Protection from Gentamicin Ototoxicity by Iron Chelators in Guinea Pig In Vivo\(^1,2\)

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ABSTRACT

This study details the prevention of gentamicin-induced hearing loss in guinea pig in vivo. The approach is based on our recent demonstrations of a redox-active gentamicin-iron complex in vitro and partial attenuation of gentamicin-induced hearing loss by the iron chelators defereroxamine and 2,3-dihydroxybenzoate. In our study, guinea pigs receiving injections of gentamicin (120 mg/kg body weight daily \(\times\) 19 days) developed a progressive threshold shift reaching 50 to 70 dB at 18 kHz. Concurrent treatment with different doses of 2,3-dihydroxybenzoate (30–300 mg/kg/day) reduced the threshold shift to 25 to 15 dB. Coinjection of gentamicin with dihydroxybenzoate (100 mg/kg/day) plus mannitol (15 mg/kg/day) yielded complete functional and morphological protection from gentamicin ototoxicity although partial protection was observed with combinations of dihydroxybenzoate and defereroxamine. Dihydroxybenzoate also attenuated gentamicin-induced vestibular toxicity. The iron chelators and radical scavengers affected neither serum levels nor the antimicrobial efficacy of gentamicin against Escherichia coli. These results confirm that iron and free radicals play a crucial role in the toxic side effects of gentamicin. Furthermore, they suggest that iron chelators, which are well-established drugs in clinical therapy, may be promising therapeutic agents to reduce aminoglycoside ototoxicity.

A serious limitation in the use of aminoglycoside antibiotics is their potential ototoxicity and nephrotoxicity (Schacht, 1993; Garetz and Schacht, 1996; Begg and Barclay, 1995). Attempts to attenuate or prevent these side effects have met with varying success and no proposed therapy has yet found acceptance. For example, several lines of evidence suggest that free radicals are involved in the ototoxic side effects of aminoglycoside antibiotics. WR2721, a sulfhydryl-containing radioprotectant, was shown to reduce ototoxicity of kanamycin (Pierson and Moller, 1981), but this finding was tempered by the demonstration that N-acetyl cysteine, another sulfhydryl-containing free radical scavenger, was ineffective (Bock et al., 1983). GSH also significantly attenuated the ototoxic effects of GM, but this protection was effective in sick or malnourished animals only (Garetz et al., 1994a; Lautermann et al., 1995).

The theory of participation of free radicals in the nephrotoxic side effects of aminoglycosides is similarly controversial. Support for their participation includes the demonstration that iron supplementation (i.e., augmentation of free radical production) potentiated GM nephrotoxicity in rats (Kays et al., 1991; Ben Ismail et al., 1994), and that the iron chelators DFO and DHB reduced GM-induced damage to kidney (Walker and Shah, 1988). However, others have maintained that oxygen-derived free radicals are not a causal factor in GM nephrotoxicity (Stratta et al., 1994), and that a participation of iron is unlikely (Durak et al., 1995). Furthermore, most studies fail to report the effect of preventive therapy on drug serum levels and antibacterial efficacy. Without such knowledge the clinical potential of suggested treatments cannot be evaluated.

Another major obstacle in both the design of protective therapy and the acceptance of empirical prevention is the fact that no rational mechanism for aminoglycoside toxicity has been established. A series of recent observations from our laboratory has led us to conclude that the ototoxic effects of gentamicin require an “activated” form of the drug (Schacht, 1993; Huang and Schacht, 1990; Crann et al., 1994), and that the iron-chelator complex GM possesses the ability of certain radical scavengers and iron chelators to

ABBREVIATIONS: ABR, auditory brainstem responses; DHB, 2,3-dihydroxybenzoic acid; DFO, defereroxamine mesylate; GM, gentamicin; GSH, glutathione; MANN, mannitol; OHC, outer hair cell; PBS, phosphate-buffered saline.
reduce GM-induced hearing loss in the guinea pig (Song and Schacht, 1996). The guinea pig is the experimental animal of choice for studies of ototoxic drugs. A well-defined auditory physiology is combined with easy access to the cochlea and a pattern of drug-induced pathology that closely resembles that seen in human (Garett and Schacht, 1966). The aim of our study was to investigate in detail the protective effect of iron chelators on GM-induced ototoxicity and to optimize conditions for therapeutic prevention.

Materials and Methods

Experimental groups and drug administration. Pigmented male guinea pigs initially weighing 200 to 300 g (Murphy’s Breeding Labs Inc., Plainfield, NJ) were given free access to water and a regular guinea pig diet (no. 5025; Purina, St. Louis, MO). The animals were allowed 1 wk of acclimation before treatment was begun. All experimental protocols on animal use were approved by the University of Michigan Committee on Use and Care of Animals. Animal care was under the supervision of the University of Michigan’s Unit for Laboratory Animal Medicine.

Two separate studies were conducted. The first study was comprised of eight groups of four animals each in which GM was tested alone and in combination with DHB, DFO or MANN. Details of the treatment protocol are given in table 1. In the second study, treatment with DHB continued for another week after the last injection of GM. Body weight of animals was monitored daily and the doses were adjusted accordingly.

Evaluation of auditory function. Auditory thresholds were measured as evoked ABR. Thresholds were determined for each animal before the beginning of the study, again at day 17, and then weekly for up to 8 wk after the beginning of treatment as stated in the figure legends.

Animals were anesthetized with an i.p. injection of 40 mg ketamine and 10 mg xylazine/kg body weight. ABR measurements at 3, 8 and 18 kHz were performed as described previously (Lautermann et al., 1995; Song and Schacht, 1996). In brief, tone bursts of 3, 8 and 18 kHz (3-msec duration, 1 msec rise and fall time) were generated using a Fordham Audio Generator (Fordham Radio Supply, Hauppauge, NY) (model AG-298) and presented to the left external auditory meatus in a closed acoustic system through an ear bar connected to a Beyer (Beyer Dynamic, Farmingdale, NY) (DT-48) transducer. Needle electrodes were placed s.c. below the ipsilateral right pinna (negative electrode) and the contralateral pinna (ground electrode). The positive electrode was located at the vertex. The output was fed to an amplifier (10^5 gain, 300–3 kHz filter setting) and viewed on an oscilloscope (Kenwood, CS-4035) (Kenwood Corp., Long Beach, CA). The average responses from 512 stimuli were obtained at 5-dB intervals near threshold. Thresholds at each frequency were verified at least twice and defined as the lowest intensity to yield a reproducible deflection in the evoked response trace. Threshold shifts were calculated for individual animals by comparison to their prestudy thresholds.

Evaluation of vestibular function. Three months after the last GM injection, animals from the second study were tested for rotatory vestibular and optokinetic nystagmus. The animals were awake and confined in a restraining box placed in the center of a rotation table (Neurokinetics Inc., Pittsburgh, PA). Nystagmus was induced by abruptly starting or stopping the turntable and recorded in the dark via periorcular needle electrodes with the ground electrode fixed to the contralateral pinna. Clockwise and counterclockwise rotations were carried out alternately at 27.3 rpm for 11 sec each. The signal was fed to a preamplifier and a computerized graphic recording system and displayed on an oscilloscope screen (Tektronix Inc., Beaverton, OR). The duration of postrotatory nystagmus was measured with a manually assisted computer program, and the total number of beats throughout the duration of nystagmus was counted. Both the start and stop nystagmus responses were similar, but the responses at the second stop were more robust and therefore used for analysis.

As a control for the vestibular origin of the recorded response, optokinetic nystagmus was measured in the light. The table rotated counterclockwise at 1.7 rpm for 30 sec. The nystagmus was recorded as above.

Serum gentamicin. Gentamicin levels were determined at 1 hr after gentamicin injections on day 8 and 19. Blood was obtained by nail clipping after light anesthesia of the animals by metofane inhalation. Blood cells were removed by centrifugation at 1000 g for 15 min, and sera were stored at −20°C. GM levels were measured using a commercial fluorescence polarization immunoassay (Abbott Diagnostics, Abbott Park, IL).

Serum albumin, BUN and creatinine. Sera were obtained as above. Albumin, BUN and creatinine were determined by standard

### TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th>GM (mg/kg body weight)</th>
<th>DHB</th>
<th>DFO</th>
<th>MANN</th>
<th>Day 15 Weight Gain (g)</th>
<th>Day 28 Weight Gain (g)</th>
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<tbody>
<tr>
<td>Study 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>1. Control</td>
<td>153 ± 42</td>
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<td></td>
<td>269 ± 48</td>
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<tr>
<td>2</td>
<td>2. GM</td>
<td>94 ± 41</td>
<td></td>
<td></td>
<td></td>
<td>117 ± 50              *</td>
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<tr>
<td>3</td>
<td>3. GM + DHB (30)</td>
<td>120</td>
<td>30</td>
<td></td>
<td></td>
<td>80 ± 52</td>
<td>124 ± 59</td>
</tr>
<tr>
<td>4</td>
<td>4. GM + DHB (100)</td>
<td>120</td>
<td>100</td>
<td></td>
<td></td>
<td>107 ± 5</td>
<td>111 ± 14              *</td>
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<tr>
<td>5</td>
<td>5. GM + DHB (300)</td>
<td>120</td>
<td>300</td>
<td></td>
<td></td>
<td>41 ± 26</td>
<td>108 ± 33              *</td>
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<tr>
<td>6</td>
<td>6. DHB (300)</td>
<td>65 ± 45</td>
<td></td>
<td></td>
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<td>146 ± 120</td>
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<td>7</td>
<td>7. GM + DHB + DFO</td>
<td>120</td>
<td>100</td>
<td>100</td>
<td></td>
<td>93 ± 46</td>
<td>150 ± 56</td>
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<td>8</td>
<td>8. GM + DHB + MANN</td>
<td>120</td>
<td>100</td>
<td>100</td>
<td></td>
<td>133 ± 10</td>
<td>168 ± 29</td>
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<tr>
<td>Study 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1. Control</td>
<td>155 ± 30</td>
<td></td>
<td></td>
<td></td>
<td>286 ± 56</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2. GM alone</td>
<td>92 ± 46</td>
<td></td>
<td></td>
<td></td>
<td>124 ± 50              *</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3. GM + DHB</td>
<td>122 ± 15              *</td>
<td></td>
<td></td>
<td></td>
<td>128 ± 64              *</td>
<td></td>
</tr>
</tbody>
</table>

GM, DFO and MANN were dissolved in 0.9% saline, and DHB was dissolved in 5% NaHCO$_3$. All reagents for each treatment were mixed, then immediately injected s.c. except 300 mg/kg DHB. The limited solubility of DHB precluded s.c. injection at the required concentration; instead, it was given orally in a slurry with 0.1 M sucrose immediately before the GM injection. GM alone or with other drugs was administered for 19 days, and controls received injections daily with the same volume of saline per kg body weight. In the second study, treatment with DHB continued for another week after the last injection of GM. Conditions of GM treatment were chosen for their documented effect on auditory function; concentrations of other drugs were based on a survey of efficacy of scavengers and chelators against GM ototoxicity (Song and Schacht, 1996). Body weight of animals was monitored daily and the doses were adjusted accordingly. Numbers for body weight are means ± SD; n = 4 in all groups except n = 3 in groups receiving DHB at 300 mg/kg alone and GM plus DHB at 30 mg/kg.

* Significantly different from saline control animals (P < .05).
techniques adapted for automated analysis on a Kodak Ektachem 700 XR Clinical Chemistry Analyzer (Clinical Products Division, Eastman Kodak, Rochester, NY).

Antimicrobial activity. Efficacy of gentamicin alone and in the presence of DFO, DHB and mannitol was tested against *Escherichia coli* (ATCC no. 25922) in a standardized microbiological assay (Chapin-Robertson and Edberg, 1991). Twenty μl of gentamicin (50 μg/ml) were dispensed onto 6-mm disks, and allowed to air-dry. Subsequently 20 μl of different concentrations of each interventional agent were added to the appropriate disks. Disks were then placed on the surface of a 150-mm culture plate containing Mueller-Hinton agar at a depth of 4 mm. The surface of the plates had previously been inoculated with a standardized concentration of *E. coli*. The inoculated plates were incubated overnight at 35°C in room air. The diameter of the inhibition zones was measured to the nearest millimeter across each disk.

Serum total iron. Sera were obtained as above. The concentration of total serum iron was determined colorimetrically by reaction with ferrozine (Ruutu, 1975). The color reagent contained 250 mg of ferrozine (0.5 mM), 10 g of ascorbic acid and 30 g of Triton X-100 in 1 liter of 0.4 M glycine/HCl buffer, pH 3.1. Iron was determined by mixing 30 μl of standard iron solutions or serum with 80 μl of the color reagent. Blanks contained all assay components except ferrozine. After 45 min, absorbance was read at 560 nm. No adjustments were necessary for the presence of iron chelators in the serum. DFO or DHB in concentrations as high as 10 mM did not interfere with the assay.

Histopathology of cochlea, kidney and liver. Five wk after the final drug administration, two animals each from seven groups in the first study (groups 1, 2, 3, 4, 5, 6 and 8; table 1) were deeply anesthetized in a CO₂ chamber and decapitated. The temporal bones were removed. The round and oval windows and the apex of the cochlea were opened and perfused with 2% paraformaldehyde in 10 mM PBS (pH 7.4) for about 2 hr, then washed with cold PBS three times for 10 min each. After fixation, the surface of the organ of Corti was stained with rhodamine phalloidin (Raphael and Altschuler, 1991). Cochleae were microdissected, and individual turns of the organ of Corti were mounted on glass slides in glycerol. Using fluorescence microscopy, present and missing outer hair cells (scars) were counted. Cytocochleograms were plotted for the percentage of outer hair cell loss using The New York State University at Buffalo software (Nicholas Powers, Hearing Research Labs, Buffalo, NY). Two other animals in each group were killed for histopathological examination of kidney and liver. Tissues were processed by standard histological methods. Samples (1 × 1 cm) were fixed in 10% formalin, embedded in paraffin, sectioned at 6 μm and stained with H&E, and then evaluated (in blinded fashion) for pathological changes by light microscopy. The autopsy report was submitted by Clarence Chrisp, D.V.M. (Unit for Laboratory Animal Medicine, University of Michigan).

Statistical analysis. Data were statistically evaluated by Student’s t test and by analyses of variance with Tukey’s post hoc test for significance (P < .05) using INSTAT Biostatistic software (Graph Pad Software, San Diego, CA).

Materials. Gentamicin sulfate was purchased from Spectrum Chemical Mfg. Corp. (Gardena, CA), 2,3-DHB from Aldrich Chemical Co. Inc. (Milwaukee, WI), MANN from Mallinckrodt Inc. (Paris, KY), metofane from Pitman-Moore Inc. (Mundelein, IL), ketamine (Keta-set) from Fort Dodge Laboratories Inc. (Fort Dodge, IA), xylazine from Lloyd Laboratories Inc. (Shenandoah, IA), rhodamine phalloidin from Molecular Probes Inc. (Eugene, OR); DFO mesylate and all other reagents were from Sigma Chemical Co. (St. Louis, MO).

Results

Weight gain and mortality. In both studies, significantly lower weight gain was evident in GM-treated as compared to saline-injected animals (table 1; P < .05). Cotreatment with chelators improved weight gain somewhat. The weight gain in these groups fell between that of the control groups and the groups receiving GM. One animal in each group died receiving 30 mg/kg DHB plus GM and 300 mg/kg DHB alone.

Auditory evoked brainstem responses. Figure 1 shows tracings of typical responses: a normal response (A), and a response after GM treatment (B). The magnitude of the response decreases as the intensity of the stimulus is attenuated from 90 to 10 dB. Simultaneously, the latency of the response (time interval between stimulus presentation and onset of the response) increases as indicated by arrows in A. Threshold—defined as the lowest intensity to yield a reproducible deflection in the evoked response trace—is seen at 20 dB in the normal response. In contrast, the GM-treated animal (B) exhibits a threshold of 70 dB. For figures 2 to 6, threshold shifts after drug treatment were calculated for individual animals by comparison to their individual pre-study thresholds.

Auditory thresholds were comparable in all groups at the beginning of the studies. Saline-injected animals maintained essentially stable thresholds throughout the course of treatment, indicating a high interest reliability. Animals receiving GM alone consistently developed a progressive hearing loss (figs. 2 and 3). Auditory thresholds were significantly

**Fig. 1.** ABR in guinea pig. ABR recordings were obtained as described in “Materials and Methods.” A shows a normal response, B a response after GM treatment. As the intensity of the stimulus is attenuated from 90 to 10 dB, the magnitude of the response decreases. Arrows on the most prominent wave illustrate the increase in latency with decreasing stimulus intensity.
elevated after 17 days of treatment and continued to deteriorate after cessation of treatment. In agreement with the established pattern of chronic GM ototoxicity (Garetz and Schacht, 1996), the higher frequencies were more affected. By the end of 4 wk, the magnitude of threshold shifts ranged from about 10 to 20 dB at 3 kHz to about 50 to 70 dB at 18 kHz.

Concurrent treatment with DHB significantly attenuated GM-induced threshold shifts (fig. 2). At a dose of 100 mg DHB/kg body weight, the final threshold shifts were reduced to 10 to 20 dB at both 8 and 18 kHz. Continuation of treatment with the iron chelator for another week after cessation of gentamicin injections induced a further recovery of hearing loss (fig. 3).

The efficacy of DHB against gentamicin appeared dose-dependent (fig. 4), and a lesser but still significant attenuation was achieved with 30 mg DHB/kg body weight than with 100 mg/kg. Increasing the chelator concentration to 300 mg/kg did not diminish the threshold shift any further. However, it should be cautioned that this dose of DHB had to be given orally because of the limited solubility of this drug in aqueous solutions. DHB alone, up to 300 mg/kg, had no effect on auditory thresholds.

Combining different the two chelators yielded an ambiguous result. DFO coadministered with DHB led to complete protection of GM-induced hearing loss in three of four animals (fig. 5). However, the remaining animal only showed a moderate attenuation.

The most striking result was achieved with the coinjection of GM with DHB and mannitol. This regimen yielded complete protection at all measured frequencies in all animals (fig. 6).

**Vestibular and optokinetic nystagmus responses.** In control animals, the vestibular response was pronounced and
similar between animals (fig. 7; table 2). GM treatment significantly reduced the duration of nystagmus and the number of nystagmus beats ($P < .05$). Administration of DHB together with GM improved the performance of the vestibular response. Optokinetic nystagmus was comparable between groups except for one animal treated with GM (data not shown).

**Serum albumin, BUN and creatinine levels.** No obvious signs of nephrotoxicity were observed with any of the treatments. The mean values of serum albumin (controls: $1.9 \pm 0.1$ g/dl; $n = 7$), BUN (14.4 $\pm$ 1.4 mg/dl) and creatinine (0.3 mg/dl) were not significantly different in any of the treatment groups ($P > .05$).

**Histopathology.** Cochlear pathology (fig. 8) was consistent with the functional results obtained from the ABR measurements. Control animals had a normal complement of outer hair cells with a loss of about 10% scattered throughout the length of the cochlea (A). GM-treated animals exhibited severe to almost complete hair cell loss in all three rows of cells in the basal turns of the cochlea (B) corresponding with the pathophysiology at high frequencies (18 kHz). Hair cells in a more apical location were less affected, a result again corresponding with the lesser effect of GM on the physiological response at low frequencies (3 kHz).

Coadministration of 100 mg/kg DHB with GM significantly reduced damage to hair cells although some loss was still observed toward the base (C). Coadministration of DHB and MANN with GM yielded almost complete protection from hair cell loss (D). In these animals, scattered loss remained at around 10% of cells but seemed more uniform throughout the
cochlea than in controls. DHB alone at 300 mg/kg did not cause any damage to the cochlear hair cells (E).

Treatment-related renal lesions were apparently absent with the possible exception of animals receiving the higher dose of DHB. Histopathological evaluation of renal tissue was confounded by a probable infection of the animals with Encephalitozoon cuniculi, a common parasite in guinea pigs that causes kidney lesions. Mild to moderate interstitial fibrosis and tubular atrophy was found in all groups as well as in an untreated control animal. Beyond this, a somewhat more pronounced interstitial fibrosis and tubular atrophy was noted in one animal receiving 300 mg/kg DHB, one of two animals receiving 300 mg/kg DHB plus GM.

Liver tissue appeared to be essentially normal. One minimal focus of subcapsular necrosis was found in each of three animals. There was no pattern to the appearance of this lesion because different treatment groups (GM alone and 300 mg/kg DHB plus GM) were affected as well as a saline control. Furthermore, the second animal in each group showed no liver pathology.

Serum GM levels. Serum levels of GM were measured in all animals receiving GM in the first study (fig. 9). None of the treatments lowered drug serum levels. Rather, treatment with DHB appeared to increase serum levels. At 1 hr after injections on day 8, the group receiving 30 mg/kg DHB plus GM showed a moderately higher serum GM concentration than GM alone (P < .05). At 1 hr on day 19, there were markedly higher serum levels of GM in the 100 mg/kg DHB and 15 mg/kg MANN; E, 300 mg/kg DHB alone.

Serum iron levels. Total iron in serum was analyzed at 1 hr after GM injections on days 8 and 19 (fig. 10). GM alone had no influence on serum iron levels. Similarly, coadministration of MANN with GM had no effect. There was a trend toward decreased iron levels with 300 mg/kg DHB alone (low iron levels of 1.23 and 1.14 mg/ml were observed in two animals on day 8) and DHB combined with GM (0.88 and 0.95 μg/ml of iron in two animals on day 8). However, the group means did not differ significantly. In contrast, combined GM/DHB/DFO treatment significantly lowered iron levels (P < .05).

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**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Beats (n)</th>
<th>Duration (sec)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.0 ± 13.5</td>
<td>5.7 ± 2.5</td>
</tr>
<tr>
<td>GM alone</td>
<td>3.7 ± 3.2*</td>
<td>1.1 ± 1.0*</td>
</tr>
<tr>
<td>GM + DHB</td>
<td>10.8 ± 2.8b</td>
<td>3.7 ± 1.3b</td>
</tr>
</tbody>
</table>

*Animals were treated and nystagmus was measured as described in “Materials and Methods.” Four control animals and three animals in each drug treatment group were each tested twice. Numbers are means ± S.D.

*Significantly different from control (P < .05) by analysis of variance.

b GM vs. GM/DHB, P < .05 by χ² analysis.

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**Fig. 7.** Effects of DHB on GM-induced vestibular toxicity. Drugs were administered as described in table 1, and vestibular nystagmus was recorded as described in “Materials and Methods.” Typical tracings of vestibular nystagmus are shown from one animal in each group. Nystagmus beats are triggered at each change in rotatory stimulus (start, stop). A, Saline control; B, GM treatment; C, GM combined with DHB. Duration and number of beats was significantly affected by GM and protected by combined treatment with DHB (table 2).

**Fig. 8.** Cytocochleograms. Cytocochleograms were obtained as described in “Materials and Methods.” The percentage of missing hair cells is plotted for the entire length of the cochlea. OHC1, OHC2 and OHC3 represent cochlear outer hair cells of rows 1, 2 and 3, respectively. A, Saline controls; B, GM treatment; C, GM combined with 100 mg/kg DHB; D, GM combined with 100 mg/kg DHB and 15 mg/kg MANN; E, 300 mg/kg DHB alone.
Antimicrobial efficacy. GM efficacy against E. coli was tested in a standard antimicrobial assay. The iron chelators (DFO and DHB) and radical scavenger (MANN) did not influence the inhibition zones produced by 1 mg GM (table 3) even at a 100-fold molar excess over GM. The interventional agents alone had no antibacterial activity.

### Table 3

Effect of interventional agents on GM antimicrobial activity against E. coli

<table>
<thead>
<tr>
<th>Molar ratio of drug added to Gm</th>
<th>Inhibition Zones (mm)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>GM alone</td>
</tr>
<tr>
<td></td>
<td>1x</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>GM alone</td>
<td>23.0</td>
</tr>
<tr>
<td>GM + DFO</td>
<td>22.8</td>
</tr>
<tr>
<td>GM + DHB</td>
<td>24.0</td>
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</table>

Growth inhibition zones produced by gentamicin on an E. coli-inoculated agar dish were measured in a microbiological assay as described in “Materials and Methods.”

**Discussion**

Combined iron chelator and antioxidant therapy appears to be a most promising avenue to alleviate the adverse side effects caused by treatment with aminoglycoside antibiotics. We have previously shown that antioxidants alone were rather ineffective protectants and that individual iron chelators significantly but incompletely attenuated GM-induced hearing loss (Song and Schacht, 1996). Furthermore, the efficacy of an individual iron chelator could not be improved when its dose was increased, as shown here for DHB, or when the stronger chelator DFO was substituted for the weaker DHB (Song and Schacht, 1996). In contrast, the combination of chelators and radical scavengers in the present study appears to afford essentially complete protection. This success of a combined treatment can best be explained by multiple sites and mechanisms of action in the prevention of GM-induced toxicity. For example, the additive protection provided by combined administration of DHB and DFO may reflect the fact that these two compounds can chelate iron from different pools (Graziano et al., 1978). The additive protection by DHB and MANN suggests complementary mechanisms. Protection by iron chelators is generally accepted as evidence for the participation of hydroxyl radical in tissue injury (Gutteridge et al., 1979; Starke and Farber, 1985). MANN may scavenge hydroxyl radicals whose formation has not been blocked by DHB.

There is excellent agreement between the functional and the morphological assessments of cochlear damage and protection. GM-induced hair cell destruction is massive at the base of the cochlea, corresponding to high-frequency hearing loss; the apical region is less affected, consistent with less functional loss at the low frequency of 3 kHz. The pattern of hair cell preservation by the different treatments is also consistent with the observed protection against functional loss. Furthermore, protection also extends to the vestibular system, another target of GM toxicity.

The protective effect of iron chelators reinforces the hypothesis that GM exerts its toxic effects via the formation of an iron complex catalyzing free-radical production (Priuska and Schacht, 1995; Wang et al., 1996; Sha and Schacht, 1996). This proposed mechanism of free radical formation may also underlie the nephrotoxic side effects of aminoglycosides, a notion that has repeatedly been challenged (Ali, 1995). In agreement with the postulate of a single basic mechanism responsible for both ototoxicity and nephrotoxicity are the results presented here and those of Walker and Shah (1988) demonstrating attenuation of by iron chelators of renal damage induced by GM.
Several points speak for the clinical relevance of the proposed treatment. The daily dosage of GM used here is common in animal experimentation but approximately 20 times higher than used clinically in patients. Yet, even under these conditions, chelators and scavengers afford protection at dosages equal to (DHB) or only two to three times higher (DFO) than established clinical usage. Importantly, the protective treatment does not compromise the efficacy of the antibiotics. First, chelators and radical scavengers do not lower GM serum levels. Thus, there is no enhanced renal clearance that could “mimic” protection. Second, they do not affect the antibacterial efficacy of the drug even in great excess to GM. Third, there is evidence that DFO and DHB may increase aminoglycoside antimicrobial activity synergistically (van Asbeck et al., 1983; Pearce et al., 1985).

Both DFO and DHB are potential antidotes to aminoglycoside toxicity. DFO has been recognized as an effective chelation agent for long-term treatment of iron overload (Hershko, 1992), but a number of clinical studies have also noticed its potential visual and auditory neurotoxicity in patients (Cohen et al., 1990; Voest et al., 1994; Bentur et al., 1990; Gallant et al., 1987). These effects may be dose related. In animal studies, no significant changes in cochlear function or morphology were observed in chinchillas receiving chronic DFO treatment at 100 mg/kg/day, 5 days/wk, for 3 mo (Shirane and Harrison, 1987). However, at higher doses (600 mg/kg/day for 30 days) ototoxicity was noted in guinea pig (Kanno et al., 1995).

DHB was initially tested clinically as an aspirin analogue with no apparent toxicity at dosages of approximately 100 mg/kg/day for 11 to 21 days (Clarke et al., 1958). It was evaluated in the 1970s as a chelating agent for treatment of patients with iron overload (Graziano, 1978). A double-blind study in 15 patients suffering from thalassemia major found no significant side effects and good patient tolerance to this drug at a dose of 100 mg/kg/day for 1 yr (Peterson et al., 1979). Chronic administration of DHB to mice, rats and dogs has also shown minimal toxicity only (Graziano, 1978; Martini and Ponzo, 1952). Consequently, our study focused on DHB as a protective agent because of its potentially lower intrinsic toxicity. Our observations support the previous findings. At a dose of 100 mg/kg, DHB protected from GM toxicity without apparent detrimental effects on cochlea, kidney and liver. At a dose of 300 mg/kg DHB, no functional defects were observed in the cochlea and the kidney, and liver morphology was essentially normal.

In addition to intrinsic toxicity, the effect of the chelating drugs on serum iron levels need to be considered. Decreased serum total iron could become a potentially confounding factor in clinical treatment. However, apparently prevention of GM ototoxicity is not predicated on reduction of total serum iron. DHB, even at 300 mg/kg, does not induce any significant changes and neither does the most successful preventive treatment, the combination of DHB with MANN. In terms of the mechanism of prevention, this further argues against a simpler explanation of a reduction in total iron concentration mediating the protection and supports more complex interactions. Serum, furthermore, may not be a major or the only site where interactions between GM and the protective agents occur. Other potential sites are the supporting cells of the inner ear that are capable of activating GM to a toxin (Crann and Schacht, 1996) or the outer hair cells that are the preferred targets of destruction.

In summary, our study presents a mechanism-based strategy to prevent the toxic side effects of GM with probable extension to other aminoglycoside antibiotics (Crann and Schacht, 1996). The application of the protective treatment is simple and efficient because the drugs can be combined in a single injection. Furthermore, because the suggested drugs are well-established in clinical therapy, clinical trials are warranted.

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