Variation in Gentamicin-Induced Death Among Independent Cultures of Proximal Tubule Cells*

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ABSTRACT

This study determined the sensitivity of 12 independent isolates of cultured human proximal tubule (HPT) cells to gentamicin. The TD50 (dose [μg per ml]) for each isolate was determined by linear regression analysis of cells exposed to a wide concentration range of gentamicin over a 16-day period of exposure. The results of this determination clearly showed that within a series of HPT cell cultures there was significant diversity in sensitivity to gentamicin. Three groups of toxic response were delineated. A single isolate, isolate I, was extremely resistant to gentamicin toxicity and was the sole member of group 1. The majority (9 of 12) of the isolates demonstrated intermediate sensitivity to gentamicin and were classified as group 2. Group 3 included two isolates that were very sensitive to gentamicin, with relatively low doses producing cell death. These results provide initial evidence that a portion of the variability seen in nephrotoxicity among patients receiving the aminoglycoside antibiotics might be mediated by factors intrinsic to each individual's proximal tubule cells.

Introduction

The aminoglycoside antibiotics are widely utilized in the clinical management of gram-negative infections; however, their use is associated with nephrotoxicity localized specifically to cells of the proximal tubule.1,6 This nephrotoxicity occurs even when aminoglycoside concentrations are maintained within the recommended therapeutic peak and trough values, with 10 to 15 percent of all courses of treatment being complicated by clinically detectable alterations in renal function.1,12 In a large prospective study, it was shown that 11 percent of hospital acquired cases of acute renal failure were attributable to aminoglycoside administration.5 Likewise, it has been estimated that up to 53 percent of patients treated with gentamicin suffer some form of renal damage.10 This high level of nephrotoxicity has stimulated...
studies designed to identify risk factors associated with aminoglycoside usage (for review\textsuperscript{1,6,14}).

Several factors have been identified that increase the probability that a patient will develop nephrotoxicity including: increasing age; pre-existing renal insufficiency; concomitant administration of intrinsically nephrotoxic agents; pre-existing liver disease; severe hypertension; and, fluid and electrolyte abnormalities. However, the identification of these risk factors has not eliminated aminoglycoside nephrotoxicity. An additional factor, one not easily identified or defined, is the possibility that certain individuals are simply more susceptible than others to aminoglycoside nephrotoxicity. That is, within the "normal" population there exist intrinsic renal determinants that create a spectrum of sensitivity to the aminoglycoside antibiotics.

One of the few means to provide evidence that such a variation in susceptibility might exist would be a comparison of the toxicity of the aminoglycosides among a series of human proximal tubule (HPT) cell cultures where, unlike in vivo studies, experimental conditions can be tightly controlled. Such an analysis is feasible since HPT cells have been isolated that are homogeneous in composition and retain many of the differentiated functions expected of cells derived from this nephron segment.\textsuperscript{2,3,13,19} In the present study, a series of 12 such isolates were employed to determine if the sensitivity of the HPT cells to the aminoglycoside, gentamicin, does vary appreciably among a series of human proximal tubule cell cultures.

**Methods**

**Tissue Culture**

Stock cultures of HPT cells were grown in 75 cm\textsuperscript{2} T-flasks using procedures described previously by this labo-

<table>
<thead>
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* Collaborative Research.
† U.S. Biochemicals.
tissue obtained for cell culture purposes is normal cortical tissue remote from any pathological alteration. Experimental protocols were initiated by subculturing confluent stock cultures at a 1:2 subculture ratio into a series of 12 well (4.2 cm² per well) tissue culture dishes. Each well contained two ml of growth media. The cells were fed fresh growth medium every three days for six days, and all isolates reached confluence within three days of subculture. On culture day 7, (gentamicin exposure day 0), the cells were fed fresh growth medium containing the selected concentrations of gentamicin. The concentrations of gentamicin utilized were: 50; 100; 250; 500; 1,000; 2,000; and 4,000 µg per ml of growth medium. The effect of gentamicin on cell growth was determined on exposure day 1, 4, 7, 10, 13, and 16.

Statistical Analysis of Growth Data

Cell counts utilized the nuclear stain DAPI (4',6-diamino-2-phenylindole) and an automatic counting program executed on a Zeiss‡ IBAS 2000 image analysis computer.¹⁷,¹⁸ Briefly, following exposure to gentamicin at a given concentration for a given time, the wells containing the monolayers were rinsed with phosphate buffered sulfate (PBS), fixed for 15 minutes in 70 percent ethanol, rehydrated with two ml of PBS and stained with 50 µl of DAPI (10 µg per ml in distilled water). The well was examined under fluorescent illumination at 320× magnification on the Zeiss‡ IM35, an inverted fluorescent microscope linked to the Zeiss‡ IBAS 2000 image processor, revealing fluorescent nuclei which could readily be quantitated utilizing an automatic counting program. For each concentration and time point, a minimum of 20 fields per well and three wells per data point were determined. Both nuclear counts and total nuclear area were obtained from the program and gave equivalent results. Viability for a given gentamicin exposure was expressed as a percent of control value (cells not exposed to gentamicin) for a given isolate and time point.

The TD₅₀ (dose [µg per ml] producing 50 percent lethality) and the standard error of this estimate was calculated utilizing least squares linear regression analysis (Systat for Windows® 5.01 software)§ for each isolate and each experimental day. In general, the regressions on isolates exposed to gentamicin for more than six days produced R values exceeding 0.85 (exceptions noted in results). Residuals were examined from these regressions, and they did not have a heteroscedastic distribution. The errors were normally distributed, and the studentized residuals were less than 2.5 absolute magnitude, with most falling between -1 and +1. The errors were independent and not serially correlated. The Cooks distance for the residuals demonstrated that the linear model satisfactorily described all members of the regression. From the TD₅₀ analysis, three groups of isolates were identified (see results). Significant differences between these three groups were analyzed by the non-parametric Krusal-Wallis test, utilizing Systat® software for both the TD₅₀ and viability data. For these calculations, a values of 0.05 were considered significant.

Results

The results of the present study clearly demonstrate that a series of HPT cell cultures exhibit a significant diversity in their sensitivity to gentamicin (figure 1). The toxic response of gentamicin was not

‡ Carl Zeiss, Inc., Thornwood, NY.
§ Systat, Inc., Evanston, IL.
immediate; little toxicity was seen until after seven days of exposure, and R values obtained from linear regression analysis to calculate the TD$_{50}$ at early time points were very low, most less than 0.3. This temporal pattern of gentamicin toxicity is in agreement with clinical and in vivo animal studies (for review$^{1,6,14}$).

At day 7, five isolates (A, C, F, G, N) demonstrated gentamicin toxicity, and the R values for the TD$_{50}$ of these isolates exceeded 0.85. At the next experimental day (day 10), the remainder of the isolates demonstrated toxicity with the R values exceeding 0.85 for all isolates with the exception of isolate B, D, and E where R values were less than 0.5. Isolate I on day 7 had a TD$_{50}$ of over 10,000 µg per ml and an R value of 0.667; it was omitted from figure 1 to avoid further expansion of the y axis.

After the toxic response to gentamicin was evident in an isolate, further time points for that isolate demonstrated continued toxicity, and R values for the regression analysis exceeded 0.85; the analysis of the residuals from these regressions met the diagnostic tests described in the methods. The temporal onset of toxicity was not related to the degree of toxicity, i.e., isolates B, D, E demonstrated little toxicity until after day

![Diagram](image-url)

**Figure 1.** The TD$_{50}$ of 12 independent isolates of HPT cells. The TD$_{50}$ was determined by linear regression analysis and employed HPT cells exposed to the various concentrations of gentamicin for 16 days. Points are the mean ± the standard error; however, owing to the expanded scale of the graph, the error bars, which are in the range of 30 to 60 µg for an individual data point, are barely perceptible. The open circle (day 16, isolate N) has a low R value (0.466) because few data points available for this analysis owing to toxicity.
10 of exposure, but once apparent, the TD$_{50}$s were similar to other group 2 isolates demonstrating earlier toxicity.

Three groups of gentamicin response were clearly distinguished. Two isolates, G and N, demonstrated extreme sensitivity to gentamicin, with low TD$_{50}$s for the time period examined. These isolates demonstrated decreased viability at relatively low doses of gentamicin, had TD$_{50}$ values that were significantly lower than most other isolates, and are designated as group 3, gentamicin sensitive isolates. At the last day of exposure (day 16), isolate N exhibited such toxicity that only four concentrations of gentamicin were available to calculate the TD$_{50}$; gentamicin concentrations above 500 µg per ml produced total cell death. Since there were so few points available for the regression analysis the resulting R value for this point had a low value (0.466) and the estimated TD$_{50}$ was below 450 µg per ml. Most isolates (9 of 12) were intermediate in toxic response to gentamicin and are designated as group 2. A single isolate, isolate I, manifested resistance to gentamicin exposure with relatively high exposure needed to elicit a toxic response. This isolate is the sole member of group 1, the gentamicin-resistant group. Krusal-Wallis analysis of the TD$_{50}$ measurements between these gentamicin response groups (table II) demonstrated that after seven days of exposure, highly significant differences ($\alpha < 0.001$) existed between these three groups.

A Krusal-Wallis analysis of these groups utilizing the viability of the cells, expressed as a percent of control, for each gentamicin concentration and each experimental day was also done to complement the data obtained by the TD$_{50}$ measurements. The viability measurement was utilized to correct for any plating differences between experiments that could result in absolute cell number differences between isolates that was not related to gentamicin exposure. The results of this analysis were in agreement with the TD$_{50}$ measurements (table II); the lower probabilities in this analysis are likely related to the greater N. Finally, Krusal-Wallis analysis was performed between the gentamicin response groups for each separate experimental day, utilizing both TD$_{50}$ measurements and the percent viability. This analysis confirmed significant differences ($\alpha = 0.05$) between the gentamicin response groups at days 7 to 16 and no significant differences between groups at days 1 and 4 (data not shown).

**Discussion**

The mechanism underlying these differences in HPT cell sensitivity to gentamicin has not yet been explored. However, several explanations that center on the use of cell culture techniques can be ruled out. The characteristics of the individual tissue as regards age, race, or sex did not appear to have an influence on the HPT cell isolates' sensitivity to gentamicin—although the current sample size is limited. Likewise, the passage number (culture age) of the cell isolates had no relationship to gentamicin sensitivity as all were utilized between passage 3 and 6, and previous investigations in this laboratory have demonstrated no differ-

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DIVERSITY IN GENTAMICIN TOXICITY

ences in several experimental parameters\textsuperscript{2,4,13,17,18,19} in this passage range. Additionally, while no means exist to prove absolutely the constant origin of cultured cells, the technique employed to isolate the individual cell cultures herein has been shown to produce cells with characteristics consistent with a homogeneous population and an origin from the proximal tubule. Specifically, a constant enzyme histochemical and immunohistochemical profile,\textsuperscript{3} a characteristic electrical and ultrastructural profile,\textsuperscript{2,19} and consistent cAMP stimulation as a result of exposure to parathyroid hormone.\textsuperscript{4,13} Thus, the results obtained do not appear to arise from an easily identifiable artifact of cell culture technique.

The finding of a variability in gentamicin toxicity among the HPT cell isolates affords the possibility of defining a linkage between human sensitivity in vivo to these in vitro findings. This linkage would require a comparison of aminoglycoside binding between brush-border membrane (BBM) isolated from each donor human kidney with the BBM binding and toxicity from each matched donor-derived HPT cell culture. The rationale for this is that the BBM of the renal proximal tubule appears to be intimately involved in the binding and transport of aminoglycosides into the proximal tubule.\textsuperscript{11,16,21} The binding of aminoglycosides to isolated renal BBM of rats\textsuperscript{7,8,9} and rabbits,\textsuperscript{11} but not humans, has been described. These analyses indicate that aminoglycoside binding to BBM is rapid, saturable, and specific in these species.

There is also evidence that renal BBM binding affinity of the aminoglycoside is correlated to toxicity. When the renal BBM binding affinity of the aminoglycoside gentamicin is compared in male and female rats, the aminoglycoside binding affinity to the male BBM is greater than the binding to female BBM.\textsuperscript{21} This correlates to the greater sensitivity of male rats to the nephrotoxicity of aminoglycosides.\textsuperscript{21} Likewise, it has also been demonstrated that immature rats are less sensitive to the nephrotoxicity of aminoglycosides than older mature rats.\textsuperscript{21} Once again, the binding affinity correlates with the nephrotoxicity; the renal BBM of immature rats have less binding affinity for aminoglycosides than mature renal BBM.\textsuperscript{20}

Furthermore, the ranking of the binding affinity of different aminoglycosides to renal BBM of rats correlates with the in vivo nephrotoxicity ranking of these antibiotics in rats.\textsuperscript{20} These results define that the kinetic parameters and characteristics of BBM binding may represent intimate details associated with the ultimate or penultimate toxin/toxic receptor interaction. Thus, compelling evidence suggests that a comparison of donor-matched BBM from in vivo and in vitro sources could define a human component to renal aminoglycoside susceptibility.

In summary, while two other reports have appeared using cultured human proximal tubule cells in studies that assess the toxicity of gentamicin,\textsuperscript{15,22} the present report is the first to determine if an inherent diversity in sensitivity might be present within a series of independent cell culture isolates. The present results provide evidence that at least a portion of the variability seen in nephrotoxicity among patients receiving the aminoglycoside antibiotics might be mediated by factors intrinsic to the individual patient and not due to compromising secondary complications. The definition of this variability in HPT cell isolates may allow a linkage to human renal toxicity through a comparison of renal BBM aminoglycoside binding.

Acknowledgments

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References


