ELSEVIER

Hearing Research 77 (1994) 75-80

HEARING RESEARCH

Sulfhydryl compounds and antioxidants inhibit cytotoxicity to outer hair cells of a gentamicin metabolite in vitro [1]

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(Received 10 November 1993; Revision received 3 March 1994; Accepted 16 March 1994)

Abstract

Aminoglycoside antibiotics such as gentamicin have long been known to destroy cochlear and vestibular hair cells in vivo. In the cochlea outer hair cells are preferentially affected. In contrast, gentamicin will not damage outer hair cells in vitro unless it has been enzymatically converted to a cytotoxic metabolite. Several potential inhibitors of this enzymatic reaction were tested in an in vitro assay against outer hair cells isolated from the guinea pig cochlea. Viability of hair cells (viable cells as per cent of total number of cells observed) averaged about 70% under control conditions. Addition of metabolized gentamicin significantly reduced viability to less than 50% in one hour. Sulfhydryl compounds (glutathione, dithioerythritol) and antioxidants (vitamin C, phenylene diamine, trolox) prevented the cytotoxic actions of the gentamicin metabolite. Inhibitors of amine oxidases and compounds reportedly protective against renal and acute lethal toxicity of aminoglycosides (poly-L-aspartate and pyridoxal phosphate, respectively) were ineffective as protectants. The results reinforce the hypothesis that gentamicin is enzymatically converted to a cytotoxin and imply the participation of sulfhydryl-sensitive groups or free radicals in this reaction. Alternatively or additionally, sulfhydryl compounds or antioxidants may participate in detoxification reactions.

Key words: Aminoglycosides; Ototoxicity; Protection; Glutathione; Free radicals; Scavengers

1. Introduction

Aminoglycoside antibiotics are essential drugs in therapy against gram-negative infections. The major side effects associated with aminoglycoside treatment both in patients and experimental animals are impairment of kidney and inner ear function. Histologically, drug-induced damage is manifested as progressive and irreversible destruction of the sensory cells of the vestibular and auditory systems whereby the primary site of insult depends on the type of aminoglycoside. In the mammalian cochlea, outer hair cell death occurs first in the basal turn and progresses toward the apex. Correlating with the morphological damage, the sensorineural hearing loss begins in the higher frequencies, advances into the speech range and is most often permanent (for reviews see Hawkins, 1976; Garetz and Schacht, 1994).

Contrasting with the well-documented morphological and physiological pathology, attempts to establish a biochemical mechanism of the toxic action of these drugs have had limited success (Schacht, 1993; Garetz and Schacht, 1994). It was believed that aminoglycoside antibiotics did not require metabolism to exert either their therapeutic or deleterious effects. This notion was based largely on studies showing that the drugs were excreted in the urine apparently without being metabolized (Schentag and Jusko, 1977). However, it has been recently shown in our laboratory that gentamicin does not of itself damage outer hair cells in vitro (Dulon et al., 1989). It is rendered acutely cytotoxic when first incubated with an enzyme preparation from liver (Huang and Schacht, 1990; Crann et al., 1992) suggesting that an enzymatic reaction precedes the ototoxicity. This theory has opened new investigations into the characterization of the enzyme(s) involved, the reaction mechanism, the structure of the metabolite

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[1] A preliminary report of this research has appeared in Abstract form. Garetz and Schacht, Abstr. Res. Otolaryngol. 15, 110, (1992).

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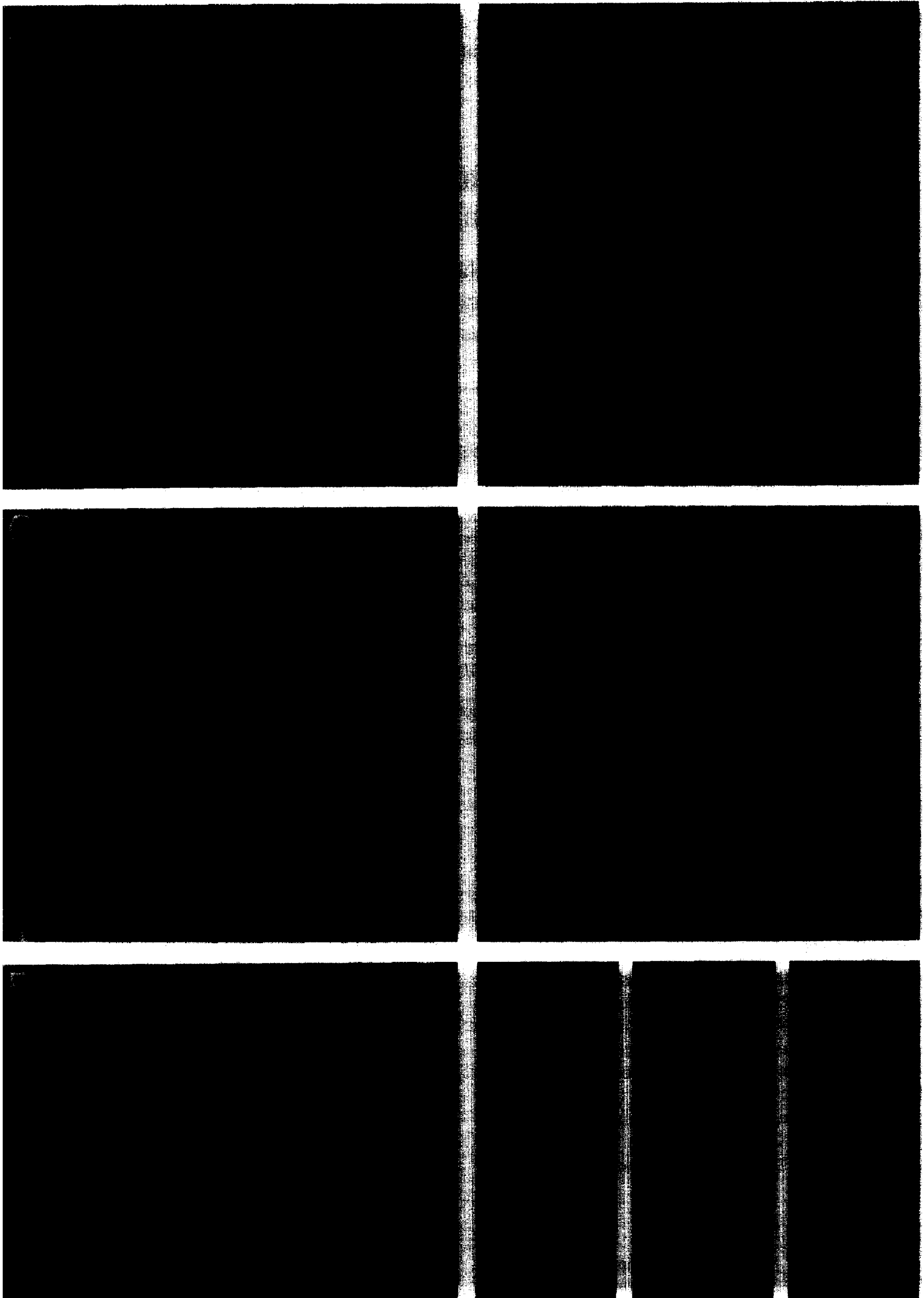
SSDI 0378-5955(94)00048-U



and its mechanism of action. Furthermore, it prompted the search for agents capable of blocking the cytotoxic mechanism at the enzymatic level (Garetz and Schacht, 1992; Garetz et al., 1993).

The current study reports on the efficacy of several compounds to interfere with hair cell destruction by the metabolite of gentamicin (tentatively termed 'gentatoxin') in vitro. Drugs were chosen based on suspected properties of the enzymatic reaction and the toxic metabolite, and on previous attempts to ameliorate aminoglycoside ototoxicity (Federspil, 1979; Schacht, 1993) or drug-induced nephrotoxicity (Kishore et al., 1992; Jones et al., 1992). In particular, we investigated sulfhydryl compounds, antioxidants and free-radical scavengers for their capacity to protect isolated outer hair cells from the acute cytotoxicity of gentatoxin.

2. Materials and methods

Preparation of the gentamicin metabolite

Fresh guinea pig liver (pigmented guinea pigs from Murphy, Plainfield, NJ) and HBSS (Hanks' Balanced Salt Solution without bicarbonate from Gibco Laboratories, Grand Island, NY; buffered with 5 mM HEPES to pH 7.40, and osmolality adjusted to 300 mOsM with NaCl) were mixed 1:3 (w/v) and homogenized with a Polytron (Brinkman, Westbury, NY) for 10 s. The suspension was centrifuged for 10 min at $1000 \times g$ to remove nuclei and gross sediment, and the supernatant ('post-nuclear fraction') used for the incubation with gentamicin. Alternatively, liver was homogenized in HBSS in a teflon-glass homogenizer (two passes of the pestle at 1000 rpm), and a 'cytosolic fraction' was obtained by centrifugation at $10{,}000 \times g$ for 20 min followed by centrifugation of the supernatant at $100{,}000 \times g$ for 90 min. The enzyme fractions were incubated for 1 hr at 37°C with or without 10 mM gentamicin as previously described (Huang and Schacht, 1990; Crann et al., 1992) and detailed in the figure legends. Reactions were terminated by heating at 55°C for 90 s. After 5 min on ice, precipitated proteins were removed by centrifugation at $10{,}000 \times g$ for 10 min and the supernatants assayed directly or stored at −20°C for no longer than one week prior to use.

Trolox was purchased from Aldrich Chemical Co (Milwaukee, WI), other chemicals from Sigma (St. Louis, MO). MDL 72-527 was a gift from Merrell Dow Research Laboratories, Cincinnati, OH.

Toxicity assay with isolated outer hair cells

Cochleae were immediately excised from decapitated guinea pigs and outer hair cells isolated essentially as described previously (Zenner et al., 1985; Zajic and Schacht, 1987). The sensory epithelium from the three upper turns was removed by microdissection, transferred into HBSS containing type IV collagenase (0.5 mg/ml; Sigma, St. Louis, MO) for 15 min after which cells were mechanically dissociated. For each assay, approximately 100 isolated hair cells were placed in a 45-μl droplet of HBSS in a Petri dish with controlled humidity. Five μl of the deproteinated incubation mixture were added yielding a final drug concentration of 1 mM. After 1 hr at room temperature, outer hair cells were carefully examined by light microscopy ($20\times$) by a blinded observer. Cells were classified according to previously established morphological criteria (Zajic and Schacht, 1987) as either healthy (long, cylindrical cells with smooth cytoplasm and central nucleus) or damaged (ruptured, swollen, constricted with a displaced or absent nucleus). The percentage of healthy cells was the index of cell viability.

3. Results

Controls

Outer hair cells exposed to HBSS alone or incubation media without gentamicin generally maintained a viability of 65 to 75% following the isolation procedure and the subsequent 1-h assay at room temperature (Figs. 1a, f, g, h; 2 and 3). Likewise, when gentamicin was added to the hair cell assay only and not to the preceding enzymatic incubation, cells maintained their normal appearance. However, when gentamicin was first metabolized, its addition decreased hair cell viability to approximately 40% within 1 h (Figs. 2 and 3). Both enzyme preparations, post-nuclear fraction and cytosol, were equally effective in converting gentamicin to a cytotoxin (Figs. 2 and 3).

Each experiment included a negative control for the quality and viability of the hair cell preparation and a positive control (with gentamicin) for the successful formation of a cytotoxin. A typical experiment additionally accommodated tests of a potential inhibitor with and without gentamicin for a total of four to eight

Fig. 1. Effect of metabolized gentamicin on isolated outer hair cells. Gentatoxin was prepared by incubating gentamicin with a cytosolic fraction (see legend to fig. 3) and assayed against isolated hair cells as described in 'Methods'. The same group of hair cells is seen exposed to toxin for 2 min (panel a), 8 min (b), 14 min (c), 35 min (d) and 45 min (e). Panels f, g and h show representative control cells exposed for 45 min to incubation medium to which gentamicin was added *after* incubation and heat precipitation of proteins.

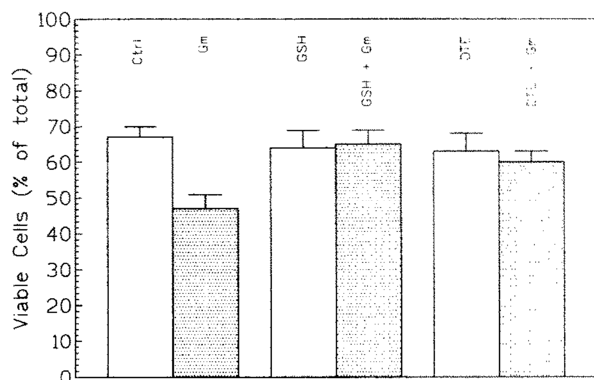


Fig. 2. Sulfhydryl compounds protect from cytotoxicity. Reagents were incubated with a hepatic post-nuclear fraction and tested against isolated outer hair cells as described in 'Methods'. The incubation medium contained the enzyme fraction, 100 mM sodium phosphate (pH 7.4), 4 mM NADPH, 30 mM KCl and 7 mM $MgCl_2$. Concentrations of gentamicin, glutathione or dithioerythritol were 10 mM each. Results represent means ± s.d. of an average of 6 independent experiments on 918 cells (control: Ctrl), 753 cells (gentamicin: Gm), 333 cells (glutathione: GSH), 533 cells (GSH plus gm), 359 cells (dithioerythritol: DTE), and 788 cells (DTE plus Gm). Differences between gentamicin and control, and between gentamicin alone and each gentamicin/drug combination were statistically significant at $P < 0.01$ (Fisher's Least Significant Difference).

conditions. Five to eleven independent experiments were performed to examine the efficacy of a drug as an inhibitor.

A complication in assessing viability could arise if the toxin would reduce affected cells to debris which then might escape scoring. In this case, the total number of cells observed in gentatoxin-treated incubations should be consistently and significantly lower. This, however, was not observed. Even within a limited number of experiments (Fig. 3), total cells in the absence and presence of toxin were within 10% of each other (3739 vs. 3414).

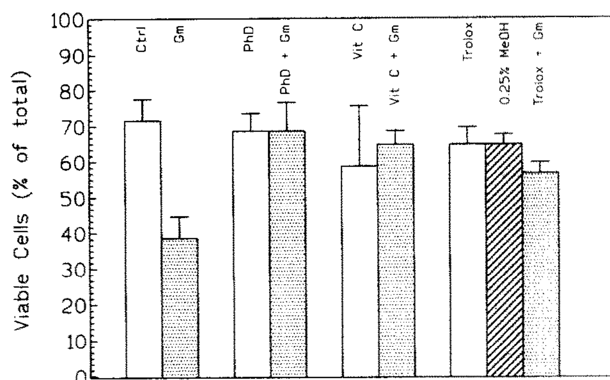


Fig. 3. Antioxidants protect from cytotoxicity. Antioxidants were incubated with a hepatic cytosol without added cofactors and tested against isolated outer hair cells as described in 'Methods'. Concentrations in the incubations were 10 mM for all reagents except Trolox (4 mM). Results represent means ± s.d. of an average of 6 independent experiments on 3739 cells (control: Ctrl), 3414 cells (gentamicin: Gm), 393 cells (phenylene diamine: PhD), 1049 cells (PhD plus Gm), 667 cells (vitamin C: Vit C), 546 cells (Vit C plus Gm), 370 cells (trolox), 418 cells (0.25% methanol: MeOH; vehicle for trolox), and 527 cells (trolox plus Gm). Differences between gentamicin and control, and between gentamicin alone and each gentamicin/drug combination were statistically significant at $P < 0.01$ (Fisher's Least Significant Difference).

Morphology

Loss of viability was initially seen as granulation of the cell cytoplasm, loss of birefringence and swelling or distortion of the cylindrical shape (Figs. 1 a–e). These changes became evident at different times in individual cells, visible in some after a few minutes (Figs. 1b and c), while other cells appeared normal throughout much of the assay period (Figs. 1 d and e). In the absence of gentatoxin, hair cells survived without apparent alterations of their morphology (Figs. 1f, g and h).

Effects of sulfhydryl compounds and antioxidants

The morphology of hair cells remained unaffected by glutathione or dithioerythritol or the antioxidants phenylene diamine, vitamin C or trolox. When these compounds were incubated with the enzymatic fractions in the absence of gentamicin and added to isolated hair cells, viability was maintained at control levels (Figs. 2 and 3). Vitamin C appeared to compromise cell integrity in some of the experiments, but the cumulative observations showed no significant difference from controls. However, each one of these compounds prevented the expected decrease in viability when added to enzymatic incubations with gentamicin. All assays in the additional presence of the protective drugs differed significantly from assays with metabolized gentamicin alone and were not different from assays with the protective agents without gentamicin (Figs. 2 and 3). A quantitative difference between the tested agents could not be inferred from the results since all effects were of similar magnitude and drugs were only studied at a single concentration. Glycine, glutamate and cysteine, the amino acid constituents of glutathione, were tested in combination in two experiments (121 cells) where they did not attenuate gentamicin toxicity.

In a related series of experiments, glutathione or dithioerythritol were absent from the enzymatic incubation with gentamicin but subsequently added to the hair cell assay. The resulting protective effect was similar to that achieved when these agents were included in the enzymatic incubation. Viability was maintained at 63 ± 10% in the presence of glutathione and at 62 ± 14% in the presence of dithioerythritol (observations on 669 and 586 cells, respectively).

Other agents

Inhibitors of polyamine and serum diamine oxidases, aminoguanidine and MDL 72-527, were incu-

bated and tested on 326 and 202 cells, respectively. At 1 and 2 mM final concentrations, they were ineffective at preventing the cytotoxic actions of the gentamicin metabolite.

Preliminary experiments also included agents previously tested against other side effects of aminoglycosides. Pyridoxal phosphate can antagonize lethal actions of gentamicin in the rat (Kenniston et al., 1987), and differential effects of poly-L-aspartate, poly-L-glutamate and poly-D-glutamate on renal aminoglycoside toxicity have been reported (Kishore at al., 1992). Pyridoxal phosphate (10 mM) was investigated in six, each poly-amino acid (10 mM with respect to amino acid residues) in two experiments. None of these agents altered the toxicity of the gentamicin metabolite.

4. Discussion

Sulfhydryl compounds, antioxidants and free-radical scavengers protect isolated guinea pig outer hair cells from the damaging effects of the toxic gentamicin metabolite. At least two mechanisms can account for this effect. Firstly, the formation of the cytotoxin can be inhibited by these agents. In this case, sulfhydryl or disulfide groups (e.g. on enzymes) or free radicals should participate in the reaction converting gentamicin to gentatoxin. This does not imply that the metabolite itself is a free radical. In fact, the ability to recover it from the incubation medium after heating or filtration (Crann et al., 1992) would seem to argue against this. Peroxides or endoperoxides are examples of chemicals which are not radicals but whose formation is susceptible to the influence of radical scavengers and antioxidants. Such compounds can, in turn, generate free radicals and thus, initiate chain reactions that may lead to cell damage or cell death.

Alternatively, the observed protection can arise from a detoxification of the cytotoxin rather than from the prevention of its formation. The fact that sulfhydryl reagents also prevent hair cell destruction when added after the enzymatic incubation makes detoxification a possibility. This sulfhydryl-mediated attenuation of toxicity is intriguing in view of established cellular biochemical defense mechanisms. Glutathione, a thiol-containing tripeptide found in virtually all mammalian cells, plays a key role in a number of reactions related to detoxification of xenobiotics and protection against reactive oxygen species (Jakoby, 1978; Listowsky et al., 1988; Meister, 1991). It is thus possible that glutathione inactivates the gentamicin metabolite in an enzymatic reaction involving glutathione transferase or glutathione peroxidase. This, however, remains speculative as it is currently unknown whether the inactivation proceeds enzymatically or non-enzymatically. Furthermore, it is conceivable that the protective agents act at both the level of formation and detoxification of the gentamicin metabolite.

Hypersensitivity to aminoglycoside-induced hearing loss has recently been linked to a mutation in a specific mitochondrial gene in several patient populations (Hutchin et al., 1993; Prezant et al., 1993). Until the functional consequences of this mutation are known, any association with the formation or inactivation of the proposed metabolite remains speculative. However, oxidative metabolism in mitochondria is the major source of reactive oxygen radicals; and mitochondria are a site of enzymes of detoxification and a major glutathione reserve. Enhanced mitochondrial leakage of reactive oxygen species or defects in detoxification mechanisms are possible links between mitochondrial DNA polymorphism and sensitivity to aminoglycosides.

While the reaction mechanisms of gentamicin metabolism remain speculative, at least one pathway may be ruled out. Aminoglycosides are structurally characterized by the presence of primary and secondary amine groups. Thus, they could serve as substrates for enzymes of amine or polyamine metabolism yielding, for example, reactive aldehydes. The inability of oxidase inhibitors to prevent cytotoxicity makes this possibility unlikely.

In addition to ototoxic side effects, the spectrum of aminoglycoside toxicity includes acute effects and chronic renal impairment. There is evidence to suggest that the underlying mechanisms differ from one another (Schacht, 1993; Garetz and Schacht, 1994). The lack of protection afforded here by pyridoxal phosphate, a blocker of acute lethal toxicity (Kenniston et al., 1987), and poly-L-aspartate, a blocker of nephrotoxicity (Kishore et al., 1992) could be indicative of such a dissociation of acute, nephrotoxic and ototoxic mechanisms. It still remains possible, however, that these compounds exert their effects at different steps in the sequence of aminoglycoside toxicity.

The successful protection of isolated hair cells from gentamicin cytotoxicity in vitro prompts the question of a possible protection against gentamicin ototoxicity in vivo. Attenuation of aminoglycoside ototoxicity by free radical scavengers has been claimed in animal experiments (Pierson and Møller, 1981) but has also met with failure (Bock et al., 1983). Likewise, sulfhydryl compounds have been employed in vivo with varying success and were eventually considered ineffective (reviewed by Federspil, 1979). However, support for an influence of glutathione in the expression of aminoglycoside toxicity has come from experiments with the glutathione-synthesis inhibitor buthionine sulfoximide. Lowering of glutathione levels with this drug in guinea pigs potentiated the ototoxicity caused by the combined administration of the aminoglycoside kanamycin and the diuretic ethacrynic acid (Hoffman et al., 1988). Preliminary experiments suggest that the converse is

true, namely attenuation of ototoxicity by elevating glutathione levels (Garetz et al., 1993, 1994). This renders the current in vitro observations relevant for both the elucidation of mechanisms of gentamicin metabolism and therapeutic intervention in aminoglycoside ototoxicity.

Acknowledgment

The authors wish to thank Sherry Crann for her contribution to the tests in this study and Gary Zajic for the micrographs. This research was supported by NIH research grant DC-00124 and training grant DC-00024.

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