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Relationship of monoclonal antibody (KHRI 3 epitope) to cochlear supporting cell microvilli in the guinea pig

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Summary. As reported previously, monoclonal antibodies can be generated that bind against guinea pig cochlear structures. Preliminary immunohistochemical characterization revealed that one of these monoclonal antibodies (KHRI 3) most probably binds against a surface structure of guinea pig cochlear supporting cells. This study was undertaken to further characterize the KHRI 3 epitope in the cochlea. Since KHRI 3 immunolabeling appeared to be punctate and epitope expression was most pronounced in the reticular lamina, we hypothesized that KHRI 3 epitopes are related to microvilli. To prove this hypothesis immunoelectron microscopy was used. Also investigated was how epitope expression is altered in the reticular lamina microvilli following drug or noise-induced changes. When immunocytochemical results were compared to scanning electron microscopy findings, a striking correlation could be seen between changes in KRHI 3 immunolabeling and changes in the distribution of microvilli. These findings support the assumption that KHRI 3 epitopes are related to microvilli of inner ear supporting cells.

Key words: Cochlear supporting cells – Microvilli – Monoclonal antibodies – Guinea pig

Introduction

We previously reported that murine monoclonal antibodies against guinea pig cochlear epithelium were generated with the goal of differentiating subsets of cells in the auditory end organ based on antigen characteristics [11, 16]. In these studies, homogenates of cochlear tissue containing hair cells and supporting cells were used as the antigen in two immunization procedures utilizing an intrasplenic antigen injection designed to compensate for the limited amount of antigenic material. Based on ELISA screening for antibody production in hybridoma supernatants and for binding to homogenates from cochlea, liver, lung, kidney and brain, three monoclonal antibodies designated KHRI 1, 2, and 3 were selected for further study because they were reactive only with cochlear homogenates.

KHRI 3, an IgG1 antibody, was found to stain the phalangeal processes of outer pillar cells and the apical processes of phalangeal cells. The antigen recognized by KHRI 3 was partially characterized by sodium dodecyl sulfate gel electrophoresis, and immunoblotting on nitrocellulose. KHRI 3 detected a broad band of M_r 70–75 kDa in detergent extracts of the cochlea. A discrete band, M_r 70 kDa, was stained by KHRI 3 in extracts of tongue and brain. The antibody also reacted with peripheral nerves in unfixed frozen sections of other tissues, but the antigenic site was not stable to fixation. In contrast, the epitope detected in the cochlea was stable to fixation by 2% paraformaldehyde. Although immunoreactive antigen was detectable in Western blots of brain, no discrete staining in brain sections could be identified. The results of our previous investigations indicated that monoclonal antibody KHRI 3 defines epitopes specifically expressed by distinct populations of supporting cells in the guinea pig cochlea [16]. The present study was thus carried out to further characterize the KHRI 3 epitope in these populations of supporting cells. In general the KHRI 3 epitope seemed to be expressed in the reticular lamina where immunolabeling was prominent and where immunolabeled punctae could be seen. To add supporting evidence that KHRI 3 labels microvilli, morphological changes in the reticular lamina were also studied after ototoxic drug exposure, in order to determine if changes in the distribution of microvilli reflected changes in KHRI 3 immunolabeling. To exclude immunolabeling changes due only to drug effects, cochleae were used from guinea pigs exposed to noise and exhibiting morphological changes in the reticular lamina.

Materials and methods

Animals

Young healthy pigmented guinea pigs (Murphy's Breeding Lab, Plainfield, Ind., USA) weighing approximately 200 g were maintained in the Unit of Animal Medicine, University of Michigan.

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Deafening

Twenty-two guinea pigs were anesthetized with ketamine (Ketalar; Parke-Davis, New York, N.Y., USA) 25–40 mg/kg body wt. and xylazine (Rompun; Mobay Corp., Animal Health Division, Shawne, Kan., USA) 5–10 mg/kg body wt. Fifteen minutes after anesthesia, kanamycin sulfate (USP, 1 g/3 ml; Quad Phamaceuticals, Indianapolis, Ind., USA) was injected i.m. at a dose of 400 mg/kg body wt. Fifteen minutes later, ethacrynate sodium (Intravenous Sodium Edecrin; Merck-Sharp-Dohme, West Point, Pa., USA) 40 mg/kg body wt. was injected intracardially [14, 15].

Noise exposure

Five animals were placed in a sound-proof box and exposed to 100 dB white noise for 5 h each day for 10 days. The animals were then allowed to recover for 21 days, at which time they were killed as discussed in the experimental design.

Experimental design

Two animals each were killed at 6, 12, 18 and 24 h and 2, 4, 6, 8, 10, 14 and 21 days after drug treatment, while the five guinea pigs exposed to noise were killed 21 days after noise exposure. Immediately after death of the animal the cochleae were removed and one cochlea of each drug-deafened animal was used for immunocytochemical assays, the other for scanning electron microscopy.

Immunocytochemical assays

KHRI 3 was applied to segments of the guinea pig cochlea using immunofluorescence and immunoperoxidase procedures respectively. Tricolored guinea pigs were deeply anesthetized with urethane and perfused transcardially with phosphate buffered saline (PBS) followed by 2% paraformaldehyde in PBS (pH 7.4) at 4°C. Temporal bones including cochleae were removed and fixed locally with the same fixative flushed through the round window. For immunoperoxidase and immunofluorescence procedures, the bony shell of the cochlea, vascular stria, tectorial membranes and Reissner's membranes were removed, leaving whole mount surface preparations with the organ of Corti exposed. These latter specimens were rinsed in PBS for at least 1 h, as described in detail previously [1, 2, 4–6].

Immunoperoxidase procedures. Prior to each incubation specimens were washed three times for 3 min in PBS. Blocking of nonspecific binding sites was achieved by incubation with 3% horse serum for 10 min. Excess horse serum was removed from free floating whole mount preparations or blotted from slides. Specimens were then incubated for 30 min with KHRI 3 (diluted 1:10). The second biotinylated anti-mouse IgG antibody was diluted 1:200 and incubation time was 30 min. Specimens were next incubated with Vectastain ABC reagent [8] for 45 min. Finally, specimens were incubated for 5 min with peroxidase substrate solution (diaminobenzidine 10 mg diluted in 20 ml PBS containing 70 µl 3% H_2O_2). Whole mount preparations were then dehydrated in ethanol (70% for 5 min and 100% twice for 5 min after which they were dipped in xylene) and mounted wet on slides in Krystalon (EM Diagnostic Systems, Gibbstown, N.J., USA).

Immunofluorescence. Labeling was performed following the protocol described for immunoperoxidase, but incubation with ABC complex was substituted by incubation with avidin-conjugated rhodamine (Vector Laboratories, Burlingame, Calif., USA), followed by three washes in PBS. Filamentous actin was decorated and visualized by 30 min incubation of whole mounts with fluorescein-phalloidin. Specimens were washed again and mounted as described above.

Documentation

Slides were examined and photographed on a Leitz Dialux microscope with Kodak T-max 100 film. Fluorescence slides were examined using a Leitz Orthoplan fluroescence microscope and photographed with Kodak T-max 400 film.

Scanning electron microscopy

Surface preparations of the cochlea were fixed with 4% paraformaldehyde for 1 h. After several rinses with PBS, preparations were dehydrated as described and incubated three times with hexamethydisalizane (HMDS; Polysciences, Warrington, Pa., USA), replacing critical point drying. Specimens were then coated with 15–20 nm gold, using a Polaron SEM Coating sputter system at 1.5 kV, and examined on a ISI DS 130 dual-stage scanning electron microscope.

Transmission electron microscopy

Specimens were prepared as described for immunocytochemistry (immunoperoxidase procedure). To enhance labeling, specimens were postfixed with 1% OsO_4 for 1 h at 4°C. After dehydration in alcohol, tissue was embedded in EmBed 812 (Electron Microscopy Sciences, Washington Pa., USA). After polymerization blocks were trimmed and ultrathin sections were cut at 90 nm on a Reichert Mikrotom and mounted on 400 mesh grids for examination under a JEOL 1200 transmission electron microscope.

Results

Examination of the organ of Corti from healthy animals showed that KHRI 3 most strongly immunostained the phalangeal processes of outer pillar cells (Fig. 1a) and the inner border cells (Fig. 1b). There was no immunostaining associated with inner (IHC) or outer (OHC) hair cells. In frozen sections of the organ of Corti immunofluorescence labeling was found weakly around the apex of IHC and adjacent to the apex of OHC (Fig. 1c). Immunoperoxidase staining was found in a similar appearance at the phalangeal processes of outer pillar cells (Fig. 1d) and inner border cells (Fig. 1e). Additionally nonspecific staining of stereocilia (as seen in Fig. 1d) was sometimes observed. In semi-thin sections using immunoperoxidase, labeling appeared to be more widely distributed, since the whole reticular lamina exhibited peroxidase labeling (Fig. 1f). Even at a high magnification (× 1000), immunolabeling was strictly restricted to surface structures.

With respect to inner border cells TEM results matched frozen section light microscopic findings. Figure 2a shows dark immunoperoxidase staining of inner border cell microvilli of normal animals. Immunolabeling is absent from IHC stereocilia and from other cell membrane structures. However, immunoperoxidase findings in phalangeal processes of outer pillar cells were not as conclusive as staining in inner border cells. In Fig. 2b immunolabeling is seen as a dark stripe at the surface of outer pillar phalangeal processes although individual microvilli are not readily distinguished.

On surface preparations from normal guinea pigs and from most drug-deafened animals 6 h after drug administration, a striking "wine-glass" pattern of immunostain-

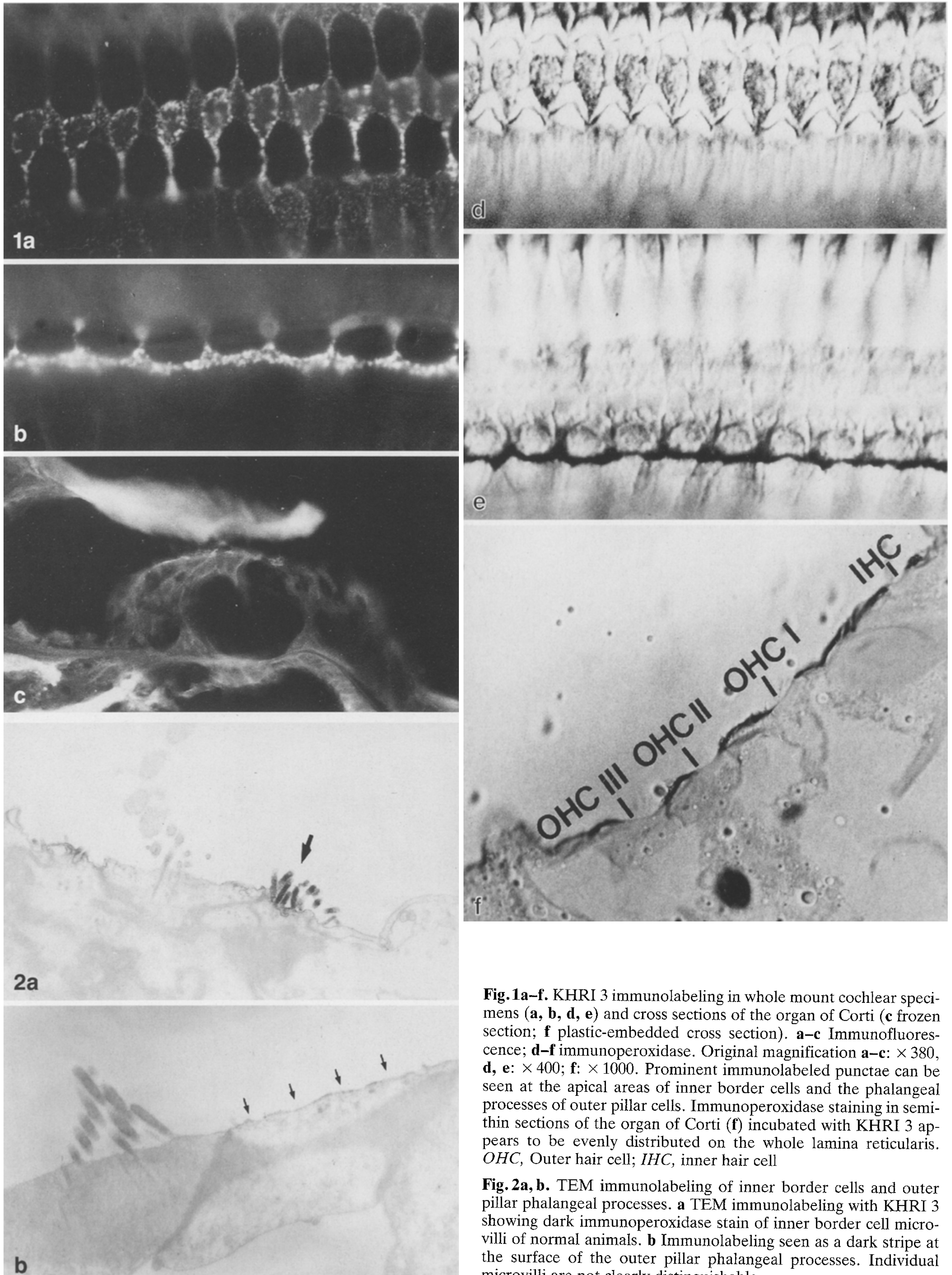
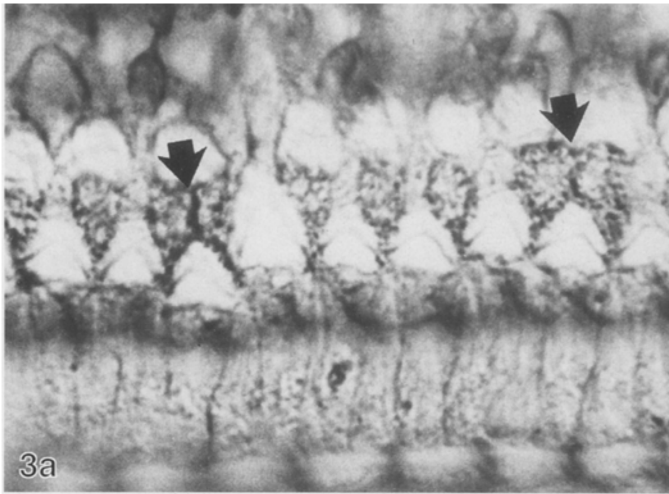


Fig. 1a–f. KHRI 3 immunolabeling in whole mount cochlear specimens (**a, b, d, e**) and cross sections of the organ of Corti (**c** frozen section; **f** plastic-embedded cross section). **a–c** Immunofluorescence; **d–f** immunoperoxidase. Original magnification **a–c**: × 380, **d, e**: × 400; **f**: × 1000. Prominent immunolabeled punctae can be seen at the apical areas of inner border cells and the phalangeal processes of outer pillar cells. Immunoperoxidase staining in semithin sections of the organ of Corti (**f**) incubated with KHRI 3 appears to be evenly distributed on the whole lamina reticularis. *OHC,* Outer hair cell; *IHC,* inner hair cell

Fig. 2a, b. TEM immunolabeling of inner border cells and outer pillar phalangeal processes. **a** TEM immunolabeling with KHRI 3 showing dark immunoperoxidase stain of inner border cell microvilli of normal animals. **b** Immunolabeling seen as a dark stripe at the surface of the outer pillar phalangeal processes. Individual microvilli are not clearly distinguishable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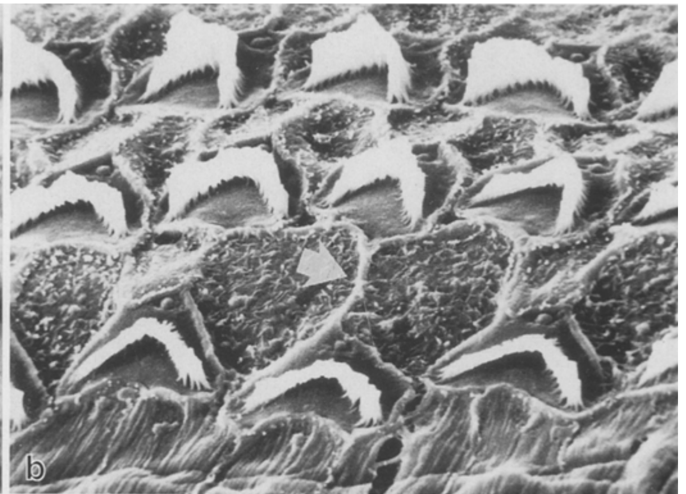
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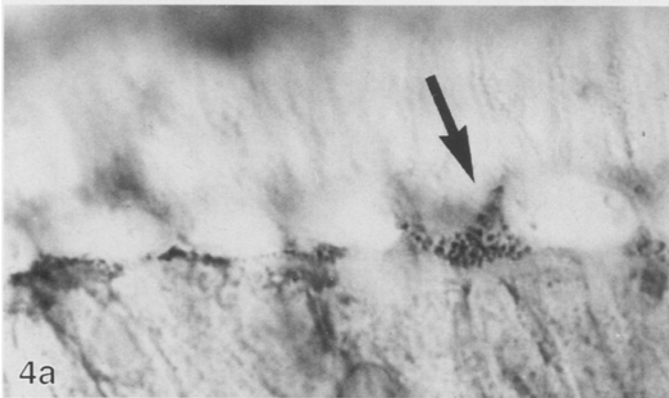
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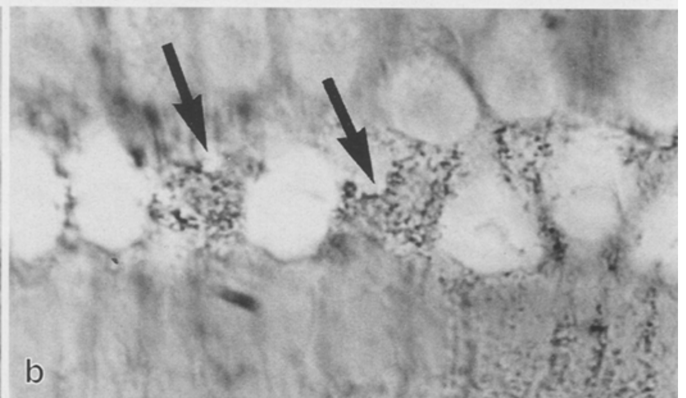
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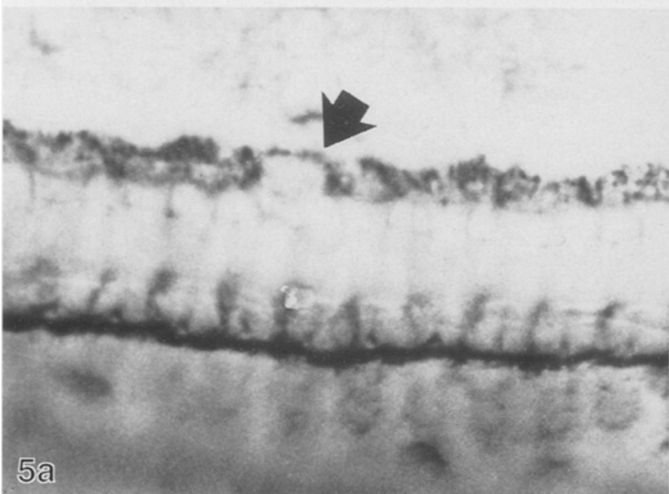
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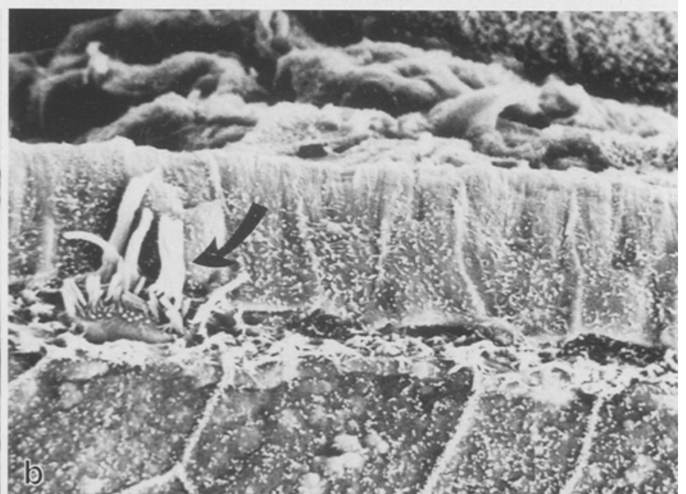
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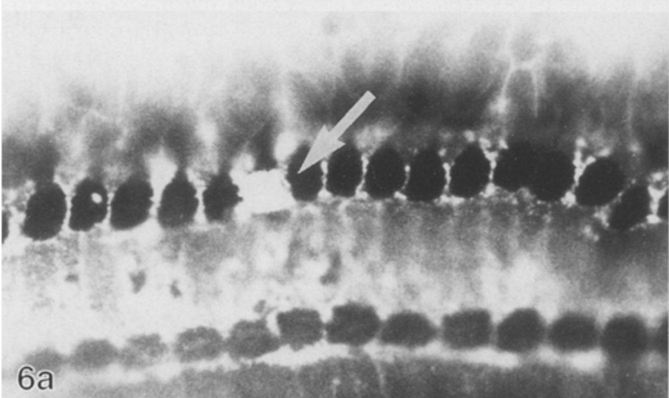
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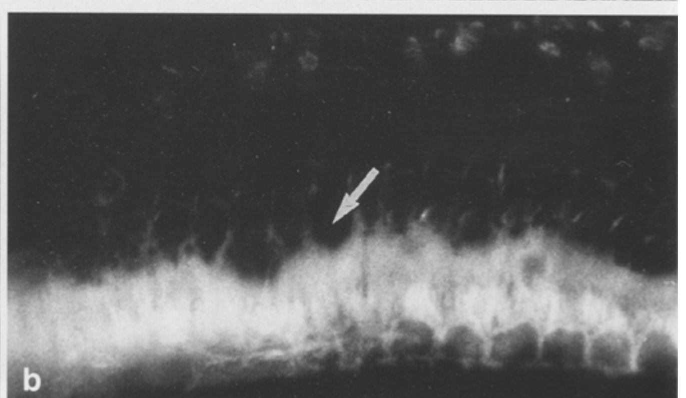
b

ing was observed in the reticular lamina with either immunoperoxidase or immunofluorescence techniques (Fig. 1). On scanning electron microscopy (SEM), morphology of most specimens seemed to be normal. However, changes in KHRI 3 immunolabeling were detectable as early as 6 h after drug deafening in some specimens. SEM at this time revealed that some phalangeal processes of outer pillar cells, which were normally separated by a phalangeal process of a Deiters cell, were now in contact with each other (Fig. 3b). Corresponding immunolabeling of KHRI 3 showed the same immunolabeling distribution pattern (Fig. 3a).

Subsequently, typical IHC and OHC cuticular plates with stereocilia disappeared, indicating cell death. As a "replacement" for cuticular plates, flat epithelial surfaces carrying microvilli were now seen. In comparison, KHRI 3-immunolabeled punctae were observed in areas presumably once having accommodated cuticular plates. Figure 4a shows an increase in an area formerly occupied by an IHC that now exhibits KHRI 3 immunolabeling. In places formerly occupied by OHC, KHRI 3 immunolabeling could also be observed. After 2–3 weeks most of the IHC seemed to be extinct as well. Occasional residues of IHC cuticular plates could be detected (Fig. 5a, b), in which the broad band of immunolabeled punctae was interrupted every so often. SEM also revealed residues of IHC, while all other cuticular plates of these cells seemed to be replaced by cell surfaces with long microvilli.

These changes were seen in all specimens, although the time course varied inter-individually.

The damage observed in noise-treated animals was considerably smaller than in drug-treated cochleae. Only a few OHC and IHC seemed to have vanished. Missing hair cells were easily detected by absence of typical actin-labeling of the cuticular plate and stereocilia. In areas with apparently missing hair cells KHRI 3 immunolabeling seemed to invade the area of IHC or OHC. As seen in Fig. 6b, absence of phalloidin staining indicated that one first-row OHC was missing. Using double labeling, KHRI 3 immunolabeling was observed at this very spot (Fig. 6a).

Discussion

Specialized membrane components enable cells to respond to specific signals, such as hormones, neurotransmitters or mechanical stimuli. In the organ of Corti, each of the diversified cell types is likely to play a unique functional role. The precise contribution of each of these elements to cochlear function under physiological and pathological conditions is not always known. In particular, the role of supporting cells during or after hair cell damage, before a final scar is formed, is still controversial. Recent studies indicate that supporting cells increase in size as damaged hair cells decrease in size, thus sealing the reticular lamina and preventing merging of the endolymph and perilymph [13]. Candidates for cells to increase in size after hair cell death are supporting cells adjacent to sensory cells. Among these are inner border cells and outer pillar cells.

From previous studies it is now known that inner border cells and the phalangeal processes of outer pillar cells possess numerous microvilli [10]. Other studies have found that microvilli increase in number following procedures to damage the sensory epithelium of the cochlea [3, 7, 9, 12]. KHRI 3 immunolabeling can be found in a punctate fashion at the apical surfaces of these cells. Thus, it has been tempting to hypothesize that KHRI 3 immunolabels a microvilli-related antigen. The most accountable way to prove this hypothesis involves immunoelectron-microscopic investigations. As shown in our present study, EM results confirmed that KHRI 3 immunolabeling is associated with a structure related to microvilli. However, we felt that supporting evidence was needed for the hypothesis that KHRI 3 also labels outer pillar cell microvilli. To obtain such evidence the drug-deafened animal model was used, especially since it is now known that drug deafening can cause dramatic changes in the structure of the lamina reticularis and in the distribution of microvilli. By using this model we were able to compare KHRI 3 immunolabeling to SEM findings. Changes in the distribution of F-actin also gave additional hints to the fate of sensory cells and cells invading the space formerly occupied by the sensory cells.

Our results showed a time course for morphological changes of sensory cells and supporting cells identical to changes in the organ of Corti described by other investigators [9, 12]. During the first days after drug damage an increase in the area exhibiting immunolabeled punctae was seen. (It was our impression that the number of immunolabeled punctae was increased, but immunolabeled punctae were not counted to valididate this assumption).

The changes seen were followed by a decrease in the area carrying immunolabeled punctae. A comparison of SEM morphology with KHRI 3 immunolabeling showed a striking correlation between the area carrying microvilli and the area exhibiting KHRI 3 immunopositive punctae. These results support the hypothesis that KHRI

Fig. 3a, b. Specimens 6 h after drug deafening. KHRI 3 immunolabeling shows an approximation of outer pillar cell phalangeal processes *(arrows)* **a** Immunoperoxidase stain, original magnification × 300; **b** SEM specimen

Fig. 4a, b. Immunoperoxidase-labeled specimen after 2 or more days, demonstrating KHRI 3 immunolabeling *(arrows)* in areas formerly occupied by inner hair cells (**a**) or outer hair cells (**b**). Original magnification × 420

Fig. 5a, b. Specimens 2 weeks after drug deafening. KHRI 3 immunolabeling (**a**) is seen as broad band of labeled punctae in the region thought to accommodate inner hair cells (original magnification × 400). On SEM (**b**) only a few inner hair cell residues can be seen *(arrow)*. The splace formerly occupied by inner hair cells is now covered by surfaces of cells with long microvilli

Fig. 6a, b. Surface preparation of guinea pig organ of Corti following noise deafening. KHRI 3 immunolabeling (**a**) is detected in areas thought to accomodate outer hair cells *(arrow)*. Double labeling with phalloidin staining (**b**) reveals absence of typical actin staining pattern of outer hair cells. (Original magnification × 400)

3 binds to an epitope expressed in microvilli of a subset of cochlea-supporting cells (i.e. outer pillar cells, inner border cells).

The structure and the structure-function relationship of the antigenic molecule remain to be investigated. Few studies have attempted to investigate the function or molecular composition of supporting cell microvilli in general, although much is known about microvilli in other organs, where they are usually involved in resorption processes (e.g. in the gut). It has been suggested that microvilli in the cochlea resorb debris after hair cell damage [7]. After inner ear damage by ototoxic drugs, an increase in the microvilli surface area and increased KHRI 3 immunolabeling were seen. The correlation between this pattern of immunostaining and microvilli suggest a change in microvilli – associated functions after hair cell insult. Further studies will have to determine whether KHRI 3 epitopes are expressed in other parts of the inner ear, whether KHRI 3 epitopes can be found in other species and whether KHRI 3 epitope can be characterized on a molecular level.

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