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THE EFFECT OF BACTERIAL ENDOTOXIN UPON THE MORPHOLOGY OF THE TECTORIAL MEMBRANE AND STEREOCILIA IN THE GUINEA PIG COCHLEA

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Abstract

Endotoxin of E coli was microperfused into scala tympani or injected into the cerebrospinal fluid in anaesthetised pigmented guinea pigs. The effects of endotoxin on the cochlea were studied using electrophysiological techniques and scanning electron microscopy. We found a drop in the amplitude of the cochlear microphonics and compound action potentials 2 to 2.5 hours after injection. There were also changes in the morphology of stereocilia and the tectorial membrane. The stereocilia lost their rigidity and the tectorial membrane appeared swollen. These effects were less severe in animals which were pretreated with dexamethasone.

Key Words: Bacterial endotoxin, cochlea, guinea pig, hair cells, tectorial membrane, stereocilia, dexamethasone, steroids.

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Introduction

Sensorineural deafness is a well known complication of bacterial meningitis. A possible target of the disease is the cochlea. Our recent studies have shown that the cell wall lipopolysaccharide (LPS) of E coli (endotoxin) when perfused into the cochlea, produces both electrophysiological and morphological changes (see Tarlow *et al.*, 1991). However the precise mechanism(s) which are responsible for these changes still remain to be elucidated. It is well established that endotoxin, a cell wall component of Gram negative bacteria is not in general deleterious to mammalian cells *in vitro*. However, *in vivo* endotoxin initiates the release of cytokines such as tumour necrosis factor (TNF) and interleukin-1 (IL1) which are thought to be responsible for the inflammatory response (Mustafa *et al.*, 1989). TNF in particular has been shown to kill a variety of cells *in vivo* and *in vitro* (see Larrick and Wright, 1990). The release of cytokines can be suppressed by dexamethasone (Beutler and Cerami, 1987) and in a recent study dexamethasone, administered concurrently with antibiotic therapy in cases of meningitis, reduced the incidence of deafness (Lebel *et al.*, 1988, 1989; Girgis *et al.*, 1989). This would indicate that the deleterious sequelae of bacterial meningitis may be due to released cytokines.

We set out to investigate whether the changes observed in the cochlea could be attributed to the inflammatory response which follows endotoxin challenge *in vivo*. The effects of LPS upon the morphology of the cochlea as revealed by scanning electron microscopy, are reported here.

Methods

Pigmented guinea pigs weighing between 500 and 700 g were anaesthetised with urethane injected intraperitoneally. The trachea was cannulated and the left cochlea exposed. A hole of about 50 μm was drilled in the basal turn to allow microperfusion of scala tympani. A slightly larger hole was drilled at the apex to allow the perfusate to flow out. A glass micropipette (tip

diameter < 50 μm), coupled via polythene tubing to a microinfusion pump (Vickers IP-3), was inserted into the basal hole so as to form a tight fluid seal. Endotoxin was dissolved in artificial perilymph (Konishi and Kelsey, 1973) and perfused at a rate of 17 μl per minute for one minute. The amount of endotoxin perfused varied from 100 ng to 100 μg. Similar control perfusions were performed using artificial perilymph alone. A platinum wire electrode sealed into the micropipette allowed measurements to be taken of sound evoked cochlear potentials. Cochlear microphonics were elicited by 5 kHz 500 ms tone pips, and the compound action potential was elicited by 10 kHz 1 ms tone pips. The peak to peak height of the microphonics and the peak height of the N1 component of the compound action potential were measured before and immediately after endotoxin perfusion, and at 30 minute intervals thereafter for 2 to 3 hours. In the case of the compound action potential, the increase in level (dB) of the tone pip required to restore the height to the pre-perfusion control value, was determined. In some experiments, the endotoxin was injected into the cerebrospinal fluid through cisterna magna. Some animals were injected with dexamethasone (1 mg/kg) one hour before cochlear microperfusion with endotoxin.

At the end of the experiment the left cochlea was intravitally fixed by microperfusion of cold (4 °C) fixative containing 2.5% glutaraldehyde, 0.05 M BES (2-hydroxyethyl-2-aminoethane sulphonic acid) and 0.05 M sucrose. The pH was adjusted to 7.4 with NaOH. The right cochlea was fixed by a similar perfusion immediately post mortem. The temporal bones containing the cochleae were immersed in the fixative and stored for a minimum of 24-48 hours at 4 °C. After dissection the bony modiolus, including the organ of Corti, was dehydrated in acetone and dried by the critical point method in liquid CO_2. The specimens were sputter coated with platinum to a depth of 12 nm and examined in scanning mode under a JEOL 120 CXII electron microscope (for more details see Osborne and Comis, 1991).

Results

The time course of action of endotoxin was fairly consistent in these experiments and the effect reached a peak between 2 to 2.5 hours. We were able to show that both the cochlear microphonics and the compound action potential declined in height at this time. There was however a marked inter-animal difference in sensitivity to endotoxin. In some animals considerable losses (approximately 20 dB) were obtained for example by microperfusion of 0.5 to 1 mg into the cochlea, whereas in others much smaller effects were seen even with 50 to 100 mg. Control microperfusions with artificial perilymph had no effect on sound evoked responses or on the morphology of the cochlea.

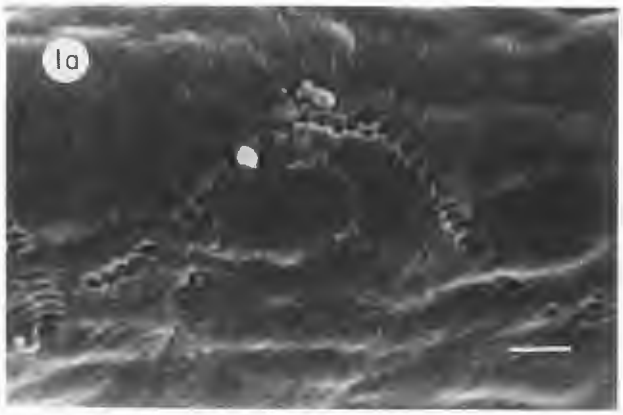
Figure 1a, 1b. Inferior surface of tectorial membrane showing: **a)** showing clearly defined footprints of stereocilia of outer hair cells in a control specimen (bar = 1 μm); and **b)** almost complete obliteration of outer hair cell stereocilial footprints, note also the coarser texture of the surface of the tectorial membrane (bar = 500 nm).

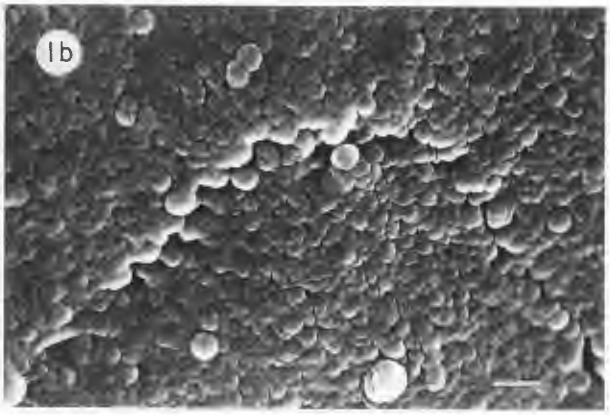
Figure 2a, 2b. Endotoxin treated. **a)** Stereocilia from inner hair cells have become detached and adhered to the inferior surface of the tectorial membrane (bar = 500 nm); and **b)** fragments of stereocilia from an outer hair cell adhering to the inferior surface of the tectorial membrane, note occlusion of footprints (bar = 500 nm).

Figure 3a, 3b. Endotoxin treated. **a)** A hair bundle of an outer hair cell from row 2 showing disruption and splaying of stereocilia (bar = 1 μm); and **b)** as (a) above, but showing hair bundles of inner hair cells, note splaying and disruption of stereocilia (bar = 1 μm).

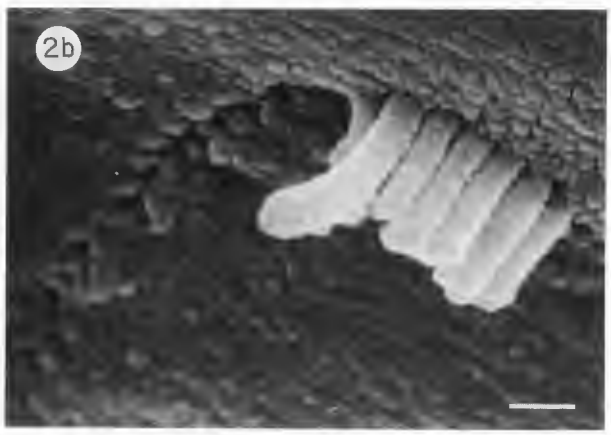
Morphological examination by scanning electron microscopy of the hair cell apical surfaces and the tectorial membrane, revealed characteristic changes. In particular, we observed swelling and a change in the apparent texture of the tectorial membrane (Fig. 1b). For comparison, Fig. 1a shows the appearance of the normal tectorial membrane. The surface texture in endotoxin-treated specimens is coarser than normal almost obliterating the stereocilial footprints at their point of attachment to the tectorial membrane. In normal specimens the stereocilia of inner hairs cells do not touch the inferior surface of this membrane. In specimens from endotoxin-treated cochleae, however, not only did these stereocilia touch the tectorial membrane, but in some cases were attached to it (Fig. 2a). The hair bundles of outer hair cells were also found attached to the tectorial membrane (Fig. 2b). We were able to show that the stereocilia lost their rigidity. In mild cases of damage, stereocilia could be seen to bend and splay out (Fig. 3a,b) whereas, in more serious cases the stereocilia lay flat upon the cuticular surface of the hair cell (Fig. 4). In some cases stereocilia fractured near their bases. Adhesion of the stereocilia to the inferior surface of the tectorial surface apparently caused them to break away from the hair cell leaving remnant stubs behind (Fig. 5).

These results were produced whether the endotoxin was administered by intracochlear microperfusion or by injection into the cerebrospinal fluid. It must be stressed


1a


1b


2a


2b


3a


3b

Figure 4. Endotoxin treated. Inner hair cell showing severe disruption: stereocilia have collapsed onto the apical cell surface. Bar = 1 μm.

Figure 5. As Fig. 4. Note stereocilia have broken away leaving remnants of points of attachment at the apical cell surface. Bar = 500 nm.

Figure 6. Dexamethasone treated, followed by endotoxin: inner hair cell showing virtually normal appearance of stereocilia and cross-links. Bar = 200 nm.

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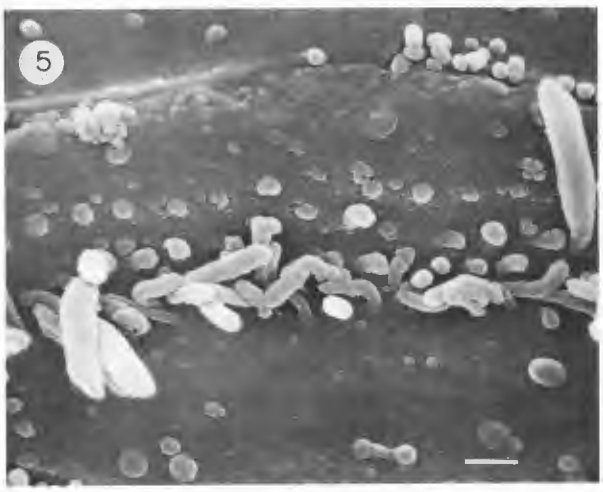
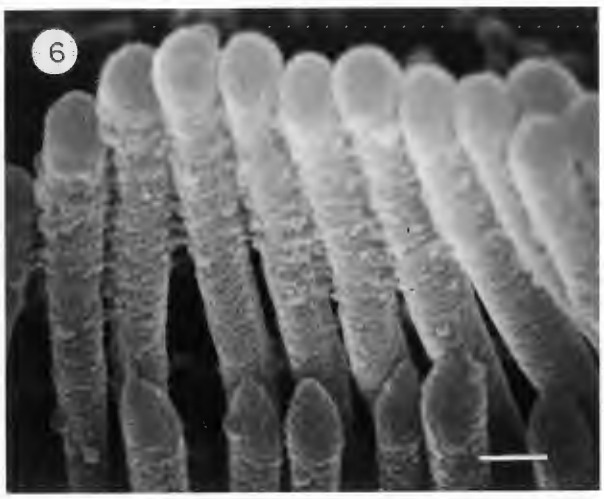
however, that the results obtained following intracisternal injection into the cerebrospinal fluid were more variable. In animals which were pretreated with dexamethasone, the effects described above were less severe and widespread (Fig. 6).





Discussion

Endotoxin is one of the most powerful inducers of cytokine release yet recognised. Among others, these cytokines include tumour necrosis factor (TNF) and interleukin 1 (IL-1): they have profound effects on the immune system and are responsible for the sequelae associated with the inflammatory response. Passive immunisation against TNF (Beutler *et al.*, 1985) or pretreatment with dexamethasone can protect an animal against the deleterious effects of endotoxin. TNF is also known to be cytotoxic, but the mechanisms underlying this cytotoxicity are complex (Larrick and Wright, 1990).

The precise site(s) responsible for the deafness in bacterial meningitis are not known. Many workers believe that the cochlea is a likely target of the inflammatory response following bacterial meningitis but it may well be that other structures along the auditory pathway such as the auditory nerve or more central nuclei are also involved. It is, however, clear from the observations described in this work that the apical surfaces of hair cells and the tectorial membrane are targets of the endotoxin-induced response. Particularly noteworthy are the changes seen among stereocilia which appear to go limp, bend and splay apart and often fracture. TNF is known to cause an increase in intracellular calcium (see Larrick and Wright, 1990). An increase in the level of calcium in the cytosol activates actin-severing proteins (Matsudeira and Janmey, 1988) and alters hair cell mechanics (Orman and Flock, 1983). Whether such a mechanism is responsible for the observed effects on stereocilia reported in this study, remains to be elucidated. We are in the process of examining stereocilia under transmission electron microscopy to see whether

the actin core of stereocilia shows signs of disruption following endotoxin challenge. It is not certain as yet whether the changes which take place in the tectorial membrane following microperfusion with endotoxin are due to a direct effect of endotoxin or of released cytokines. The possibility of an effect upon stria vascularis leading secondarily to changes in the ionic composition of endolymph and their effects upon hair cells should also be considered.

Our preliminary studies involving dexamethasone pretreatment indicate that the morphological damage resulting from intracochlear or intracisternal administration of endotoxin, can be considerably attenuated. This is in agreement with recent work which showed that steroids administered before or concurrently with antibiotics suppressed the inflammatory response in meningitis and also reduced the incidence of deafness (Mustafa *et al.*, 1989; Lebel *et al.*, 1988, 1989; Girgis *et al.*, 1989). Antibiotics produce a surge in the release of endotoxin due to the lysis of Gram negative organisms. In experimental animals and in patients with meningitis the CSF concentrations of endotoxin rise rapidly following antibiotic treatment (Mustafa *et al.*, 1989; Arditi *et al.*, 1989).

The time course of action of endotoxin in our study is similar to that observed following endotoxin injections into the CSF of rabbits. Such injections are known to cause the release of TNF which reaches a peak after about 2 hours (Mustafa *et al.*, 1989). We suggest therefore that the release of TNF and/or other cytokines could be responsible for the morphological damage observed in our experiments. It is known that within the CNS astrocytes and microglia are cytokine producing cells (Lee *et al.*, 1989; Fontana *et al.*, 1987). Cytokines released into the CSF could gain access into the cochlea via the cochlear aqueduct. Alternatively there may be cytokine-producing cells within the cochlea. We are currently using immunocytochemical techniques in conjunction with light and electron microscopy to discover whether any cell types within the cochlea are capable of synthesising cytokines.

Acknowledgements

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Discussion with Reviewers

A. Forge: Does the endotoxin affect CM and AP across all frequencies and are the morphological effects uniform along the length of the cochlea, or there preferential sites of damage?

S. Rydmarker: Was the endotoxin caused hair cell damage found in the apical of basal region of the

cochlea?
Authors: This is an interesting question which we have not yet addressed. We routinely elicit the CM using a 5 kHz tone pip, and the CAP with a short 10 kHz tone pip. So far as we can tell the damage appears to be restricted to the upper basal and middle turns. However, we have not carried out detailed cochleograms.

A. Forge: Is there any relationship between the extent of the damage to the apex of the hair cells and the amount of endotoxin administered?
Authors: This is a difficult question to answer because of the inter-animal variability to endotoxin challenge.

A. Forge: Are the effects confined to the apex of the hair cell?
Authors: We are currently studying this aspect using transmission electron microscopy. At present we have only found damage to the apex of the hair cells.

A. Forge: Is there any evidence that any of the endotoxin induced derangements observed in this work are reversible, or do the hair cells ultimately degenerate completely?
Authors: We are currently not able to answer these questions.

S. Rydmarker: How many animals were included? As you mention a marked inter-animal difference this is interesting to know.
Authors: In this study to date we have used 47 animals.

S. Rydmarker: Concerning Fig. 5, you say that the stereocilia are broken off. Could they be atrophied instead?
Authors: Two points here. First, the stereocilia in some cases are undoubtedly broken off since there remnants are stuck on the inferior surface of the tectorial membrane. Second, these are acute experiments, and the time scale would appear to be too short for atrophy of stereocilia to take place.

S. Rydmarker: What do you think will happen if you start the dexamethasone treatment after the administration of endotoxin and not before?
Authors: The inference from clinical studies is that you want to prevent as much as possible endotoxin induced release of cytokines, therefore we would predict that if dexamethasone was administered some time after endotoxin challenge, its protective action would be less. However, it is an important point for future study.

S. Rydmarker: The hair cell damage caused by endotoxin, is it restricted to a certain row of hair cells or including all rows?
Authors: As far as we can tell endotoxin can induce damage in all rows.

S. Rydmarker: In guinea pigs with induced endolymphatic hydrops, a rise in the concentration of endolymphatic Ca^{2+} is found. The tectorial membrane is, in that case, found to be shrunken. You found the tectorial membrane swollen. Can you explain the difference?
Authors: Without having any direct evidence that endotoxin produces ionic changes in the endolymph, we do not feel in a position to comment. It would, however, be interesting to investigate changes in the ratio of free:bound calcium in the various cochlear compartments following endotoxin challenge.