Nomarski optics and a 10 × 10 eyepiece reticule. The location of each labeled cell was plotted onto an outline of the whole utricular macula or basilar papilla drawn to scale. Only labeled cells whose nuclei were present within the SE were counted. For the basilar papilla, care was taken to ensure that extrasensory cells (e.g., hyaline cells, clear cells, homogene cells) and cells within the fibrocartilaginous stroma were not included in the counts. Similarly for the utricle, care was taken to exclude labeled cells outside the SE, such as those

Fig. 2. LT40+ macrophages in chicken inner ear sensory epithelia. Photomicrographs of LT40 immunohistochemical staining for macrophages in chicken basilar papilla and utricular macula. **A:** Normal control basilar papilla with a rare LT40-positive macrophage (arrow) located superficial to the hair cell layer. The arrowhead points to a stereociliary bundle arising from the lumenal surface of a hair cell. **B:** Basilar papilla 2 days postgentamicin, demonstrating a significantly increased number of LT40-immunostained macrophages in the damaged proximal region. Note that many sensory epithelial (SE) nuclei are missing in the damaged region. The plane of focus is at the level of the hair cell nuclei. **Inset:** Higher magnification showing the single nucleus and characteristic granular cytoplasm of the labeled macrophage. **C:** Normal control utricular macula with a rare LT40-positive macrophage. The plane of focus is slightly superior to the hair cell nuclei. **D:** Utricular macula 1 day poststreptomycin, demonstrating a significantly increased number of LT40-positive macrophages in the damaged region. The plane of focus is at the level of the hair cell nuclei. Scale bars = 6 µm in A,B, 8 µm C,D.

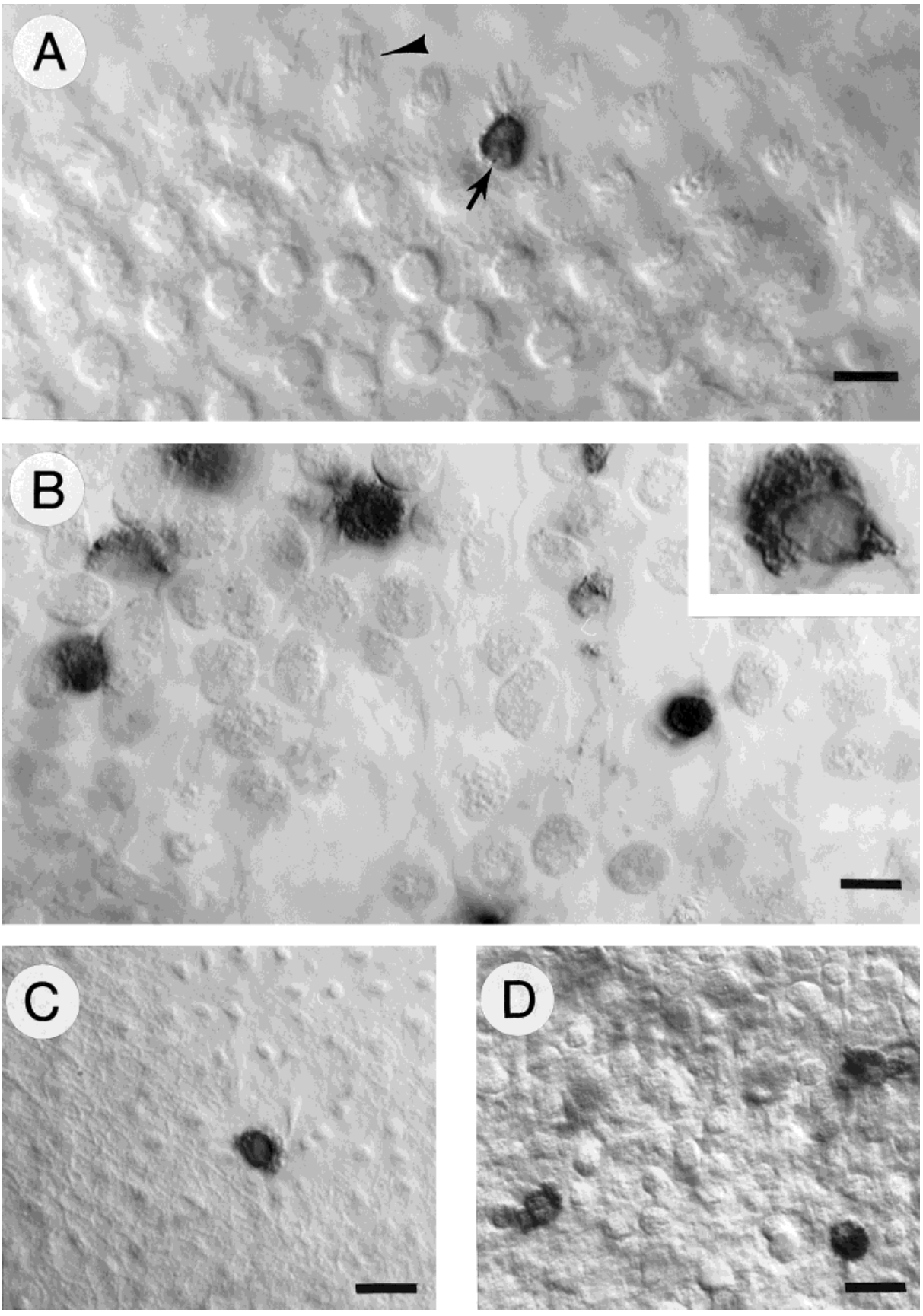

Figure 2

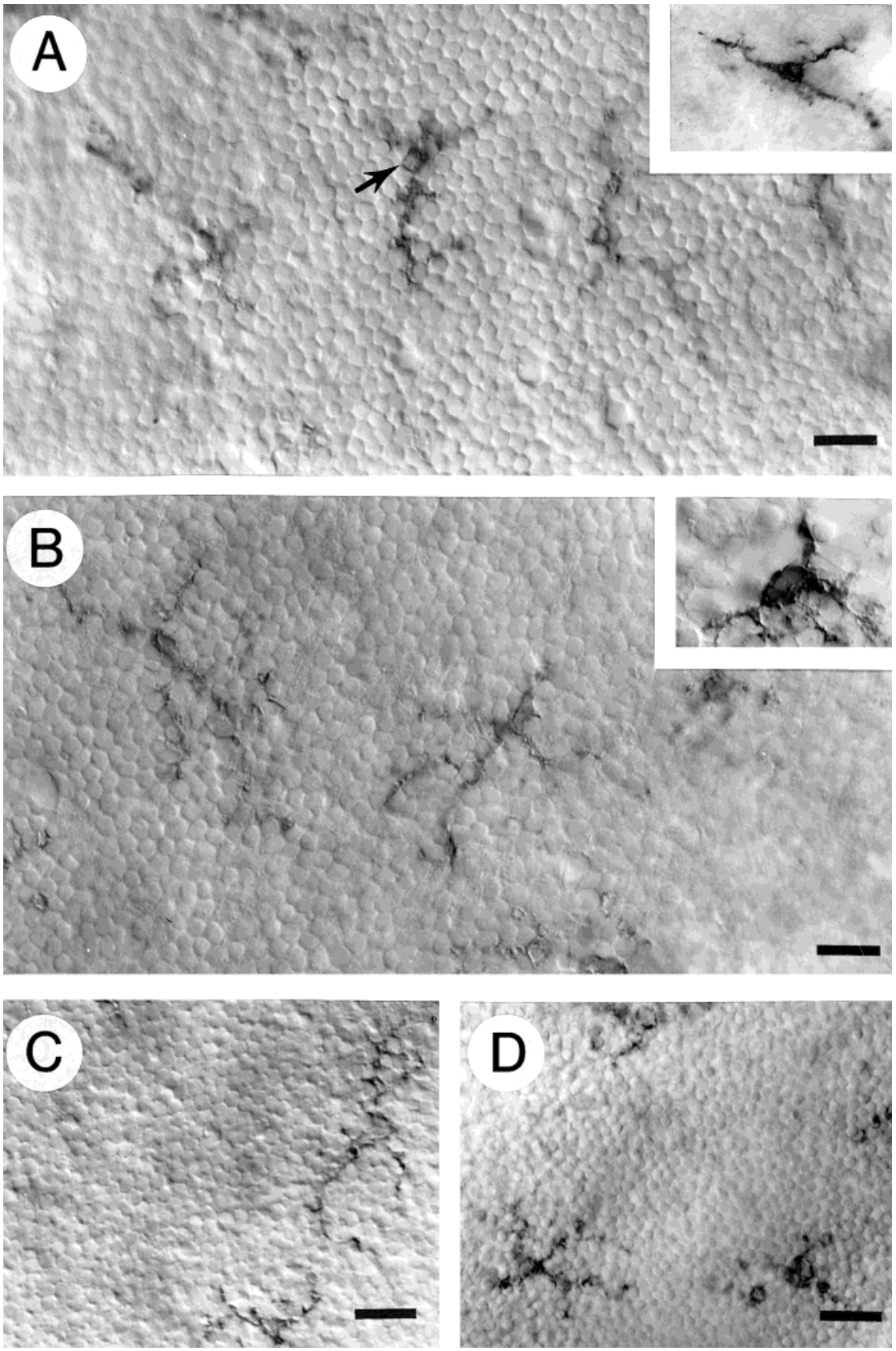

Figure 3

in the transitional epithelium and fibrocartilaginous stroma.

Statistical analysis of the data included a one-way analysis of variance (ANOVA) using the SUPERANOVA software package (Abacus Concepts, Berkley, CA). Post-hoc comparisons, when appropriate, used the Fisher's Protected Least Significant Difference or Dunnett's test.

RESULTS

Positive controls

Sections of gut from experimental chicks served as positive controls for the BrdU immunohistochemistry studies. In these sections, BrdU-labeled cells were present in the mucosal layer at the base of the gut crypts (data not shown). LT-40 (CD45)-labeled microglia were identified in sections of brain parenchyma and retina by their LT40-immunostained elongated cell body with 3 to 5 primary radiating processes and second-order branches extending out from the cell body (Fig. 3A, inset). The LT40-immunostained microglia strongly resemble the ramified form of microglia (Ling and Wong, 1993). Large mononuclear macrophage-type cells labeled with the LT40 antibody were identified in the spleen along the sinusoids (data not shown).

Control basilar papilla: Histology

The cellular organization of the chicken basilar papilla has been described in great detail by others (Hirokawa, 1978; Tanaka and Smith, 1978; Smith, 1981, 1985; Tilney and Saunders, 1983; Manley, 1990). The basilar papilla is composed of sensory hair cells (tall, short, and intermediate hair cells), supporting cells, and unmyelinated nerve endings of the 8th cranial nerve. Hair cells are located in the lumenal portion of the sensory epithelium, whereas supporting cells extend from the basement membrane to the lumenal surface of the sensory epithelium, the reticular lamina (Fig. 4B). In wholemount and SEM preparations of the basilar papilla, the lumenal surface of the papilla is sickle-shaped; it has a narrow proximal (high frequency) end (Fig. 4A), a broad distal (low frequency) end, a convex superior edge, and concave inferior edge. Along the convex superior edge, nonsensory epithelial cells, including clear cells and homogene cells, can be identified by their location and shape. Irregularly shaped border cells (Oesterle et al., 1992) form the inferior edge of the sensory epithelium, and cuboidal-shaped hyaline cells lie inferior to the border cells. Stereocilia bundles rise from the lumenal surfaces of the basilar papilla hair cells, and the hair cells themselves are arranged in a hexagonal pattern (Fig. 4E,F). Hair cells are surrounded by supporting cells, which can be identified by their smaller apical surface and numerous microvilli. Hair cell nuclei are round and organized in rows visible at a focal plane immediately beneath the reticular lamina. Supporting cell nuclei are smaller than hair cell nuclei and oval. Supporting cell nuclei are more numerous than the hair cell nuclei and lie at deeper focal planes, closer to the basement membrane.

Control basilar papilla: Immunohistochemistry

Very few BrdU-labeled cells are seen in the normal, undamaged basilar papilla (mean = 4 $\pm$ 2 labeled cells/organ, S.D., Fig. 1A; Table 1). As can be seen in the distribution map of Figure 6B, the BrdU-labeled SE cells are located in the distal half of the papilla. Occasional BrdU labeling is seen in the peripheral extrasensory epithelium, and numerous BrdU-labeled cells are present among the fibroblasts of the basilar membrane, chondrocytes of the fibrocartilaginous stroma, and in the vascular endothelium.

A small number of macrophages are seen in the normal basilar papilla superficial to the hair cell nuclear layer (mean = 2 $\pm$ 1 labeled cells/organ, S.D.; Fig. 2A, Table 1). Typically, they are located in the distal one-third of the papilla. As described in Materials and Methods, the macrophages are identified by their large round shape, presence of a single large nucleus, and LT40-immunostained granular cytoplasm.

A few microglia-like cells are also present in the normal, undamaged basilar papilla. They are located in the supporting cell layer (mean = 4 $\pm$ 3 labeled cells/organ, S.D.; Fig. 3A, Table 1). They tend to be located in the distal half of the papilla (Fig. 6A). They are identified by their star shape, with a characteristic LT40-labeled small oval cell body and fine, radiating processes. Morphologically, these microglia-like cells resemble the ramified form of central nervous system (CNS) microglia identified in sections of brain parenchyma and retina (Fig. 3A, inset).

Drug-damaged basilar papilla: Histology

Following gentamicin treatment, extensive hair cell loss is detected by 3 days postinjection (Fig. 4A,C,D; Bhave et al., 1995; Janas et al., 1995; Stone et al., 1996). At all survival times, hair cell loss is mainly limited to the proximal 1,000 μm of the basilar papilla, the region where high frequency sound information is encoded by the hair cells. The distal region (where low frequency sound information is processed) does not demonstrate any visible hair cell loss at the dosage levels used.

Drug-damaged basilar papilla: immunohistochemistry

In drug-damaged basilar papillas, a significant increase in the number of BrdU-labeled SE cells is not seen until day 3 (mean = 75 $\pm$ 43 labeled cells/organ, S.D.; $P < 0.01$; Figs. 1B, 6D; Table 1). The number of BrdU-labeled SE cells declines by day 7, and by day 15 the number of labeled SE cells approaches control values (mean = 7 $\pm$ 6 labeled cells/organ, S.D.; Table 1). BrdU-labeled SE cells are

Fig. 3. LT40+ microglia-like cells in chicken inner ear sensory epithelia. Photomicrographs of LT40 immunohistochemical staining for microglia-like cells in chicken basilar papilla and utricular macula. **A:** Normal control basilar papilla demonstrating several LT40-positive microglia-like cells within the supporting cell layer. The arrow points to the cell body of a labeled microglia-like cell. The dendritic processes appear discontinuous in places because of changes in their focal plane. **Inset:** An LT40-labeled microglia cell in the chicken brain, demonstrating the elongated cell body with primary radiating processes and second-order branches. **B:** Basilar papilla 3 days postgentamicin showing increased numbers of LT40-positive microglia-like cells in the damaged proximal region. **Inset:** Higher magnification demonstrating the plumper cell body and shortened dendritic processes seen in many microglia-like cells located in areas of damage. **C:** Normal control utricular macula. LT40-positive microglia-like cells are present within the supporting cell layer. **D:** Utricular macula 2 days poststreptomycin, demonstrating an increased number of LT40-positive microglia-like cells in the damaged striolar region. Scale bars = 12 μm in A,B, 14 μm in C,D.

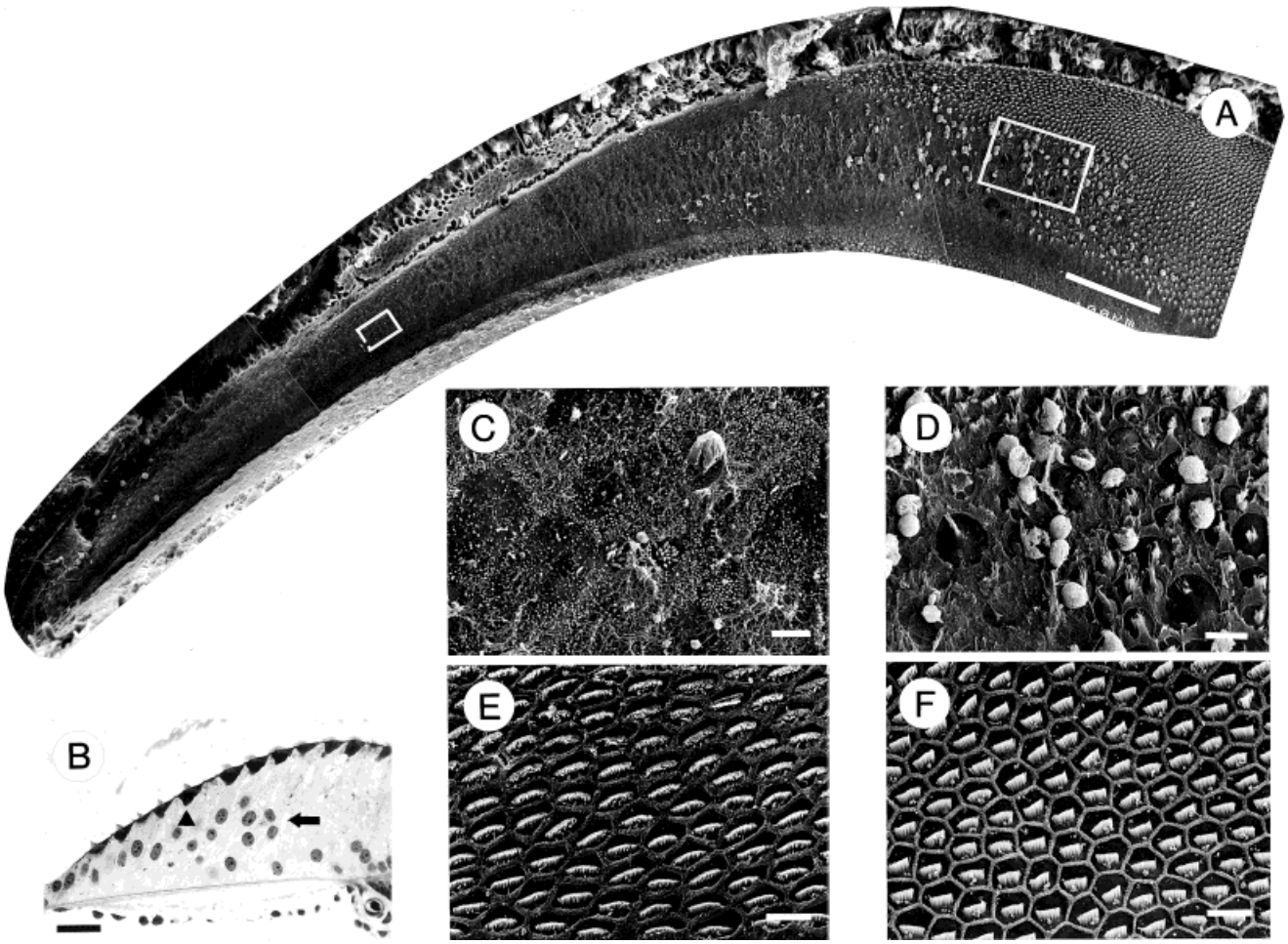

Fig. 4. Normal and drug-damaged chicken basilar papilla. **A:** Lumenal surface of the proximal two-thirds of a drug-damaged basilar papilla, 3 days postgentamicin. The proximal (basal) region of the papilla is on the left, and the more distal (apical) region of the papilla is on the right. Higher magnifications of the areas delineated by the small and large boxes are shown in C and D, respectively. Nearly all hair cell stereociliary bundles have been lost from the proximal region of the drug-damaged papilla (**C**), the area where high frequency sound information is encoded by the hair cells. At this time, more distally located hair cells are still being extruded from the zone of transition (**D**) between the proximal, damaged region and the distal (not shown), undamaged region of the papilla. Some intact stereociliary bundles remain in the transition region. **B:** Cross-section of normal control basilar papilla ~200 μm from the proximal end. Hair cell nuclei (arrowhead) are located more lumenal than the more numerous supporting cell nuclei (arrow). **E,F:** High magnification scanning electron micrographs from a normal, undamaged, control papilla. The regions correspond directly to the boxed areas shown in the drug-damaged papilla (C and D). Scale bars = 100 μm in A, 20 μm B, 2 μm C, 10 μm E,F.

mainly present in the proximal 1,000 μm of the papilla, towards the concave inferior edge (Fig. 6D). BrdU-labeled SE cells are rarely detected in the distal morphologically undamaged region.

The number of macrophages in the papilla increases significantly by day 2, a day prior to the significant increase in BrdU-labeled SE cells (mean = 5 ± 4 labeled cells/organ, S.D.; $P < 0.01$; Fig. 2B, Table 1). At 2 days, the peak of the increase, twofold more macrophages are present in drug-damaged auditory SE than in control SE. The increase in macrophage number is short-lived; it is followed by an immediate decline by day 3. Macrophages are mainly found in the proximal 500–800 μm of the drug-damaged papilla, and they are chiefly located near the convex superior edge. An occasional macrophage is detected in the undamaged distal region. A large increase in the number of monocytic-type cells is also seen in the stromal vessels of damaged basilar papillas as compared with controls.

The number of microglia-like cells increases significantly by day 2 after drug treatment ($P < 0.01$) and continues to increase through day 3 (mean = 56 ± 14 labeled cells/organ, S.D.; $P < 0.01$; Fig. 3B, Table 1). At 3 days, 14 times more microglia-like cells are present in drug-damaged basilar papilla than in control papilla. Relative to day 3, the number of microglia-like cells decline by day 5, but they remain significantly higher than control values through day 15 (mean = 19 ± 9 labeled cells/organ, S.D.; Table 1). Increased numbers of microglia-like cells are detected in the proximal damaged region, as well as in the morphologically undamaged distal region (Fig. 6C). Microglia-like cells in the damaged region are almost exclusively located within the supporting cell layer, whereas microglia-like cells in the distal undamaged region

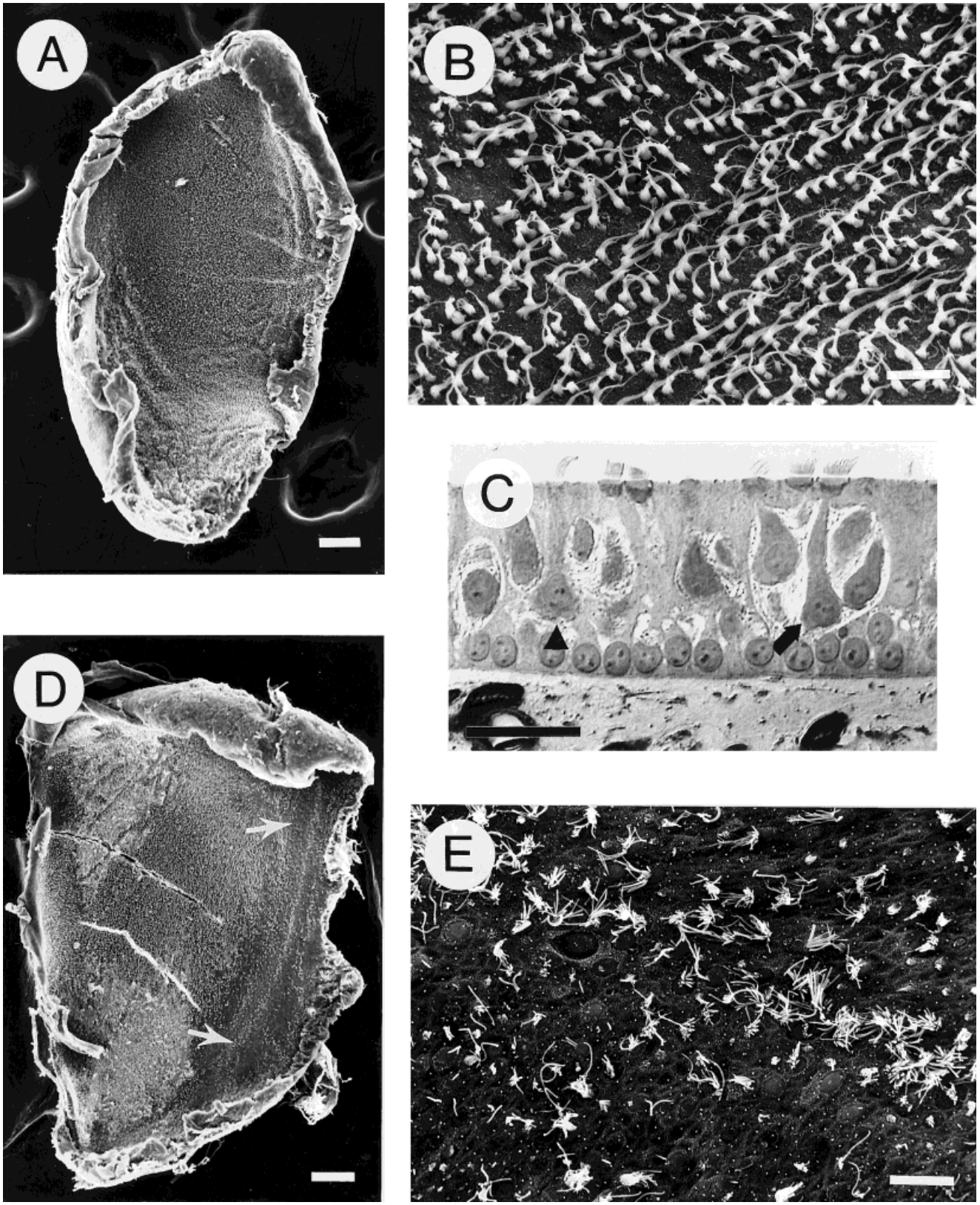

Fig. 5. Normal and drug-damaged chicken utricle. **A:** Scanning electron photomicrograph of a normal utricular macula. **B:** High power scanning electron micrograph of the striolar region of the utricular macula shown in A. Note the numerous hair cell stereociliary bundles. **C:** Cross-section of the normal vestibular sensory epithelium. Hair cell nuclei (arrow) are located more lumenal than the more numerous supporting cell nuclei. Type I (arrow) and Type II (arrowhead) hair cells can be identified. **D:** Scanning electron photomicrograph of a utricle 3 days poststreptomycin, demonstrating damage in the striolar region (arrows). **E:** High power scanning electron micrograph of the striolar region of the utricle shown in D. Note the reduced numbers of stereociliary bundles in the striolar region. Scale bars = 100 µm in A,D, 10 µm B,E, 20 µm C.

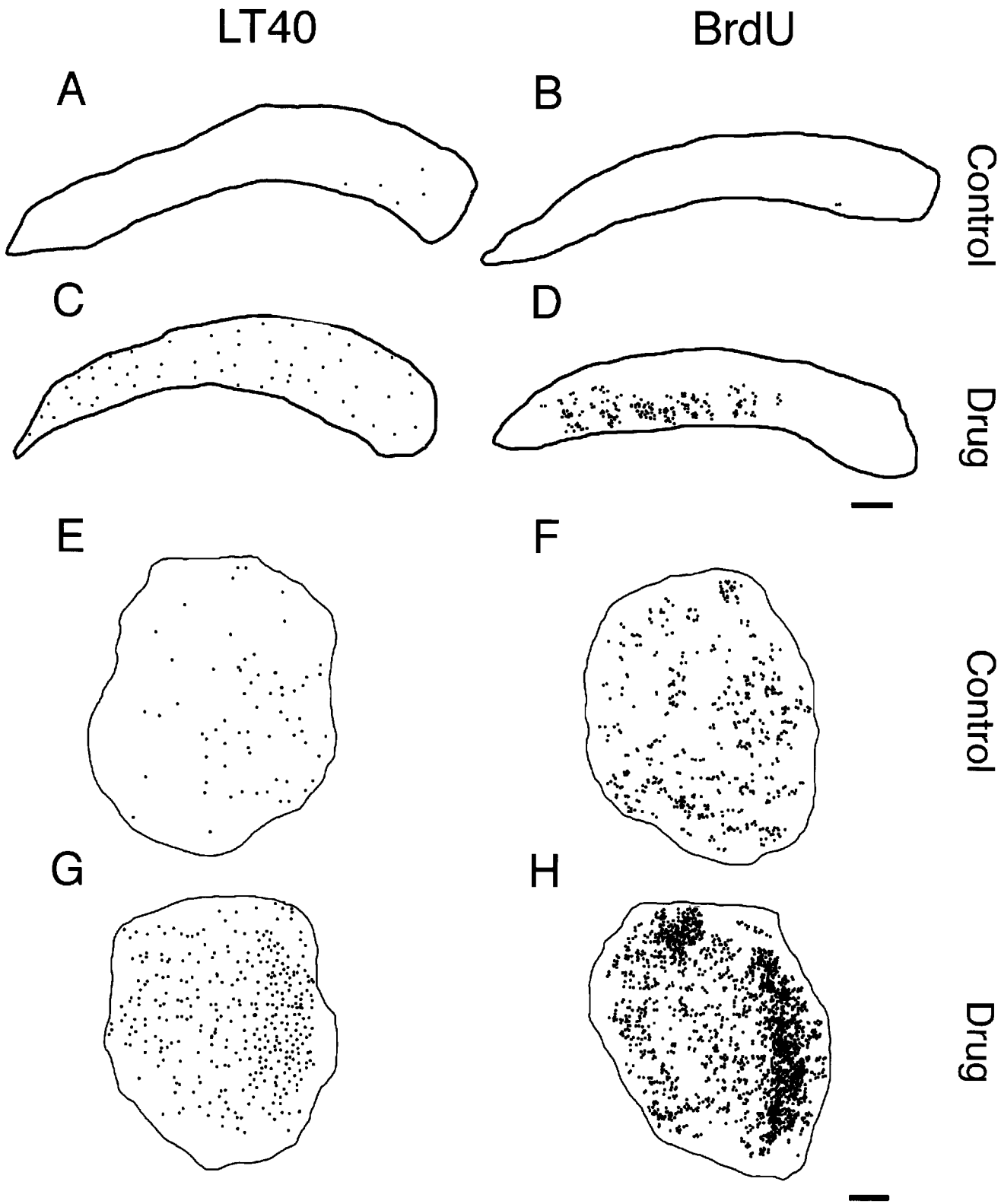

Fig. 6. Bromodeoxyuridine (BrdU) and LT40 immunostaining distribution maps for representative chicken basilar papilla and utricular macula specimens. Normal (A,B) and drug-damaged (C,D) basilar papillas oriented as in Figure 4A. The proximal (high frequency) end is on the left. BrdU (**A**) and LT40 (**B**) immunostained cells are rare, and they tend to be found in the distal region in control specimens. LT40 immunostaining at 2 days postgentamicin (**C**) is distributed throughout the papilla. BrdU immunostaining at 3 days postgentamicin (**D**) is concentrated in the proximal 1,000 µm, towards the concave inferior edge. Normal (E,F) and drug-damaged (G,H) utricular maculae oriented as in Figure 5A,D with the striolar region on the right. LT40- (**E**) and BrdU-(**F**) immunostained cells are common and are scattered throughout the normal undamaged utricular macula. LT40 immunostaining at 2 days poststreptomycin (**G**) is found throughout the utricular macula with a concentration in the striolar region. BrdU immunostaining at 3 days poststreptomycin (**H**) is found throughout the utricular macula with a concentration in the striolar region. Scale bars = 200 µm in A-D, 100 µm in E-H.

TABLE 1. Basilar Papilla: Average Number of BrdU-[1] or LT40-labeled SE[2] Cells (with S.D.) in Control and Experimental Basilar Papillas

Days following gentamicin injection	No. of BrdU-labeled SE cells	No. of LT40-labeled macrophages in the SE	No. of LT40-labeled microglia-like cells in the SE
Control (n = 12)	4 (2)	2 (1)	4 (3)
1 (n = 14)	13 (8)	3 (2)	12 (6)
2 (n = 12)	20 (14)	5 (4)**	35 (14)**
3 (n = 10)	75 (43)**	3 (2)	56 (14)**
5 (n = 6)	59 (13)**	2 (2)	29 (6)**
7 (n = 5)	12 (5)	3 (1)	35 (11)**
15 (n = 8)	7 (6)	1 (1)	19 (9)**

[1]BrdU, bromodeoxyuridine.
[2]SE, sensory epithelial.
**Significantly different from control value, $P < 0.01$.

TABLE 2. Utricle. Average Number of BrdU-[1] or LT40-labeled SE[2] Cells (with S.D.) in Control and Experimental Utricles

Days following streptomycin injection	No. of BrdU-labeled SE cells	No. of LT40-labeled macrophages in the SE	No. of LT40-labeled microglia-like cells in the SE
Control (n = 6)	391 (135)	6 (4)	110 (33)
1 (n = 6)	437 (104)	26 (22)**	153 (90)
2 (n = 6)	1,788 (470)**	7 (10)	220 (58)**
4 (n = 6)	1,225 (666)**	14 (10)	205 (65)**
10 (n = 6)	561 (143)	12 (5)	116 (39)
15 (n = 6)	552 (161)	10 (6)	174 (48)

[1]BrdU, bromodeoxyuridine.
[2]SE, sensory epithelial.
**Significantly different from control value, $P < 0.01$.

frequently lie just above the basilar membrane. Morphologically, the microglia-like cells in the damaged region appear to have plumper cell bodies and shorter processes as compared with those in the undamaged regions (Fig. 3B, inset). Like the macrophage population, the significant increase in the number of microglia-like cells occurred prior to the significant increase in BrdU-labeled SE cells.

Control utricle: Histology

The vestibular labyrinth consists of the otolithic (utricle, saccule) and ampullary organs. The basic cellular composition of the SE is similar in all chicken vestibular organs. The vestibular SE is comprised of two types of hair cells (Type I and Type II hair cells), supporting cells, and the unmyelinated endings of the 8th cranial nerve (Fig. 5C). Mature vestibular hair cells are distinguished on the basis of their morphology and innervation (Wersäll, 1956; Jørgensen and Christensen, 1989). Type I hair cells are pear-shaped and enclosed in a single nerve calyx. Type II hair cells are cylindrically shaped and have multiple bouton-type nerve endings at their basal end. Like the basilar papilla, vestibular supporting cells surround the hair cells. The supporting cells extend from the lumen to the basement membrane, and their nuclei are found below the hair cell nuclei. Extrasensory transitional epithelium, with its cuboidal transitional cells, lies at the periphery of the SE. Fibrocartilaginous stroma, a loose connective tissue, lies under the basement membrane.

In the utricle, the otolithic membrane covers most of the hair cell layer. Following removal of this membrane, hair cells with tall stereocilia bundles can be seen at the lumenal surface of the utricular macula, the sensory epithelium of the utricle (Fig. 5A,B). The lumenal surface of the supporting cells is small, and it is covered with numerous microvilli. Type I hair cells are predominantly present in the "C-shaped" striolar region of the utricular macula.

Control utricle: Immunohistochemistry

Unlike the normal basilar papilla, significant ongoing proliferation has been documented in normal, undamaged utricular macula of the chick and budgerigar (Jørgensen and Mathiesen, 1988; Roberson et al., 1992; Weisleder and Rubel, 1993). In the present study, BrdU-labeled SE cells are detected throughout the macula (mean = 391 ± 135 labeled cells/organ, S.D.; Figs. 1C, 6F, Table 2). Numerous BrdU-labeled cells are also present in the fibrocartilaginous stroma.

Morphologically, utricular macrophages and microglia-like cells look identical (under light microscopy) to their basilar papilla counterparts. A few macrophages are scattered throughout the hair cell layer of the normal utricular macula (mean = 6 ± 4 labeled cells/organ, S.D.; Fig. 2C, Table 2). In contrast, numerous microglia-like cells are present in the normal undamaged utricular macula (mean = 110 ± 33 labeled cells/organ, S.D.), largely within the supporting cell layer (Figs. 3C, 6E). The number of microglia-like cells in the normal macula is significantly greater than that seen for the normal basilar papilla ($P = 0.001$).

Drug-damaged utricle: Histology

Following a single dose of streptomycin, hair cell loss is evident in the " C-shaped" striolar region by 3 days (Fig. 5D,E). Previous studies have shown that Type I cells, the predominant hair cell type in the striolar region, are the most susceptible to aminoglycoside-induced damage (Oesterle et al., 1993; Weisleder and Rubel, 1993).

Drug-damaged utricle: Immunohistochemistry

In drug-damaged utricles, numbers of BrdU-labeled SE cells increase significantly by day 2 (mean = 1,788 ± 470 labeled cells/organ, S.D.; $P < 0.01$; Figs. 1D, 6H, Table 2) and remain elevated through day 4 (mean = 1,225 ± 666 labeled cells/organ, S.D.; $P < 0.01$; Table 2). At the peak of the proliferative increase, the number of BrdU-labeled SE cells in the drug-damaged utricles is roughly five times greater than that in age-matched control utricles. BrdU label is mainly seen in the damaged striolar region (Fig. 6H). By day 10, proliferating cells decline in number and approach control values (mean = 561 ± 143 labeled cells/organ, S.D.; Table 2). Using this experimental paradigm, significant increases in proliferation in drug-damaged utricular maculae precede significant proliferation increases in drug-damaged basilar papillas by one day (see above).

Following drug treatment, the number of macrophages located within the vestibular SE increases significantly by day 1 (mean = 26 ± 22 labeled cells/organ, S.D.; $P < 0.01$; Fig. 2D, Table 2). A fourfold increase in macrophage numbers relative to controls is seen at day 1, and the macrophages are typically located in the striolar region. Macrophages show a reduction in number by day 2 (mean = 7 ± 10 labeled cells/organ, S.D.). As for the BrdU label, the rise and fall of the macrophage number in the utricular macula following drug treatment appears to precede their counterparts in the basilar papilla by 1 day.

Numbers of microglia-like cells also increase by day 1 and are significantly increased compared to controls by day 2 (mean = 220 ± 58 labeled cells/organ, S.D.; $P < 0.01$;

Figs. 3D, 6G, Table 2) and day 4 (mean = 205 ± 65 labeled cells/organ, S.D.; $P < 0.01$; Table 2). At the peak of the increase, twofold more microglia-like cells are present in drug-damaged than control utricular maculae. Similar to the basilar papilla, numbers of microglia-like cells in the macula increase along with the rise in the macrophage numbers. Microglia-like cells are distributed throughout the drug-damaged macula, in the supporting cell layer. Though not statistically significant, the number of microglia-like cells appears to be higher than controls through day 15 (mean = 174 ± 48 labeled cells/organ, S.D.; Table 2).

DISCUSSION

Macrophages, white blood cells derived from a monocyte lineage, play multiple roles in wound repair. Injured proteins, fragmented matrix proteins, and chemotactic stimuli attract macrophages to the site of damage. Macrophages migrate from surrounding tissues and from local blood vessels where their monocytic precursors are constantly being replenished (Roessmann and Friede, 1968; van Furth et al., 1985). Upon activation, macrophages remove damaged tissue and induce repair by releasing mitogenic growth factors and other lymphokines (Baird et al., 1985; Shimokado et al., 1985; Assoian et al., 1987; Nathan, 1987; Madtes et al., 1988; Rappolee et al., 1988; Rom et al., 1988; Higashiyama et al., 1991).

Microglia, a non-neuronal cell type in the CNS with characteristic ramified, highly branched cell processes emanating from a rather small perikaryon, are a form of tissue macrophage generally believed to be specific to the CNS (Dunning, 1935; Mori and Leblond, 1969; Hume et al., 1983; Chugani et al., 1991; Ling and Wong, 1993). Microglia have been described in the brain parenchyma and in the retina (Ling, 1981; Provis et al., 1995). The branched microglial morphology and the expression of major histocompatibility complex (MHC)-class molecules by microglial cells is believed to enable microglia to establish specific neuronal connections during CNS development (Hume et al., 1983; Hayes et al., 1987; Perry et al., 1993). Microglia and macrophages are derived from a common monocytic lineage and share the same biochemical processes and immunodetectable antigens, including common leukocyte antigen also known as CD45 (Chugani et al., 1991; Provis et al., 1995). Functionally, microglia (and macrophages) play an active role in phagocytic and secretory activities. Like macrophages, microglia secrete various mitogenic factors, lymphokines, and neurotrophic factors making them important in CNS tissue repair following injury (Giulian et al., 1989; Mallat et al., 1989; Plata Salam'an, 1991; Shimojo et al., 1991; Lindholm et al., 1992; Perry et al., 1993).

To the best of our knowledge, this is the first report demonstrating the presence of microglia-like cells in the in vivo inner ear. Microglia-like cells and macrophages were identified in both normal and drug-damaged avian inner ear end organs. The inner ear sensory epithelium has typically been considered to be composed of hair cells, supporting cells, and unmyelinated endings of the eighth cranial nerve. A resident population of leukocytes also appears to be present within the inner ear sensory epithelium. Several studies have reported the presence of macrophages in vivo in normal and regenerating lateral line organs (Jones and Corwin, 1993, 1996). Increased numbers of macrophages are found in the mammalian cochlea following labyrinthine infection (Nomura et al., 1988) and acoustic trauma (Bohne, 1971; Fredelius and Rask, 1990). Histochemically and immunocytochemically identified macrophages have also been identified in cultured avian basilar papilla (Warchol, 1997). The findings of the present study indicate that a resident population of macrophages is present normally within the undamaged auditory and vestibular SE. In addition, yet another cell type, microglia-like cells, are also a constituent of the normal inner ear sensory epithelium. Studies of the inner ear sensory epithelium must take into account the presence and potential impact of these cell types on inner ear function.

The identification of a dynamic population of macrophage and microglia-like cells in the supporting cell layer raises several issues affecting this study and previous regeneration studies utilizing thymidine analogues (e.g., BrdU and tritiated thymidine). Many studies have presumed that all or most thymidine analogue-labeled cells in the supporting cell layer are in fact supporting cells (e.g., Girod et al., 1989; Hashino and Salvi, 1993; Stone and Cotanche, 1994; Warchol and Corwin, 1996). It is possible that some of these labeled cells are macrophage or microglia-like cells, particularly in the utricle where there are large increases in the absolute numbers of LT40-labeled cells following damage. Tritiated-thymidine-labeled leukocytes have been detected in cultured basilar papilla lesioned with laser microbeam (Warchol, 1997). Because of technical limitations, we did not perform BrdU/LT40 double-labeling in the wholemount specimens. The lack of double-labeling data does not change the fundamental findings of this study, nor their interpretation; a significant increase in the macrophage and microglia-like populations occurs prior to a significant increase in BrdU-labeled cells. If the initial increase in BrdU-labeled cells is due in part, or in whole, to macrophage and microglia-like cell proliferation, this would mean that the real increase in supporting cell proliferation occurs later than the raw BrdU data implies. Adjusting the BrdU counts accordingly would further underscore the build-up of the macrophage and microglia-like cell populations prior to supporting cell proliferation.

Whereas BrdU/LT40 double-labeling using the present BrdU-injection protocols could be useful, it would not resolve the origin of the burgeoning, postdamage macrophage and microglia-like cell populations. Considering the mobility of macrophages, a BrdU-labeled macrophage could have gone through the cell cycle prior to migrating to the sensory epithelia. Likewise a BrdU-labeled microglia-like cell could be derived from the differentiation of a BrdU-labeled migrating macrophage. A shorter pulse-labeling protocol, or a noncumulative proliferation marker (e.g., Histone 3 in situ mRNA detection), might be helpful in overcoming the limitations of the present study.

It is possible that some cells identified as macrophages in this study included other leukocytic cell types such as the granulocytes. Little is known about granulocytes in the inner ear epithelia. A few basophilic granulocytes have been seen with transmission electron microscopy in sound-damaged basilar papillas (Oesterle, unpublished results). Macrophages far outnumber the rare basophils in the blood (Bellanti, 1985) and in typical wound response experiments. Hematoxylin and Eosin-stained sections of damaged auditory and vestibular end-organs have not identified significant numbers of characteristic basophils either in the periphery or in the microvascular structures.

Similarly, it must be recognized that other leukocytic cell types, such as gamma delta T-cells or dendritic cells, may share the dendritic morphology and CD45-positivity of the microglia-like cells in the inner ear sensory epithelium (Thomas et al., 1993; Ulvestad et al., 1994; Ol'ah and Glick, 1995; Johnson et al., 1996). Lymphocytes have been detected in the scala media (Gloddek et al., 1991) and scala tympani (Gloddek et al., 1991; Yamane et al., 1995) of the mammalian cochlea, but they have not been identified within the sensory receptor epithelium, the organ of Corti. Work to definitively ascertain the cell type of the microglia-like cell is likely to be a fruitful direction for future research. Since these other cell types also elaborate many of the same growth factors which microglia elaborate (reviewed in Haas et al., 1993 and Yamamoto et al., 1993), the effect on end-organ repair would be similar. As it is in other instances of wound healing, the initial macrophage presence could still be the major driver for recruitment and proliferation of a putative dendritic T-cell population.

Following aminoglycoside-induced hair cell death, increased numbers of macrophages and microglia-like cells are seen in avian auditory and vestibular sensory epithelia. The preponderance of macrophage and microglia-like cells are located in the area of hair cell damage, in the proximal (high frequency) portion of the basilar papilla and in the striolar region of the utricle, demonstrating the specific nature of the response (Fig. 6). Significant increases in numbers of macrophages are detected 24 hours prior to a significant increase in SE cell proliferation, as detected by BrdU incorporation. Our findings are qualitatively similar to those of Jones and Corwin (1996) who demonstrated macrophage recruitment to laser-damaged neuromasts prior to any proliferative increase by neuromast supporting cells. Our findings are also in agreement with the recent report of Warchol (1997) in cultured basilar papilla who detected the recruitment of macrophages to sites of laser-induced hair cell lesions approximately 4 to 8 hours after the lesion. The first basilar papilla supporting cells to undergo regenerative proliferation in response to laser-induced hair cell death enter S-phase approximately 16 hours after the lesion (Warchol and Corwin, 1996). Thus, macrophages appear to be in situ early enough to undergo activation and to potentially release various factors such as PDGF, IGF-1, TGFαand EGF (Nathan, 1987; Rappolee et al., 1988). IGF-1 has been demonstrated to be mitogenic for the avian vestibular sensory epithelium (Oesterle et al., 1997). Release of these or other mitogenic factors may stimulate hair cell precursors to enter and progress through the cell cycle (Jones and Corwin, 1993, 1996). It is also possible that release of proteases by activated macrophages may play a role in modifying hair cell or supporting cell contacts with the precursor cell population, thus initiating the signal for the precursor cells to leave the G_0 phase and enter the cell cycle (Nathan, 1987; Bhave et al., 1995). Supporting cells in undamaged areas of the basilar papilla have been shown to upregulate proliferating cell nuclear antigen (PCNA) in concert with supporting cells in damaged areas, evidence for entry into the early part of the active cell cycle by cells not known to progress through S phase (Bhave et al., 1995). Increases in the numbers of macrophage and microglia-like cells throughout the basilar papilla following damage places them temporally and spatially where they could be involved in this generalized supporting cell response (Fig. 6). It should be noted, however, that there is currently no direct evidence that macrophages or microglia-like cells play a stimulatory role in hair cell regeneration.

Similar to the macrophage population trends, significant increases in the number of microglia-like cells were observed in the damaged sensory epithelium prior to an increase in SE cell proliferation. However, numbers of microglia-like cells continued to increase and remained high following a decline in macrophage numbers. This pattern is consistent with the hypothesis that the microglia-like cell population is derived, at least in part, from the migrating macrophage population (Hume et al., 1983; Perry et al., 1985). Similar to their putative role in CNS histogenesis and damage repair, an increased number of microglia-like cells might be necessary to direct repair of the sensory epithelium following structural disruption (Giulian et al., 1989). The morphologic changes noted in the sensory epithelial microglia-like cells may reflect their differentiation into activated or reactive forms as seen in response to CNS injuries (Andersson et al., 1991; Davis et al., 1994). The rapid increase in inner ear macrophage and microglia-like cell numbers postinjury is distinctly different from changes seen after CNS injury when the blood-brain barrier is intact (Davis et al., 1994). In the CNS, the blood-brain barrier prevents the influx of blood monocytes, the immediate precursors of microglia, into the brain parenchyma, and new microglia are formed in situ from their embryonic precursors. The microglia population remains stable throughout life and little turnover occurs. In contrast, the anatomical absence of a blood- brain barrier in the inner ear allows a large number of blood monocytes to infiltrate into the ear and mature into microglia-like cells. Because of the constant availability of precursor monocytes, a rapid rise in the number of microglia-like cells can occur after inner ear injury.

In normal inner ear sensory epithelia, microglia-like cells may play a role in the clearing of extracellular fluid, neurotransmitter substances, and the maintenance of normal tissue homeostasis (Murabe and Sano, 1982). Twenty-eight times more microglia-like cells were seen in the normal, undamaged utricular macula than in the normal basilar papilla. This may be related to the continuous turnover of hair cells that is thought to occur normally in the vestibular epithelia (Jørgensen and Mathiesen, 1988; Jørgensen, 1991; Roberson et al., 1992; Weisleder and Rubel, 1993; Kil et al., 1997) and to the absence of any significant hair cell turnover in the normal basilar papilla (Oesterle and Rubel, 1993; Mason et al., 1995). Microglia-like cells may play a role in phagocytosis of dying hair cells and the maintenance or repair of neural endings which interface with the constantly regenerating hair cell population (Pow et al., 1989). The phagocytic role of the macrophage population both during regeneration and in undamaged lateral line neuromasts has been demonstrated by time-lapse microscopy (Jones and Corwin, 1993, 1996), and macrophages in cultured basilar papilla have phagocytized latex beads that were added to the culture medium (Warchol, 1997). The phagocytic activity by LT40-positive microglia-like cells remains to be demonstrated. With respect to the regenerating ear, the role of the microglia-like cells may not be totally supportive in nature. Giulian demonstrated the potential for microglia to secrete neurotoxic substances during CNS repair processes. He postulated that the microglia may act as a counterbalance to other cells such as astroglia which were secreting neurotropins. It is possible that the increase in numbers of

microglia-like cells leads to an inhibition of nerve cell regrowth prior to hair cell repopulation in the inner ear end organs.

As previously noted, significant increases in the macrophage population and in SE cell proliferation in the utricular macula occurred approximately 1 day earlier than their respective counterparts in the basilar papilla. In contrast to the utricle, ongoing proliferation in the basilar papilla is rare (Oesterle and Rubel, 1993). It is possible that with the ongoing proliferation in the utricle, precursor cells may be primed to enter the active cell cycle rapidly due to the presence of inductive factors already present in the epithelium prior to damage (Jørgensen and Mathiesen, 1988; Roberson et al., 1992). These same factors, or other associated factors, may be accelerating the increase in the macrophage and microglia-like cell numbers in the utricle compared to the basilar papilla. In such a scenario, macrophages and microglia-like cells would not be necessary for the earliest changes in sensory epithelial cell cycle status, but may be required for continued progression through the cell cycle (Bhave et al., 1995). It is also possible that significant increases in the macrophage population and in SE cell proliferation in the utricular macula as compared to their respective counterparts in the basilar papilla may be the result of the different lesion paradigms that were used (streptomycin vs. gentamicin) and different aminoglycoside dosages.

In conclusion, an increase in sensory epithelial cell proliferation is seen in the in vivo avian auditory and vestibular epithelia following single-dose aminoglycoside treatment. The proliferative increase is preceded by an increase in the number of macrophages and microglia-like cells in the sensory epithelium. As such, macrophages and microglia-like cells may be playing an important role in initiating early signals for cell cycle progression in damaged inner ear sensory epithelium.

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