Decreased Cellular Toxicity of Neomycin in a Clonal Cell Line Isolated from LLC-PK$_1$

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We have previously shown in LLC-PK$_1$ cells, that apical membrane enzyme activity was inhibited by aminoglycoside antibiotics (Am. J. Physiol. 254, C251-C257, 1988). In the present study, the relationship between the lethal cytotoxic effect of aminoglycoside and its effect on apical membrane enzyme was examined by establishing aminoglycoside resistant cells. A clonal cell line, LLC-PK$_1$/NRa3, was isolated from parent LLC-PK$_1$ cells in the presence of neomycin. Neomycin inhibited colony formation and increased the number of floating dead cells in parent LLC-PK$_1$ cultures. In contrast, these cytotoxic effects of neomycin were negligible or less pronounced in NRa3 cells, indicating that NRa3 cells were more resistant to neomycin compared with the parent cells. The inhibitory effect of neomycin on apical enzyme activity was significantly weaker in NRa3 cells than in the parent cells. These results suggest that a common mechanism is involved in the aminoglycoside-induced reductions in the apical enzyme activity and in cell viability of LLC-PK$_1$ cells.

KEY WORDS: aminoglycoside nephrotoxicity; apical membrane enzymes; cell cloning; neomycin-resistance; kidney epithelial cell line.

INTRODUCTION

Aminoglycoside antibiotics are widely used in the treatment of Gram-negative infectious diseases. However, their use is sometimes associated with nephrotoxicity, such as defective urine concentrating capacity, proteinuria, enzymuria, and tubular cell necrosis, which is confined almost exclusively to the proximal tubules (1). Studies on the mechanisms of nephrotoxicity induced by aminoglycoside antibiotics showed that many structural and functional alterations occurred in lysosomes, plasma membranes, and mitochondria, during and/or after the development of nephrotoxicity (2,3). However, the biochemical events responsible for proximal tubular injury and subsequent renal failure are still unclear.

LLC-PK$_1$, a continuous cell line derived from the pig kidney, possesses many characteristics of the proximal tubular epithelium such as apical marker enzymes (4,5) and transport systems for hexose (6–8), amino acids (9), phosphate (10), and organic ions (11–13). Previously, we investigated the effects of aminoglycoside antibiotics on cellular function of LLC-PK$_1$ and found that the activity of cell-associated apical membrane enzymes such as alkaline phosphatase, aminopeptidase, and γ-glutamyltransferase is reduced by aminoglycoside treatment (14–16). Further, the order of potency of various aminoglycoside antibiotics in inhibiting such enzyme activity is correlated with their nephrotoxic potential *in vivo* (14). However, it has not been established whether the change in apical membrane enzymes is related to the lethal cytotoxic effect of the drugs in LLC-PK$_1$ cells.

Though LLC-PK$_1$ cell cultures are rather homogeneous compared to renal tubular cells, they consist of subpopulations. Several clonal sublines were nonselectively isolated from parent LLC-PK$_1$ cells and were found to be distinct in their morphology and physiology (17,18). In addition, LLC-PK$_1$ mutants were isolated using appropriate selection methods (19). The present study was undertaken to isolate clonal LLC-PK$_1$ cells with a decreased sensitivity to aminoglycoside toxicity and to examine the relationship between the cytotoxic effect of the drug and its effect on apical membrane enzyme.

MATERIALS AND METHODS

Cell Culture

LLC-PK$_1$ cells obtained from the American Type Culture Collection (ATCC CRL-1392) were grown on plastic dishes (Corning Glass Works, Corning, NY) in medium 199 (Flow Laboratories, Rockville, MD), supplemented with 10% fetal calf serum (Microbiological Associates, Bethesda, MD) without antibiotics, in an atmosphere of 5% CO_2–95% air at 37°C and were subcultured every 4–5 days using 0.02% EDTA and 0.05% trypsin. In most experiments, 60- and 100-mm dishes were inoculated with 4×10^5 cells in 5 mL and 1×10^6 cells in 10 mL of complete culture medium, respectively.

Cell Cloning

Neomycin is the most nephrotoxic of all clinically available aminoglycoside antibiotics. Therefore, in this study, we used neomycin as a selective agent for aminoglycoside resistant clones. LLC-PK$_1$ cells were seeded at a density of 3000 cells per 100-mm dish. Neomycin (1 m*M*) was added to the culture medium, and after 2 weeks, independent colonies of cells, which proliferated in the presence of neomycin, were isolated using metal cloning cylinders. Briefly, the culture medium was removed from the dishes, which were rinsed twice with calcium- and magnesium-free Dulbecco's phosphate-buffered saline, pH 7.4 (PBS buffer). Sterilized cloning cylinders were dipped into stopcock grease, then the greased end was placed down against the plastic so that the cylinder encircled a colony of cells. After detaching the cells with 1–2 drops of trypsin/EDTA, complete medium was added to the cylinders to stop the trypsinization, and the cell suspension was transferred into 24-well microplates (Corning Glass Works, Corning, NY). Several isolated clones

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were expanded in the presence of 2 m*M* neomycin. As described previously (15), the inhibition of dome formation is a manifestation of aminoglycoside toxicity in LLC-PK_1 cells. Therefore, two clonal lines, designated LLC-PK_1/NRa1 and /NRa3, which formed many domes even in the presence of 2 m*M* neomycin, were selected and subcultured in the presence of 2 m*M* neomycin until sufficient numbers of cells were obtained. We characterized NRa3 cells in this study, because the preliminary results obtained with NRa1 and NRa3 cells were essentially the same. In the present investigation, the parent cells were studied between the 219th and the 243th passages, and NRa3 cells between the 230th and the 250th passages. Mycoplasma testing was performed by Dainippon Pharmaceutical Co. (Osaka, Japan), and both parent and NRa3 cells were confirmed to be free of mycoplasmas.

Enzyme Assay

Usually, cells exposed to aminoglycosides on the day of inoculation were used on the fourth day. Cells were washed with ice-cold saline, scraped with a rubber policeman into ice-cold saline (2 mL for 60-mm dish and 4 mL for 100-mm dish), then homogenized with a Polytron (Kinematica, Kriens-Luzern, Switzerland) at a setting of 7 for 1 min. Alkaline phosphatase (EC 3.1.3.1), aminopeptidase (EC 3.4.11.2), γ-glutamyltransferase (EC 2.3.2.2), and protein in homogenate were measured as described previously (14,15).

Materials

Neomycin sulfate was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of the highest purity available.

RESULTS

Figure 1 shows the cell number and the amount of protein per dish in parent and NRa3 cells during growth in culture. The curves obtained from both parent and NRa3 cells were essentially the same, in both the number of cells and the protein per dish. Confluent monolayers of NRa3 cells as well as the parent cells exhibited dome formation, a manifestation of transepithelial transport properties.

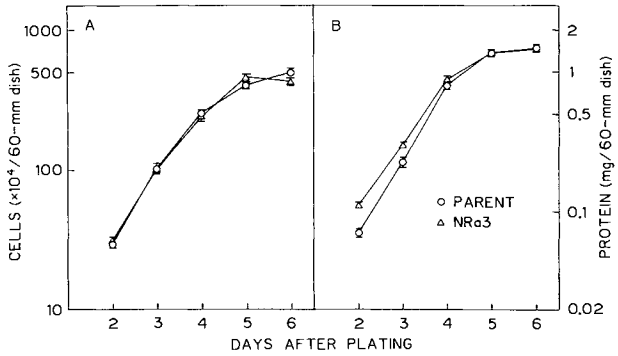

Fig. 1. The growth rate of parent and LLC-PK_1/NRa3 cells. The parent (○) and NRa3 (△) cells were plated on day 0 (15×10^4 cells/60-mm dish) and cultured for 6 days with a medium change on day 4. (A) The number of cells per dish. (B) The amount of protein per dish. Each point represents the mean ± SE of three determinations from a typical experiment.

The cytotoxic effect of neomycin on the parent and NRa3 cells was tested by colony formation assay. As shown in Fig. 2, the line for NRa3 cells shifted to the right compared with that for the parent, indicating that NRa3 is more resistant to neomycin as assessed by the colony formation assay. The apparent IC_{50} was 2.2 m*M* for parent cells and 4.0 m*M* for NRa3.

We have previously reported that treatment of LLC-PK_1 cells with gentamicin increased the number of floating cells in the culture medium (15). These floating cells were dead as assessed by trypan blue staining, indicating that the number of floating cells can also be used as a marker of aminoglycoside cytotoxicity to LLC-PK_1 cells. Therefore, the effect of neomycin on this parameter was estimated in parent and NRa3 cells. As shown in Fig. 3, the number of floating cells was markedly increased by neomycin in parent cells. In contrast, no effect was observed in NRa3 cells. The percentage of control after neomycin treatment was 286 ± 38 in parent cells and 106 ± 10 in NRa3 cells ($P < 0.01$, significantly different).

Next the effect of neomycin on the specific activity of apical marker enzymes was investigated in parent and NRa3 cells (Fig. 4). After 4 days, the specific activity of alkaline phosphatase, aminopeptidase, and γ-glutamyltransferase in parent LLC-PK_1 cells was reduced by neomycin in a dose-dependent manner. In contrast, the activity of apical marker enzymes in NRa3 cells was less affected by neomycin, even with concentrations up to 5 m*M*.

DISCUSSION

A number of possible mechanisms contributing to the pathogenesis of aminoglycoside toxicity have been reported (1–3,14–16,20–26) since aminoglycosides affect various cellular functions. Marker enzymes of brush border membranes

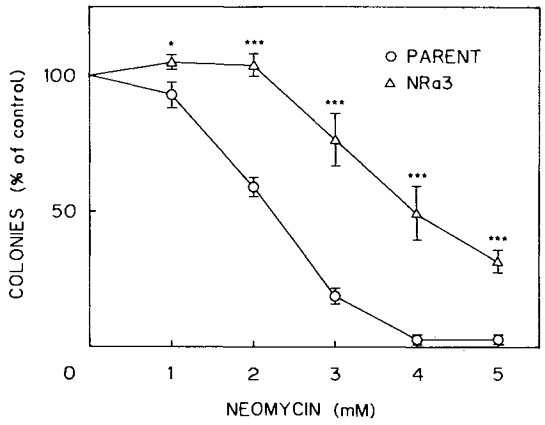

Fig. 2. The effect of neomycin on colony formation of parent and LLC-PK_1/NRa3 cells. The parent (○) and NRa3 (△) cells were plated at 300 cells per 60-mm culture dishes at various concentrations of neomycin. After 8–10 days, cells were fixed with 5% glutaraldehyde, then stained with 0.4% crystal violet, and the number of colonies was counted. Data are expressed as percentage of control (without neomycin). Control values, 100 ± 32 colonies/dish for parent cells and 104 ± 31 colonies/dish for NRa3 cells. Each point represents the mean ± SE of 6–30 determinations from 2–10 separate experiments. (*) $P < 0.05$ and (***) $P < 0.001$; significant differences from parent cells using Student's *t* test.

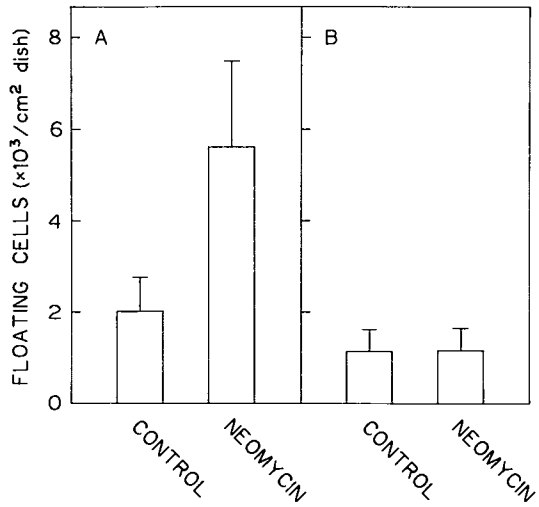

Fig. 3. The effect of neomycin on the floating cell number of parent and LLC-PK$_1$/NRa3 cells. Parent (A) and NRa3 (B) cells were cultured in the absence or presence of 2 m*M* neomycin for 4 days, and the number of floating cells in the culture medium was counted (15). The control values were not significantly different between parent and NRa3 cells. Each value represents the mean ± SE of five experiments.

such as alkaline phosphatase are detected in the urine when aminoglycosides are administered *in vivo* (1,2). Concurrently, the activity of brush border enzymes in renal cortex decreases with aminoglycoside treatment (3,20,21). Similar observations were obtained in LLC-PK$_1$ cells, that is, the activity of apical membrane enzymes decreased in the cell homogenate with aminoglycoside treatment (14,15). The objective of the present study was to isolate aminoglycoside resistant clones of LLC-PK$_1$ cells, to test the mechanism of the cytotoxicity of aminoglycoside antibiotics.

LLC-PK$_1$ cells with different phenotypes and/or genotypes were isolated (17–19). For example, Yoneyama and Lever (18) nonselectively isolated two clonal LLC-PK$_1$ cells. The expression of trehalase was induced by glucose deprivation in one clonal cell line, but not in the other. Wohlwend *et al.* (17) isolated three sublines that differ in their response to calcitonin and in their ability to form domes. On the other hand, Haggerty *et al.* (19) isolated a LLC-PK$_1$ mutant with increased Na^+–H^+ exchange and decreased sensitivity to amiloride, by selecting cells which could recover from an acid load in the presence of an inhibitor of the exchanger. In this study, a clonal cell line, LLC-PK$_1$/NRa3, with a lower sensitivity to neomycin was isolated from the parent cells in the presence of neomycin. NRa3 cells were less sensitive to neomycin when evaluated by colony formation assay and by the number of floating cells, as well as by the effects of the antibiotic on apical enzyme activity.

There may be similarities between the characteristics of NRa3 cells and the acquired resistance observed *in vivo*. Several reports indicate that gentamicin-induced renal proximal tubular necrosis is reversible despite continuous exposure to the drug (25,26). Gilbert *et al.* (26) found that, during the recovery phase, the renal histology, serum creatinine concentration, and defective urine concentrating ability returned to their pretreatment status, even though the tissue concentration of gentamicin was comparable to that, in the first toxic phase. The recovery from aminoglycoside nephrotoxicity may be related to the resistance to nephrotoxic reagents of regenerated tubular cells (26). In this context, NRa3 cells may represent a good model system to study the potential mechanisms underlying the resistance to toxins by regenerated epithelial cells observed *in vivo*, because NRa3

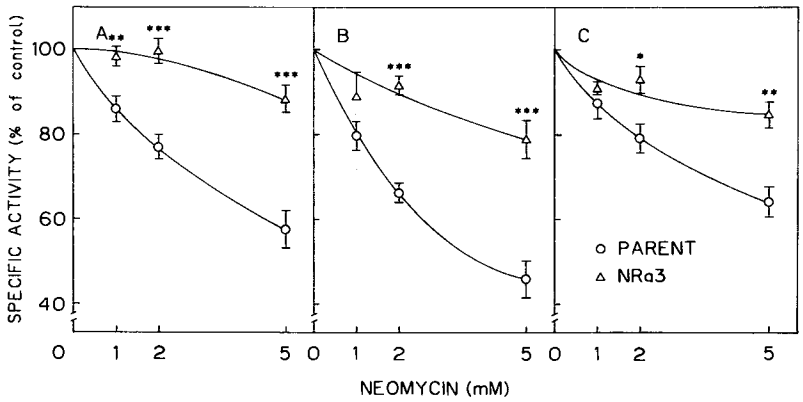

Fig. 4. Effect of neomycin on apical membrane enzyme activities of parent and LLC-PK$_1$/NRa3 cells. The parent (○) and NRa3 (△) cells were cultured in the presence of various concentrations of neomycin for 4 days. Specific activities of alkaline phosphatase (A), aminopeptidase (B), and γ-glutamyltransferase (C) in homogenates from parent and NRa3 cells were assayed as described in the text. Data are expressed as percentage of control. Control values of alkaline phosphatase, aminopeptidase, and γ-glutamyltransferase were 31.4 ± 7.7, 23.5 ± 5.8, and 48.1 ± 9.8 nmol/mg protein/min in parent cells and 26.5 ± 5.1, 14.0 ± 1.5, and 48.7 ± 10.0 nmol/mg protein/min in NRa3 cells. The control values of aminopeptidase, but not alkaline phosphatase and γ-glutamyltransferase, were significantly different ($P < 0.01$) between parent and NRa3 cells. Each point represents the mean ± SE of 6–12 experiments. (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$; significant differences from parent cells using Student's *t* test.

cells were isolated as a colony which proliferated under continuous exposure to neomycin.

In conclusion, the clonal cell line, NRa3, was less sensitive to neomycin-induced cytotoxicity. Concomitantly, the reduction in apical membrane enzyme activity by neomycin was less pronounced in NRa3 cells than in the parent. These findings suggest that a common key event induced by aminoglycoside antibiotics is involved in the reductions in apical enzyme activity and in cell viability in LLC-PK_1 cells. We have recently reported that the inhibition of protein synthesis by aminoglycoside is a possible cause of the reduced activities of apical membrane enzymes in LLC-PK_1 cells (27). Further studies are needed to clarify whether the inhibition of protein synthesis can account for these phenomena.

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