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EFFECT OF GENTAMICIN ON THE LYSOSOMAL SYSTEM OF CULTURED HUMAN PROXIMAL TUBULAR CELLS

ENDOCYTOTIC ACTIVITY, LYSOSOMAL pH AND MEMBRANE FRAGILITY

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Abstract—Gentamicin treatment results in significant changes in lysosomal morphology and enzyme activity in renal tubular epithelium both *in vivo* and *in vitro*. In this study, cultured human proximal tubular cells (PTC) were treated with gentamicin (0, 0.01, 0.1, and 1.0 mg/ml) for 3, 7, 10 and 14 days, and the endocytotic activity, pH, and membrane fragility of the lysosomal system were examined. Fluorescein isothiocyanate-labeled dextran (FITC-dextran) was used to estimate endocytotic activity and intralysosomal pH. The fragility of isolated lysosomes was estimated by the release of *N*-acetyl-β-glucosaminidase (NAG, EC 3.2.1.30) into the medium. Gentamicin content was measured and correlated with the changes seen in lysosomal function. Gentamicin treatment caused a slight decrease in the rate with which human PTC accumulated FITC-dextran and a slight increase in intralysosomal pH. Treatment of human PTC with NH_4Cl, a lysosomotropic compound, significantly increased the lysosomal pH; the NH_4Cl-induced increase in the lysosomal pH of gentamicin-treated PTC, however, was not significantly different from control (0 mg gentamicin/ml). Lysosomes isolated from human PTC cultures released NAG upon incubation for 60 min at 37°. There was no significant effect on the fragility of lysosomes isolated from cultures exposed to gentamicin for ≤ 7 days. Significantly increased fragility was seen, however, after 10 days of treatment with 1.0 mg gentamicin/ml and especially after a 14-day exposure to 0.01, 0.1, and 1.0 mg gentamicin/ml. Human PTC accumulated 0.47, 2.05 and 10.30 μg gentamicin/mg protein with 10 days of exposure to 0.01, 0.1 and 1.0 mg gentamicin/ml medium respectively. Gentamicin treatment associated with increased numbers of morphologically altered lysosomes, i.e. myeloid bodies, did not affect significantly the endocytotic activity and pH of lysosomes in cultured human PTC. Prolonged exposure (14 days) of human PTC to gentamicin, however, did increase the fragility of lysosomes after isolation. The increased numbers of morphologically altered lysosomes with increased fragility were not associated with any significant *in vitro* cell death. Therefore, it would appear that these lysosomal alterations are not directly responsible for the *in vivo* nephrotoxicity.

Gentamicin, used in the treatment of gram-negative bacterial infections, is associated with a dose-limiting nephrotoxicity [1, 2]. Various *in vivo* [3–6] and *in vitro* [7–11] animal models have been developed to study the mechanism of gentamicin-induced nephrotoxicity.

Gentamicin is known to accumulate in the lysosomes of PTC§ [12–14]. One of the earliest and most characteristic changes seen morphologically is the appearance of "myeloid bodies", concentric whorls of osmiophilic material within lysosomes [3–6, 15]. This material has been shown to contain undigested phospholipids [16] and is believed to be the result of enzyme inhibition by gentamicin [17–19]. One of the hypotheses developed to explain the pathogenesis of gentamicin-induced nephrotoxicity is that alteration of lysosomal function results in altered cellular metabolism and ultimately cell death [20].

One means by which the normal function of lysosomes could be altered is to increase the normally acidic lysosomal pH [21] and thereby decrease the optimum activity of the constituent enzymes. Because the excitation spectrum of fluorescein is extremely sensitive to changes in pH [22], uptake of FITC-dextran and its accumulation in lysosomes facilitate measurement of intralysosomal pH. This technique has been employed to determine the lysosomal pH using mouse peritoneal macrophages [23, 24], Swiss 3T3 fibroblasts [25], and human fibroblasts [26, 27].

Intralysosomal degradation, however, is also dependent upon the ability of primary lysosomes to fuse with endocytotic vesicles and/or autophagosomes. Cojocel *et al.* [28] found that 7 days of treatment with 30 mg gentamicin/kg/day causes decreased reabsorption and degradation of radiolabeled lysozyme. Later, Cojocel *et al.* [29] extended the period with which the rats were treated *in vivo* with aminoglycosides (from 7 to 21 days) and saw a similar decrease in the ability of the PTC to reabsorb filtered protein. Cojocel and Hook [30], using isolated perfused kidneys from untreated rats, demonstrated that gentamicin inhibits lysozyme reabsorption. It has been speculated that the

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§ Abbreviations: PTC, proximal tubular cells; FITC, fluorescein-isothiocyanate; NAG, *N*-acetyl-β-glucosaminidase; and Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

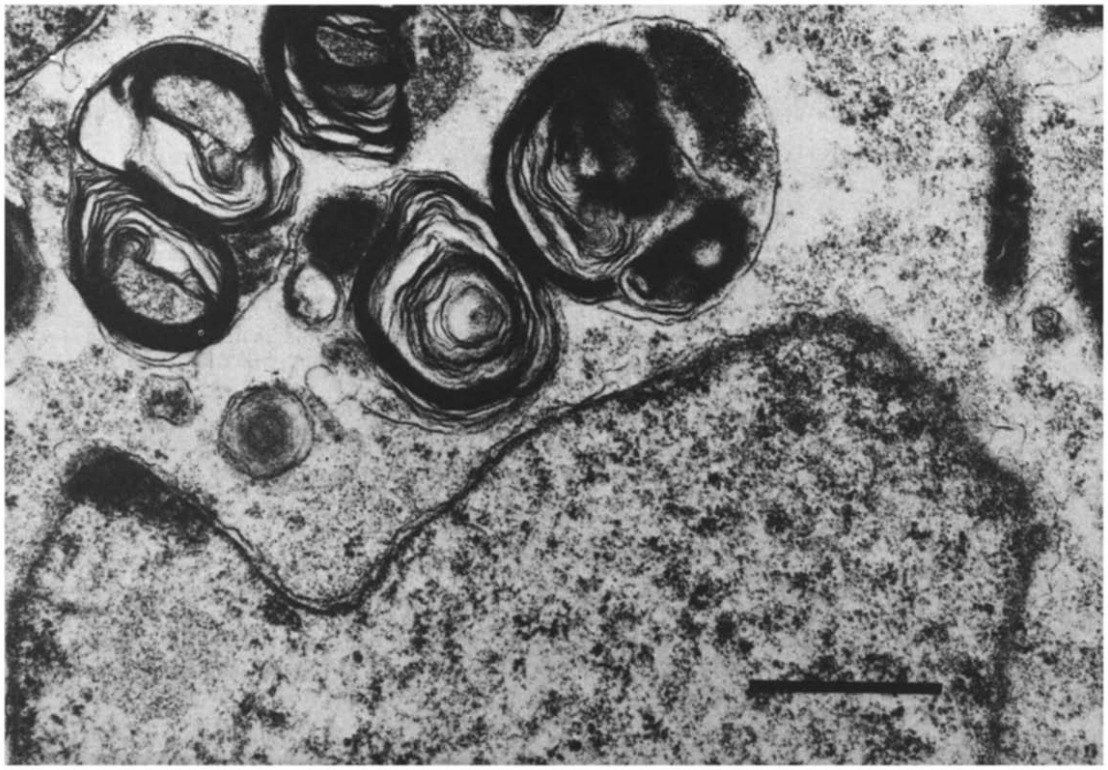


Fig. 1. Electron micrograph from a culture of human proximal tubular cells treated with 1 mg gentamicin/ml for 10 days. Numerous myelin-like inclusions can be seen in this cell. These inclusions were absent in cells of control cultures. Bar, 0.25 μm.

observed decrease in the uptake of proteins may be due to direct interaction of the aminoglycoside with the plasma membrane, i.e. competition with inducers of pinocytosis [31], or interaction of the aminoglycoside with the microtubular system within the cell [32]. The decreased catabolism of the reabsorbed proteins seen by Cojocel *et al.* may reflect not only a decrease in lysosomal enzyme activity [19], but also some gentamicin-induced interference in the fusion of endocytotic vesicles with lysosomes [33].

This paper reports the effect of gentamicin on certain aspects of lysosomal function and integrity, i.e. endocytotic activity, pH, and membrane fragility, in cultured human PTC using *in vitro* conditions associated with the morphologic observation of myeloid bodies.

MATERIALS AND METHODS

Tissue culture medium and reagents were obtained from GIBCO Laboratories (Grand Island, NY). FITC-dextran (average molecular weight = 71,600), chromogenic enzyme substrates, and other biochemicals were purchased from the Sigma Chemical Co. (St Louis, MO). Radioimmunoassay kits for ^{125}I-labeled gentamicin were purchased from Dupont-New England Nuclear (Boston, MA).

Culture of human proximal tubular cells

Human PTC were isolated and cultured as previously described [34]. Briefly, an adult cadaver kidney (postmortem time ≤ 12 hr) was flushed with a balanced salts solution. The kidney tissue was digested by perfusion with collagenase for 1 hr at 37°. After the capsule was stripped, the pale perfused cortical tissue was removed, minced, and further digested with collagenase (100 units/ml solution) in a spinner culture flask. The isolate was then filtered through sterile 40 μm nylon mesh and washed before inoculation into plastic 75 cm^2 flasks for culture in cell medium containing Hanks' balanced salts supplemented with fetal calf serum (10%), Hepes buffer (10 mM), insulin (1 unit/ml), fungizone (1.25 μg/ml), penicillin (100 units/ml) and streptomycin (100 μg/ml). Confluent monolayers were observed in 10–14 days.

Primary cultures were trypsinized and reseeded at a density of 1–2 × 10^5 cells/ml medium. Cultures were rinsed after 24 hr and incubated with fresh antibiotic-free medium for an additional 3 days prior to treatment with gentamicin (0, 0.01, 0.1, and 1.0 mg gentamicin sulfate/ml) for 3, 7, 10, and 14 days.

Electron microscopic studies

Gentamicin-treated and control human PTC cultures were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer. The fixed monolayers were routinely processed for electron microscopy. Sections were stained with uranyl acetate and OsO_4 prior to electron microscopic examination.

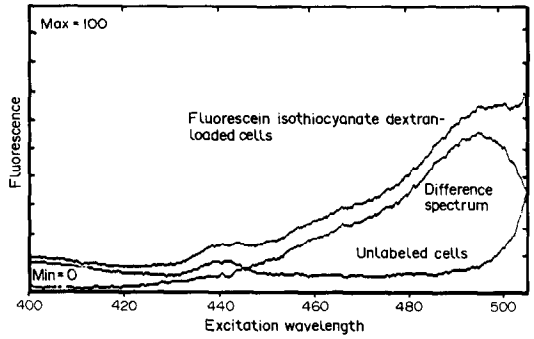


Fig. 2. Comparison of excitation spectra using human proximal tubular cells (PTC) with and without intracellularly trapped FITC-dextran. Excitation spectra were recorded from 420 to 505 nm with a constant emission wavelength of 515 nm. Subtraction of the spectrum of PTC not preincubated with FITC-dextran from spectra using PTC incubated with FITC-dextran was done using a Perkin–Elmer MPF-66 computer.

Loading with fluorescein isothiocyanate-labeled dextran

Human PTC cultures were treated for 7–14 days with gentamicin. Eighteen to twenty-four hours prior to collection, the medium was replaced with the appropriate fresh medium containing FITC-dextran (1 mg/ml). At the time of collection, monolayers were rinsed three times with a Ca^{2+}-free Hanks' balanced salt solution and trypsinized with 0.05% trypsin plus 0.53 mM EDTA for 5 min. The cells were harvested with the aid of a rubber policeman, centrifuged at 72 *g* for 5 min, and washed three times with medium containing 1.37 mM Ca^{2+}. Two milliliters of resuspended cells (0.2 to 0.4 × 10^6 cells/ml) in fresh Ca^{2+}-containing medium (pH 7.2) was used for measurement of the excitation spectrum from 420 to 505 nm with a constant emission of 515 nm in a Perkin–Elmer MPF66 spectrofluorometer [23].

Endocytotic activity. To determine the amount of FITC-dextran taken up by the human PTC, excitation spectra were recorded following addition of Triton X-100 (0.07% final concentration) to solubilize the cells; NaOH (7.5 μl of 1 M) was added to adjust the pH to 8.0. Concentrations of FITC-dextran (0 to 0.6 μg/ml) were also scanned in the presence of Triton and NaOH to determine the signal intensity at an excitation wavelength of 495 nm and an emission wavelength of 515 nm.

Lysosomal pH estimation. In addition to measurement of the baseline lysosomal pH in intact PTC suspensions, the lysosomotropic compound NH_4Cl was added to a final concentration of 0, 1, 5, 10, or 50 nM. NH_4Cl additions were made to determine if gentamicin treatment increased the sensitivity of cultured human PTC to NH_4Cl-induced elevation of the intralysosomal pH. The viability of the human PTC was also estimated by trypan blue dye exclusion.

Isolation of lysosomes

The PTC were harvested by scraping into isotonic sucrose (256 mM, pH 7.0) and homogenized with a Polytron homogenizer (Brinkmann Instruments) on ice for 3 strokes at 500 rpm. A crude lysosomal pellet was formed by centrifugation of the homogenate at

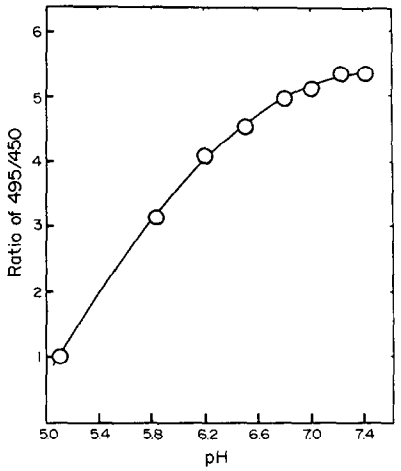


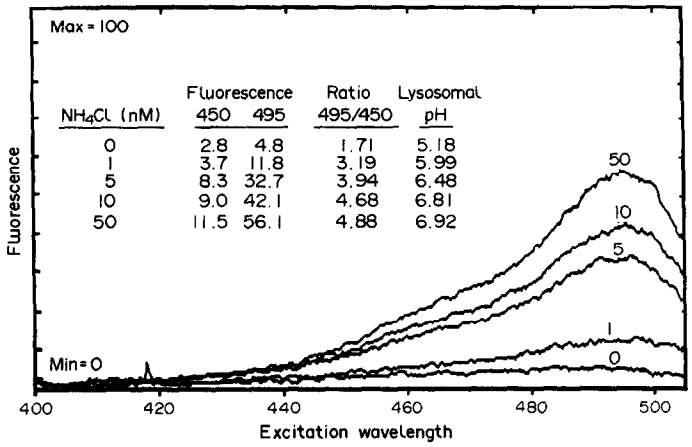
Fig. 3. Standard curve of pH versus the fluorescence ratio using FITC-dextran. A solution of FITC-dextran was titrated with NaOH and HCl. Excitation spectra of the FITC-dextran were recorded from 420 to 505 with constant emission of 515 nm to determine the effect of medium pH on the fluorescence ratio of 495 nm/450 nm.

12,000 *g* for 10 min. The isolated lysosomal pellets were resuspended in 50 mM Tris–250 mM sucrose buffer, pH 7.4. Aliquots were incubated for up to 120 min at either 0° or 37° prior to recentrifugation at 12,000 *g* for 10 min. NAG activity was determined in the supernatant fraction and pellet by the method of Moore and Morris [35]. Lysosomal fragility was estimated by calculating percent NAG release:

$$\% \text{ NAG Release} = (\text{supernatant activity}/(\text{supernatant} + \text{pellet activity})) \times 100$$

Gentamicin content

Following treatment with gentamicin (0.01, 0.1, and 1.0 mg/ml) for 7, 10 or 14 days, the monolayers were rinsed three times with phosphate-buffered saline (100 mM sodium phosphate, 150 mM NaCl, pH 7.2). The cells were disrupted by adding 0.1%



NH_4Cl (nM)	Fluorescence 450	Fluorescence 495	Ratio 495/450	Lysosomal pH
0	2.8	4.8	1.71	5.18
1	3.7	11.8	3.19	5.99
5	8.3	32.7	3.94	6.48
10	9.0	42.1	4.68	6.81
50	11.5	56.1	4.88	6.92

Fig. 4. Effect of NH_4Cl on lysosomal pH using intact human proximal tubular cells (PTC). Additions of a 2.0 M NH_4Cl stock were made to suspensions of human PTC preincubated with FITC-dextran for 18–24 hr. Excitation spectra from 420 nm to 505 nm were recorded using a constant emission wavelength of 515 nm. The ratio of fluorescence values at 495 nm and 450 nm was calculated following subtraction of the background (PTC not containing FITC-dextran) fluorescence; intralysosomal pH was estimated from a standard curve of the pH versus the fluorescence ratio.

Nonidet P-40 and then stored frozen at −70° in plastic vials until assayed using radioimmunoassay for ^{125}I-labeled gentamicin. The content of gentamicin in the treated monolayers was determined by comparison with a standard curve based on competitive binding between radiolabeled and unlabeled gentamicin with the gentamicin-specific antibody. Protein was estimated by the method of Lowry *et al.* [36] using bovine serum albumin as a standard.

Statistical analysis

Data were collected from at least three separate human renal cell cultures. Statistical comparisons were done using a two-tailed pooled Student's *t*-test. Where applicable, values are expressed as the mean ± SD.

RESULTS

As can be seen in Fig. 1, human PTC treated with gentamicin (1 mg/ml for 10 days) contained numerous myelin-like inclusions. These "myeloid bodies" were absent in untreated control cultures. In a previous study, we reported no significant cell death during 14 days of gentamicin treatment despite the presence of numerous inclusions [37].

Human PTC were found to take up 5.41 ± 3.88, 4.44 ± 3.50, 4.95 ± 2.28, and 3.42 ± 1.09 ng FITC-dextran/hr/mg protein following exposure to 0, 0.01, 0.1, and 1.0 mg gentamicin/ml respectively. Determination of FITC-dextran uptake was based on the fluorescence of known concentrations of FITC-dextran at pH 8.

The excitation spectra of FITC-dextran loaded PTC were corrected for background fluorescence as shown in Fig. 2 prior to estimation of intralysosomal pH based on the ratio of excitation wavelengths 495 nm/450 nm with a constant emission wavelength

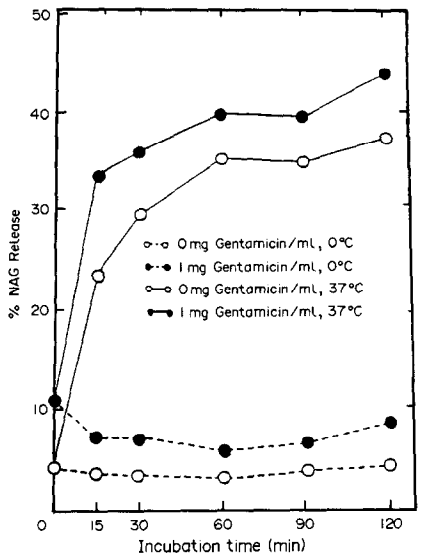


Fig. 5. Effect of incubation time and temperature on *N*-acetyl-β-glucosaminidase (NAG) release. Lysosomes were isolated from 7-day control (0 mg gentamicin/ml) and treated (1.0 mg gentamicin/ml) human proximal tubular cell (PTC) cultures. Aliquots were incubated for up to 120 min prior to recentrifugation and analysis of NAG activity.

of 515 nm (Fig. 3). Successive additions of concentrated NH_4Cl (2.0 M) to suspensions of human PTC preincubated with FITC-dextran increased the calculated 495 nm/450 nm fluorescence ratio, i.e. NH_4Cl increased the lysosomal pH as shown in Fig. 4. It was noted, however, that while final concentrations of $NH_4Cl \geq 10$ mM caused a 5–20% increase in the osmolarity, final concentrations ranging from 1 to 50 mM had no effect on medium pH

Table 1. Effect of NH_4Cl on lysosomal pH using gentamicin-treated human proximal tubular cells (PTC)*

	Lysosomal pH			
	Gentamicin dose (mg/ml)			
NH_4Cl (mM)	0.0	0.01	0.1	1.0
0	5.24 ± 0.33	5.37 ± 0.32	5.32 ± 0.51	5.70 ± 0.44
1	6.08 ± 0.05†	6.20 ± 0.31†	6.28 ± 0.28†	6.27 ± 0.25
5	6.68 ± 0.20†	6.68 ± 0.22†	6.73 ± 0.19†	6.72 ± 0.22†
10	6.78 ± 0.20†	6.84 ± 0.22†	6.95 ± 0.24†	6.85 ± 0.21†
50	7.15 ± 0.14†	7.19 ± 0.33†	7.21 ± 0.29†	7.12 ± 0.38†

* The fluorescence of FITC-dextran loaded human PTC (treated with gentamicin for 7–14 days) was recorded following sequential additions of NH_4Cl (2.0 M stock). Calculation of intralysosomal pH was based on a standard curve. Values are expressed as the mean ± SD, N = 3–5 experiments.

† Significantly different from the corresponding NH_4Cl control (0 mM), $P < 0.05$.

or cellular viability as estimated by trypan blue dye exclusion. Table 1 summarizes the effect of NH_4Cl on the lysosomal pH estimated for intact human PTC treated with gentamicin for 7–14 days; no change in lysosomal pH was seen over the 14 days of administration at each dose of gentamicin. Addition of NH_4Cl to a final concentration of 5 mM caused a significant ($P < 0.05$) elevation in lysosomal pH above control (0 mM NH_4Cl) for all gentamicin-treated cells examined. No significant difference was noted in the response of lysosomal pH to NH_4Cl perturbation, however, when gentamicin treatments were compared to the untreated (0 mg gentamicin/ml) control.

Release of NAG activity after incubation of lysosomes isolated from human PTC cultured for 7 days with 0 and 1.0 mg gentamicin/ml is shown in Fig. 5. Incubation at 0° of the lysosomal isolates did not result in any release of NAG activity. The level of NAG release increased significantly ($P < 0.05$), however, following incubation at 37° for as little as 15 min. The amount of NAG activity present in the supernatant fraction remained unchanged between 60 and 120 min incubation at 37°. Therefore, for comparative study of lysosomal fragility in gentamicin-treated and untreated cultures, a 60-min incubation at 37° was used as the thermal shock.

Lysosomal fragility estimated by NAG release is shown in Table 2. Treatment with gentamicin for ≤ 7 days caused no change in lysosomal fragility. The release of NAG from lysosomes isolated from PTC cultures treated with 1.0 mg gentamicin/ml for 10 days, in contrast, was increased significantly ($P < 0.05$) over the corresponding control, while 10-day treatment with [gentamicin] ≤ 0.1 mg/ml had no effect. By 14 days of continuous exposure, however, significant increases above control were seen in the fragility of lysosomes isolated from gentamicin-treated (0.01, 0.1 and 1.0 mg gentamicin/ml) human PTC.

The actual lysosomal content of gentamicin in treated PTC cultures is shown in Table 3. The increase in lysosomal content was dose dependent at all the time points examined with a 3- to 30-fold increase in content when the gentamicin dose was increased 10- to 100-fold.

DISCUSSION

Previous work in our laboratory has documented the appearance of myeloid bodies [38] in addition to some of the enzyme changes associated with gentamicin treatment, i.e. decreased γ-glutamyltransferase and sphingomyelinase activities and increased NAG activity [37, 39]. These morphologic and biochemical alterations, however, were not associated with any extensive necrosis or loss of human PTC numbers. The purpose of this report was to investigate more subtle changes in the function and integrity of lysosomes, i.e. endocytotic activity, lysosomal pH, and membrane stability, using cultured human PTC, and to determine if these lysosomal changes were related to gentamicin accumulation.

Cultured human PTC showed only a slight but not statistically significant decrease in the mean uptake of FITC-dextran following gentamicin treatment. There was, however, a large variation in the uptake of FITC-dextran by the control (no gentamicin) PTC isolated from different individuals; it is not known if this difference among control PTC is related to the varied incidence of gentamicin-induced nephrotoxicity seen in patients [1, 2].

Untreated human PTC cultures had a lysosomal pH of 5.24 ± 0.33. These values were comparable to the previously reported normal lysosomal pH values of 4.75, 5.40, and 5.73 estimated for mouse peritoneal macrophages [23, 24], Swiss 3T3 fibroblasts [25], and human fibroblasts, [26, 27] respectively. Gentamicin treatment (1 mg/ml) caused a slight but not significant increase in the average lysosomal pH to 5.70 ± 0.44. The lack of any significant effect of gentamicin exposure on lysosomal pH seen in the gentamicin-treated human PTC is similar to the results of Oshima *et al.* [26] in human skin fibroblast cultures. These results indicate that the significantly decreased sphingomyelinase activity reported both *in vivo* and *in vitro* [7–10, 37, 39] cannot be explained by elevation of intralysosomal pH.

Elevation of lysosomal pH by NH_4Cl has been reported by Poole and Ohkuma [24]. They found that addition of 10 mM NH_4Cl to the incubation medium increases the lysosomal pH of mouse per-

Table 2. Effects of time and dose on *N*-acetyl-β-glucosaminidase (NAG) release from lysosomes isolated from gentamicin-treated human proximal tubular cell cultures (PTC)*

Length of exposure to gentamicin (days)	% NAG released			
	Gentamicin dose (mg/ml)			
	0	0.01	0.1	1.0
0	23.3 ± 4.3			
3	23.7 ± 8.5	25.0 ± 9.9	24.4 ± 2.3	23.7 ± 2.1
7	28.7 ± 2.9	25.0 ± 1.3	27.0 ± 6.2	36.2 ± 8.1
10	29.2 ± 2.9	30.9 ± 2.1	31.0 ± 6.3	43.0 ± 6.3†
14	24.0 ± 4.6	36.3 ± 1.8†	37.7 ± 1.1†	35.5 ± 2.6†

* Lysosomes were isolated from treated human PTC cultures and incubated at 37° for 60 min. Lysosomal fragility was calculated as (NAG activity in medium/total NAG activity) 100%. Values are expressed as the mean ± SD, N = 3–5 experiments.

† Significantly different from control (0 mg gentamicin/ml), $P < 0.05$.

itoneal macrophages by 1.5 pH units. As can be seen in Table 1, addition of 10 mM NH_4Cl raised the lysosomal pH from 1.15 to 1.63 pH units above baseline pH (no NH_4Cl) in the intact human PTC. The fact that the extent to which the lysosomal pH could be altered by NH_4Cl was not influenced by prior gentamicin treatment in culture suggests that the function of the lysosomal membrane proton pump was not affected. Since no effect of gentamicin on lysosomal pH was detected, the power (for a two-tailed *t*-test at the 5% significance level) was calculated and found to be 80% for detecting a 5% change in pH. This suggests that our experiments were sensitive to slight changes in pH.

Finally, the effect of gentamicin on the stability of isolated lysosomes, as measured by release of NAG following thermal shock (60-min incubation at 37°), was also examined. Gentamicin treatment (1.0 mg/ml, 10 days) of cultured human PTC resulted in increased release of lysosomal NAG, e.g. increased lysosomal fragility following thermal shock. Decreasing the level of gentamicin in the culture medium delayed the increase in lysosomal fragility seen, i.e. 14 days of treatment with 0.01 and 0.1 mg gentamicin/ml was needed to increase significantly the lysosomal membrane fragility. Under these *in vitro* conditions, there was no evidence of significant cell death. Using conditions associated with *in vivo* histologic damage to the rat kidney (10–50 mg aminoglycoside/kg/day for 8 days), however, both Fillastre *et al.* [10] and Viotte *et al.* [19] have noted that lysosomes isolated from treated rats released more NAG than did the lysosomes from control animals. In contrast, Cowell *et al.* [40] found that lysosomes isolated from rats treated *in vivo* with gentamicin (50 mg/kg) for 12 hr released less NAG than control lysosomes. Powell and Reidenberg [41] reported that prolonged *in vivo* administration of gentamicin to rats (20 mg/kg/day for 28 days) resulted in lysosomes which, when isolated, released less NAG than lysosomal preparations from the corresponding control rats; histologically the rat PTC appeared unchanged. In an earlier study, however, Powell and Reidenberg [42] reported that 20 mg gentamicin/kg administered twice daily to rats for 28 days caused an increase in lysosomal NAG release. Again, the PTC in the kidneys of rats receiving gentamicin appeared similar morphologically to control rat PTC. The different results of these two studies may reflect the change in administration regimen, i.e. frequent administration smaller doses is more acutely toxic than a single bolus injection of the same total dosage [43–45].

Human PTC accumulated gentamicin in a dose-dependent fashion. Maximum levels (10–11 μg/mg protein) were reached with 7–10 days of treatment with 1.0 mg gentamicin/ml. Such levels were comparable to those measured *in vivo* (3 μg/mg rat kidney protein [46]; 3–4 μg/ng human renal cortical protein [47]) and *in vitro* (3–10 μg/mg fibroblast protein [48]; 33 μg/mg fibroblast protein [7]). Interestingly, the lysosomal fragility did not correlate with the gentamicin content.

The effect of aminoglycosides on the proper function of lysosomes appears to be a complex process. Possible sites of lysosomal dysfunction are the aminoglycoside-induced inhibition of the pinocytosis of the aminoglycoside itself [31] in addition to extracellular proteins [27–29], inhibition of fusion between different lysosomal elements [33], and the decreased activity of lysosomal enzymes [7, 11, 17, 25]. Alteration of lysosomal function, however, could be secondary to aminoglycoside interaction with microtubules [32] or lysosomal membranes [10, 19, 41, 49]. Indeed, lysosomal membrane permeability and/or stability may influence not only intralysosomal pH [50], but also protein catabolism [51].

Use of experimental conditions that produced significant production of myeloid bodies and alteration of enzyme activity in gentamicin-treated cultures of human PTC [37–39] did not result in any significant alteration of lysosomal function as measured by endocytotic activity or lysosomal pH. Lysosomal membrane fragility, however, increased after 14 days of treatment with gentamicin (0.01, 0.1, and 1.0 mg/ml); this increased fragility did not correlate with the content of gentamicin in the human PTC. The gentamicin-induced changes noted in the human PTC lysosomes, i.e. increased numbers of myeloid bodies, altered enzyme activities, and increased lysosomal membrane fragility, therefore, are not associated with significant *in vitro* irreversible injury, i.e. extensive necrosis and cell loss. These changes, however, may reflect metabolic adaptation and/or increased sensitivity of the human PTC to

Table 3. Effects of time and dose on the content of gentamicin in treated human proximal tubular cell cultures (PTC)*

Length of exposure to gentamicin (days)	Gentamicin content (μg/mg protein)		
	Gentamicin dose (mg/ml medium)		
	0.01	0.1	1.0
7	0.345 ± 0.127	1.77 ± 0.56	11.1 ± 4.2
10	0.472†	2.05 ± 0.76	10.3 ± 4.8
14	0.902†	2.77†	ND‡

* Gentamicin content was measured by radioimmunoassay. Values are expressed as the mean ± SD; N = 3–5 experiments unless indicated otherwise.
† N = 1 experiment.
‡ Not done.

further damage which, in turn, could lead to the nephrotoxicity seen *in vivo*.

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