Pharmaceutical Research, Vol. 11, No. 5, 1994

Research Article

Cellular Toxicity of Aminoglycoside Antibiotics in G418-Sensitive and -Resistant LLC-PK$_1$ Cells

Mikihisa Takano,[1,3] Masahiro Okuda,[1] Masato Yasuhara,[1] and Ryohei Hori[1,2]

Received August 23, 1993; accepted November 26, 1993

The effects of gentamicin and G418 on the cellular function of LLC-PK$_1$ epithelial pig kidney cells were investigated. Exposing the cells for 2 days to these aminoglycoside antibiotics inhibited the increase in cell-associated apical membrane enzyme activity (alkaline phosphatase, aminopeptidase, and γ-glutamyltransferase). Kinetic analysis revealed that the maximal activity of alkaline phosphatase was reduced by these aminoglycosides. Both aminoglycosides inhibited [^{3}H]leucine incorporation into microsomes prepared from LLC-PK$_1$ cells. The LLC-PK$_1$ cells transfected with DNA encoding aminoglycoside 3′-phosphotransferase II, designated T2000B, were resistant to G418 as assessed by colony formation assay and the number of floating dead cells and by assay of apical enzyme activity. After a 4-hr exposure to G418, [^{3}H]leucine incorporation in the host LLC-PK$_1$ cells was inhibited, whereas that in T2000B cells was relatively unaffected. Gentamicin inhibited [^{3}H]leucine incorporation similarly in both cells. The inhibition of protein synthesis by aminoglycosides occurred earlier than that of apical enzyme activity. These findings suggest that the inhibition of protein synthesis by aminoglycoside antibiotics is a possible cause of the reduction in cell viability as well as the apical enzymes in LLC-PK$_1$ cells.

KEY WORDS: aminoglycoside nephrotoxicity; apical membrane enzymes; aminoglycoside 3′-phosphotransferase II; protein synthesis inhibition; kidney epithelial cell line.

INTRODUCTION

Aminoglycoside antibiotics inhibit bacterial protein synthesis or cause mistranslation (1). They are widely used in the treatment of Gram-negative infectious diseases. Aminoglycosides are transported and accumulated by renal proximal tubular cells, where they induce necrosis in humans and experimental animals (2,3). Although aminoglycoside-induced nephrotoxicity has been extensively studied, the responsible mechanisms remain unclear.

LLC-PK$_1$ is an established epithelial cell line derived from the pig kidney (4). The cells form an oriented monolayer with microvilli at their apical side, and they exhibit the characteristics of renal proximal tubular cells, such as transport activity for hexose (5,6), amino acids (7), phosphate (8), and organic cations (9–12). Since the necrosis induced by aminoglycosides is confined to the proximal part of kidney tubules, LLC-PK$_1$ cells can serve as a model for the study of aminoglycoside-induced nephrotoxicity (13–16). In a study of aminoglycoside resistant LLC-PK$_1$ cells, we suggested that a common mechanism is involved in the aminoglycoside-induced reductions in apical enzyme activity and cell viability (14). We also found that the reduction in apical enzyme activity is caused at least in part by the inhibition of protein synthesis (16). However, whether the inhibition of protein synthesis by aminoglycosides is essential for the cytotoxic effect of the drugs in LLC-PK$_1$ cells requires clarification.

G418 is structurally similar to gentamicin. Two of the three sugar rings in G418 are the same as those of gentamicin while the third sugar ring of G418 contains one less amino group and three more hydroxyl groups (Fig. 1). G418 inhibits eukaryotic protein synthesis by interfering with 80S ribosomes (17). We compare here the effects of G418 and gentamicin on the cellular function of LLC-PK$_1$. Furthermore, G418 can be inactivated by the bacterial aminoglycoside 3′-phosphotransferase II [APH(3′)II], which is encoded on the transposon, Tn 5. Therefore, if the gene encoding APH(3′)II is introduced into LLC-PK$_1$ cells by transfection and is efficiently expressed, the gene should confer resistance to G418.

In this study, we examined and compared the effects of the two aminoglycoside antibiotics, G418 and gentamicin, on the cellular function of LLC-PK$_1$. We then transfected LLC-PK$_1$ cells with the plasmid pSV2-neo, which encodes APH(3′)II and we investigated the effects of G418 and gentamicin on cellular function in the native and transfected LLC-PK$_1$ cells.

MATERIALS AND METHODS

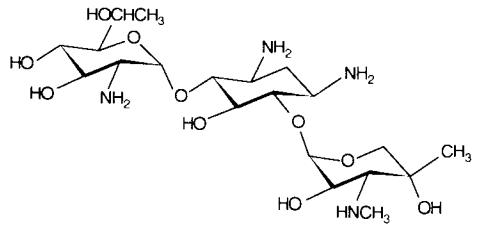
Cell Culture

LLC-PK$_1$ cells obtained from the American Type Culture Collection (ATCC CRL-1392) were grown in plastic

[1] Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan.
[2] Present address: Pharmaceutical Research and Technology Institute, Kinki University, 3-4-1 Kowakae, Higashi-osaka 577, Japan.
[3] To whom correspondence should be addressed.

0724-8741/94/0500-0609$07.00/0

G418



GENTAMICIN

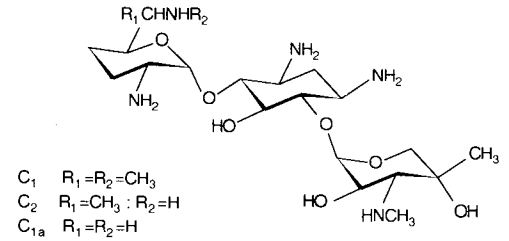


Fig. 1. Structure of G418 and gentamicin.

dishes (Corning Glass Works, Corning, NY) in medium 199 (ICN Biomedicals, Costa Mesa, CA), supplemented with 10% fetal bovine serum (Whittaker, Walkersville, MD), without antibiotics, in a 5% CO_2–95% air atmosphere at 37°C. The cells were subcultured every 4–5 days, using 0.02% EDTA and 0.05% trypsin. In most experiments, 60- and 100-mm dishes, as well as six-well plates, were seeded with 4×10^5 cells in 5 mL, 1×10^6 cells in 10 mL, and 2×10^5 cells in 2 mL of complete culture medium (medium 199 supplemented with 10% fetal bovine serum), respectively. In the present study, the LLC-PK_1 cells used were between passage 222 and passage 241.

DNA Transfection and Selection of Transformed LLC-PK_1 Cells

The plasmid, pSV2-neo, was introduced into LLC-PK_1 cells by means of calcium phosphate precipitation (18). Briefly, 60-mm dishes were seeded with 3×10^5 cells in 5 mL of complete culture medium. After 20 hr, the cells were exposed to $CaPO_4$/DNA (2.5 μg DNA per dish) and incubated for 4 hr, after which the medium was replaced with medium without $CaPO_4$/DNA. After 2 days, the cells were replated and incubated for about 2 weeks in a selective medium to obtain stable transfectants. The selective medium contained various concentrations of G418 sulfate up to 2 mg/mL. We observed that LLC-PK_1 consists of subpopulations with variable susceptibility to aminoglycoside antibiotics (14). Therefore, in this study, more than 50 clones which survived in the presence of 2 mg/mL G418 were combined and expanded. In control experiments, clones were not obtained when nontransfected LLC-PK_1 cells were cultured in medium containing 2 mg/mL G418. The G418-resistant cells, designated T2000B, were maintained in 400 μg/mL G418. However, prior to the experiments, the cells were cultured in the absence of G418 for three or four passages.

Enzyme Assays

LLC-PK_1 cells were washed twice with ice-cold saline, then removed with a rubber policeman into ice-cold saline (2 mL for a 60-mm dish and 4 mL for a 100-mm dish). The cells were homogenized with a bath-type sonicator (Model G112SP1, Laboratory Supplies, Hicksville, NY) three times for 5 sec each time. Alkaline phosphatase (EC 3.1.3.1), aminopeptidase (EC 3.4.11.2), and γ-glutamyltransferase (EC 2.3.2.2) in the homogenate were measured as described previously (13,15). Protein was measured by the method of Bradford (19), using the Bio-Rad Protein Assay Kit, with bovine γ-globulin as the standard.

Measurement of [^{3}H]Leucine Incorporation in Cells

The amount of [^{3}H]leucine incorporation was measured in LLC-PK_1 and T2000B cells grown in six-well cluster plates. Four or five days after seeding, the culture medium was replaced with Dulbecco's phosphate-buffered saline (PBS buffer; 137 m*M* NaCl, 3 m*M* KCl, 8 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 1 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$) containing 5 m*M* D-glucose and aminoglycoside. Four hours thereafter, the cells were rinsed twice with PBS buffer. The cells were then incubated with 1 mL of serum-free medium 199 containing [^{3}H]leucine (1 μCi/mL) for 30 min at 37°C in a CO_2 incubator. The medium was immediately aspirated and the wells were rapidly rinsed three times with ice-cold PBS buffer. The cells were fixed with 1 mL of 2.5% trichloroacetic acid for 30 min and rinsed twice with 2 mL of 2.5% trichloroacetic acid, then once with 2 mL of ethanol. The residues were solubilized with 0.1 *N* NaOH, and aliquots were transferred into scintillation vials to determine the amount of [^{3}H]leucine incorporation. Protein was measured as described above.

Preparation of the Microsomal Fraction from LLC-PK_1 Cells

All apparatus and buffers used were sterilized by autoclaving or with 80% ethanol. Microsomes were separated from LLC-PK_1 cells by the method of Bennett *et al.* (20) with some modifications. Samples were kept at 4°C during the preparation. Six days after seeding, LLC-PK_1 cells grown on 100-mm culture dishes were washed twice with 10 mL of ice-cold buffer comprising 150 m*M* NaCl, 20 m*M* *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), pH 7.2, and scraped with a rubber policeman. After centrifugation at 1000 rpm for 5 min, 5 mL of ice-cold buffer A [10 m*M* KCl, 1.5 m*M* Mg acetate, 10 m*M* HEPES, pH 7.5, 0.7 m*M* dithiothreitol (DTT), 0.1 m*M* (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride] was added and the cells were allowed to swell for more than 10 min. Cells were homogenized with 50 strokes of a motor-driven Teflon pestle, then 1.2 mL of buffer B (550 m*M* KCl, 2.5 m*M* spermidine, 0.35 m*M* DTT, 50% glycerol, 0.1 *M* HEPES, pH 7.5) was added and centrifuged at 27,000*g* for 10 min at 4°C. The supernatant was centrifuged at 100,000*g* for 120 min at 4°C. The top lipoprotein layer was removed by aspiration, and the supernatant (cell sap) was retained as a source of cofactors in the translation assays. The transparent microsomal pellet was gently homogenized in 0.5 mL of buffer A:B (5:1) and diluted with the same buffer to give a protein concentration of 2 mg/mL.

Measurement of [^{3}H]Leucine Incorporation into LLC-PK$_1$ Microsomes

Microsomes (50 μl, 0.1 mg protein) were combined with 100 m*M* KCl, 0.5 m*M* DDT, 30 m*M* HEPES, pH 7.5, 1 m*M* ATP, 0.25 m*M* guanosine 5′-triphosphate (GTP), 2 m*M* Mg acetate, 0.4 m*M* spermidine, 5 m*M* creatine phosphate, 54 μg creatine phosphokinase, 50 μ*M* amino acid mixture (19 amino acids without leucine), 50 μL cell sap, and 2 μCi L-[^{3}H]leucine (25 Ci/mmol) in a total volume of 200 μL and assayed at 37°C for 20 min in a water bath. The [^{3}H]leucine incorporation was terminated by adding 0.2 mL of ice-cold 6% trichloroacetic acid (TCA). The tubes were left for 10 min on ice, then centrifuged at 3000 rpm for 10 min. The pellet was dissolved in 0.1 *N* NaOH, and the protein was precipitated with TCA. This step was repeated. The supernatant was aspirated and the pellet was dissolved in 200 μL of 0.1 *N* NaOH, from which aliquots (100 μL) were transferred into scintillation vials for radioactivity counting.

Materials

Gentamicin sulfate and G418 sulfate (geneticin) were obtained from Sigma Chemical (St. Louis, MO) and GIBCO (Grand Island, NY), respectively. L-[^{3}H]Leucine (140 Ci/mmol) was obtained from Amersham International plc. (Buckinghamshire, UK). The plasmid, pSV2-neo, was a gift from Dr. Akinori Ishimoto (Institute for Virus Research, Kyoto). All other chemicals were of the highest purity available.

RESULTS

To examine the effects of G418 and gentamicin on alkaline phosphatase activity in LLC-PK$_1$ cells, we replaced the culture medium with that containing the antibiotic at the times indicated by the arrows (Fig. 2). After 2 days of incubation, the specific activity of cell-associated alkaline phosphatase was determined. A preliminary experiment to determine the effect of G418 on apical membrane enzymes showed that the potency of G418 was about 10-fold that of gentamicin (data not shown). We therefore used 0.2 m*M* G418 against 2 m*M* gentamicin. In this experiment, the cells reached confluence about 4 days after seeding. As shown in Fig. 2, G418 and gentamicin inhibited alkaline phosphatase activity when the activity in nontreated, control cells was increasing (days 3–5 and 5–7, respectively). On the other hand, the effects of the antibiotics were less pronounced when the activity in the control cells was decreasing (days 7–9 and 9–11, respectively). Studies of the other two apical membrane enzymes, aminopeptidase and γ-glutamyltransferase, also showed that G418 and gentamicin were more effective when enzyme activity was increasing than when it was decreasing or relatively constant (data not shown).

We then measured alkaline phosphatase activity in control, G418-treated, and gentamicin-treated cells at various substrate concentrations. After a 3-day incubation of LLC-PK$_1$ cells with 0.2 or 0.5 m*M* G418 or 2 m*M* gentamicin, alkaline phosphatase activity in the cell homogenate was determined in the presence of 0.005–1 m*M* *p*-nitrophenyl phosphate as a substrate (Fig. 3). G418 and gentamicin reduced the alkaline phosphatase activity at all substrate concentrations in a dose-dependent manner. The inset in Fig. 3 shows an Eadie–Hofstee analysis of the data. The maximal enzyme activity (V_{max}) of alkaline phosphatase for the control cells and for the cells treated with 0.2 and 0.5 m*M* G418 and 2 m*M* gentamicin was estimated as 60, 44, 34, and 48 nmol/mg protein/min, respectively. On the other hand, the apparent affinity constants for the substrate (K_m) were relatively unchanged, being 12, 11, 10, and 11 μ*M*, respectively.

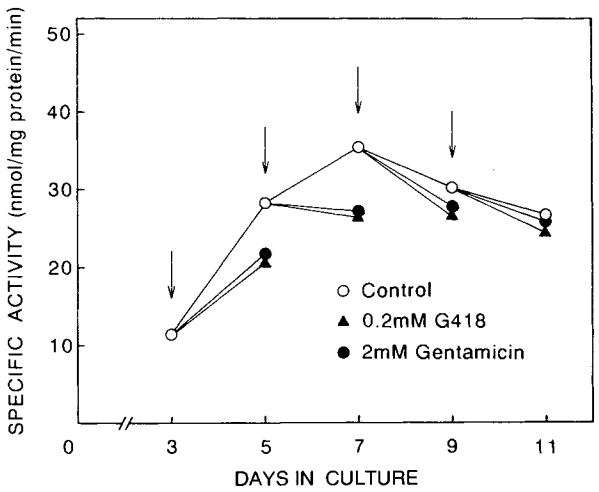


Fig. 2. The effect of G418 and gentamicin on alkaline phosphatase activity in LLC-PK$_1$ cells. LLC-PK$_1$ cells were cultured in 60-mm culture dishes. At days 3, 5, 7, and 9 after seeding (indicated by arrows), the culture medium was replaced with fresh medium or with that containing 0.2 m*M* G418 or 2 m*M* gentamicin. After a 2-day incubation, the specific activity of alkaline phosphatase in the homogenate of control, G418-treated, and gentamicin-treated cells was determined. Each point represents the mean of three separate experiments.

Figure 4 shows the effect of G418 and gentamicin on *in vitro* microsomal protein synthesis. Cycloheximide, a protein synthesis inhibitor, served as a positive control. Both G418 and gentamicin, as well as cycloheximide, inhibited [^{3}H]leucine incorporation into microsomes of LLC-PK$_1$ cells in a dose-dependent manner, and the inhibitory potency of these aminoglycosides was weaker than that of cycloheximide.

We then investigated the effects of aminoglycoside antibiotics on the cellular function of LLC-PK$_1$ cells transfected with DNA encoding APH(3′)II. The transfectant was selected in the presence of G418 and designated T2000B. APH(3′)II, when expressed in T2000B cells, should inactivate G418 by phosphorylating the antibiotic. Colony formation was assayed to test the sensitivity of the transfectant to G418, which was compared with that of the host LLC-PK$_1$ cells. Eight days after seeding T2000B and LLC-PK$_1$ cells in the presence of various concentrations of G418, the number of colonies was counted. As shown in Fig. 5A, colony formation by the host LLC-PK$_1$ cells was markedly inhibited by G418, while the effects of G418 on the transfectant were much weaker than on the host LLC-PK$_1$ cells. We reported previously that the treatment of LLC-PK$_1$ cells with gentamicin increased the number of floating cells in the culture medium (13). These floating cells were dead, as assessed by trypan blue staining, indicating that their numbers can also

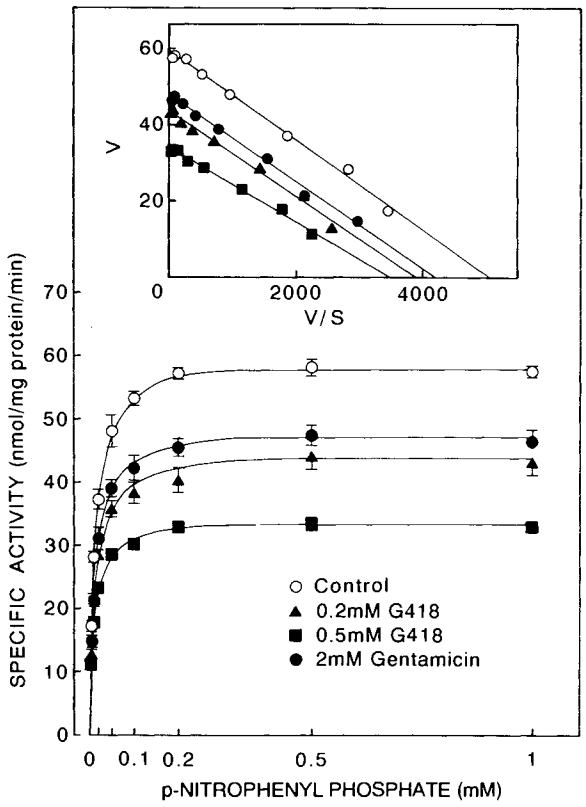


Fig. 3. The effect of G418 and gentamicin on alkaline phosphatase kinetics. LLC-PK$_1$ cells were grown on 100-mm culture dishes for 4 days, then incubated with G418 or gentamicin. After 3 days, the alkaline phosphatase activity in control, 0.2 m*M* G418-treated, 0.5 m*M* G418-treated, and 2 m*M* gentamicin-treated cells was determined at various concentrations of substrate (*p*-nitrophenyl phosphate). Each point represents the mean ±SE of three determinations from a typical experiment. The inset shows Eadie–Hofstee plots.

be used as a marker of aminoglycoside cytotoxicity to LLC-PK$_1$ cells. We therefore measured the number of floating dead cells in the culture medium after a 3-day incubation with or without 2 m*M* G418. As shown in Fig. 5B, the number of floating cells in the LLC-PK$_1$ cultures was markedly increased by G418, while no effect was observed in T2000B cells. These results show that T2000B cells are resistant to G418 cytotoxicity.

The effect of G418 on apical membrane enzymes in T2000B and LLC-PK$_1$ cells was investigated. The cells were cultured for 4 days in the absence of G418, then treated with various concentrations of G418 for 3 days. At the end of the incubation, alkaline phosphatase, aminopeptidase, and γ-glutamyltransferase activity was measured (Fig. 6). The activity of these enzymes in the host LLC-PK$_1$ cells was markedly reduced by G418 in a dose-dependent manner. In contrast, the reduction of enzyme activity by G418 was marginal in T2000B cells. Gentamicin reduced the activity of these enzymes in T2000B cells to the same extent as that observed in the host LLC-PK$_1$ cells (data not shown), indicating that T2000B cells are not resistant to gentamicin.

The effects of G418 and gentamicin on protein synthesis were evaluated in T2000B and the host LLC-PK$_1$ cells. When the cells reached confluence and apical enzyme activity was still increasing (day 4 or 5), they were incubated with various concentrations of G418 and gentamicin for 4 hr in PBS buffer containing 5 m*M* D-glucose; then the [^{3}H]leucine incorporation over 30 min was measured. In the host LLC-PK$_1$ cells, [^{3}H]leucine incorporation was reduced by G418 in a dose-dependent manner, whereas it was relatively unaffected in T2000B cells (Fig. 7). Gentamicin inhibited [^{3}H]leucine incorporation similarly in both LLC-PK$_1$ and T2000B cells.

The effect of G418 and gentamicin on apical enzyme activity was examined under the same experimental conditions as described for Fig. 7. In contrast to the results obtained when LLC-PK$_1$ cells were treated with these aminoglycosides for a longer period (Figs. 2, 3, and 6), the activity of alkaline phosphatase and aminopeptidase was not affected by a 4-hr incubation with either G418 or gentamicin (data not shown). In addition, no floating cells were observed under the light microscope after the cells were incubated for 4 hr with the antibiotics.

DISCUSSION

We investigated the effects of G418 and gentamicin using the cultured kidney epithelial cell line LLC-PK$_1$. We also transfected LLC-PK$_1$ cells with the gene encoding APH(3′)II, which inactivates some aminoglycoside antibiotics, such as G418, by phosphorylating their 3′ hydroxyl moiety. The results indicated that the inhibition of protein synthesis is a cause of the reductions in cell viability and in apical enzyme activity induced by aminoglycosides in LLC-PK$_1$ cells.

The time course and the kinetic analyses of the effect of G418 and gentamicin on apical membrane enzymes suggested that these antibiotics act on a common site in LLC-PK$_1$ cells. After LLC-PK$_1$ cells have been seeded, the activity of apical membrane enzymes develops concurrently with the differentiation of the cells during and after the formation of tight junctions (21,22). G418 as well as gentamicin inhibited the increase in the activity of apical membrane enzymes, rather than enhancing their decrease at later stages of culture incubations. Thus, the reduction of apical enzyme activity by the aminoglycosides should be due to the inhibition of enzyme synthesis (16). The finding that G418 as well as gentamicin reduced the maximal activity, but not the affinity, of apical membrane enzymes supports this notion. Further, these aminoglycosides inhibited [^{3}H]leucine incorporation into LLC-PK$_1$ cells and into microsomes isolated from the cells, confirming that these drugs potently inhibit protein synthesis.

Transfection of eukaryotic cells with the gene encoding APH(3′)II and the selection of transfectants with G418 are frequently utilized for the introduction of another gene by cotransfection (18). In the present study, LLC-PK$_1$ cells were transfected with pSV2-neo, and the transfectant, T2000B, was selected in the presence of 2 mg/mL G418. The T2000B cells were resistant to G418 when evaluated by colony formation assay and by the number of the floating cells, as well as by the effects of the antibiotic on apical enzyme activity and on protein synthesis. Gentamicin, on the other hand, inhibited apical membrane enzymes and protein synthesis similarly in the host LLC-PK$_1$ cells and in T2000B

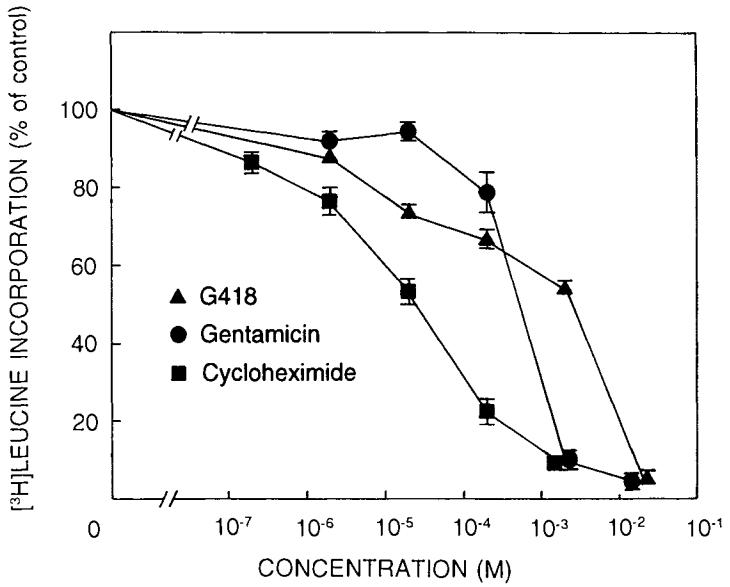


Fig. 4. The effect of G418, gentamicin, and cycloheximide on [^{3}H]leucine incorporation into microsomes isolated from LLC-PK$_1$ cells. The microsomal fraction was isolated from the host LLC-PK$_1$ cells as described under Materials and Methods. Microsomes were incubated with G418, gentamicin, and cycloheximide for 15 min at 4°C, then with 2 μCi [^{3}H]leucine for 20 min at 37°C. Each point represents the mean ±SE of six to nine determinations from two or three experiments.

cells. This result is expected, since APH(3')II phosphorylates the hydroxyl group at the 3' position of aminoglycosides, whereas gentamicin has no hydroxyl group at that position. Thus, the resistance of T2000B cells to G418 cytotoxicity should be conferred by the metabolism of G418 by aminoglycoside phosphotransferase in the cells.

Various mechanisms such as lysosomal alterations (23,24), mitochondrial energetic failure (25), and active oxygen-induced cell injury (26) reportedly contribute to the development of aminoglycoside nephrotoxicity. We therefore attempted to test the involvement of these mechanisms in the development of gentamicin cytotoxicity in LLC-PK$_1$ cells. However, our preliminary experiments (data not shown) showed that the levels of phospholipids and adeno-

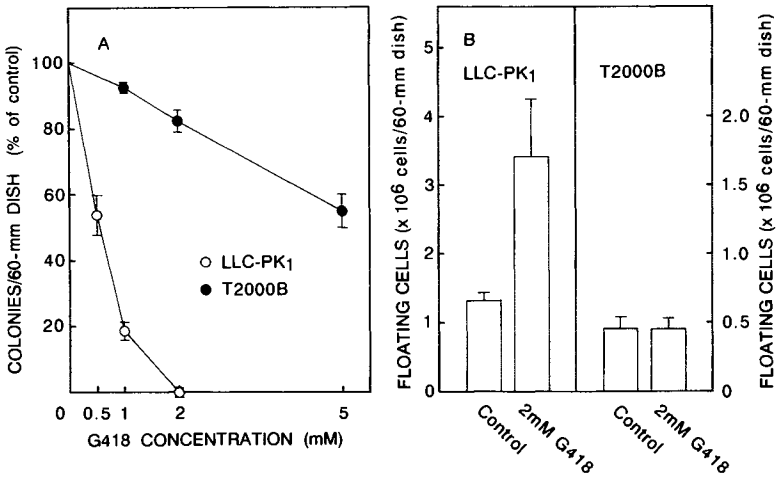


Fig. 5. The effect of G418 on colony formation (A) and floating cell number (B). (A). LLC-PK$_1$ and T2000B cells were plated (300 cells per 60-mm culture dish) in the presence of various concentrations of G418. After 8 days, the cells were fixed with 5% glutaraldehyde and stained with 0.4% crystal violet, and the number of colonies was counted. Data are expressed as percentages of control (without antibiotics). (B) After 4 days of culture in 60-mm culture dishes, both cell types were incubated with or without 2 m*M* G418. After 3 days, the number of floating cells in the culture medium was counted. Each point or column represents the mean ±SE of three experiments.

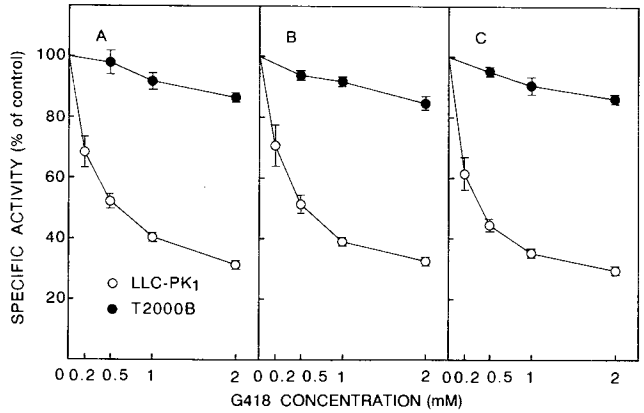


Fig. 6. The effect of G418 on apical membrane enzyme activity in LLC-PK_1 and T2000B cells. Cells were cultured for 4 days, then incubated with various concentrations of G418. After 3 days, the specific activity of alkaline phosphatase (A), aminopeptidase (B), and γ-glutamyltransferase (C) in the homogenate of LLC-PK_1 and T2000B cells was determined. Each point represents the mean ±SE of three experiments.

sine 5′-triphosphate in the gentamicin-treated LLC-PK_1 cells were not significantly different from those in control cells when the cells were treated for 4 days with 2 m*M* gentamicin, suggesting that the contribution of lysosomal alterations and mitochondrial energetic failure to aminoglycoside-induced cytotoxicity is small. Furthermore, cotreating LLC-PK_1 cells with gentamicin and radical scavengers, such as dimethyl sulfoxide, dimethylthiourea, 2,3-dihydroxybenzoic acid, and deferoxamine mesylate, did not prevent the decrease in the level of apical membrane enzymes caused by gentamicin. Therefore, although the involvement of these mechanisms in *in vivo* nephrotoxicity of aminoglycoside cannot be ruled out, they probably contributed little, if any, to the cytotoxicity of aminoglycosides in LLC-PK_1 cells under our experimental conditions.

Aminoglycoside antibiotics kill bacteria by inhibiting

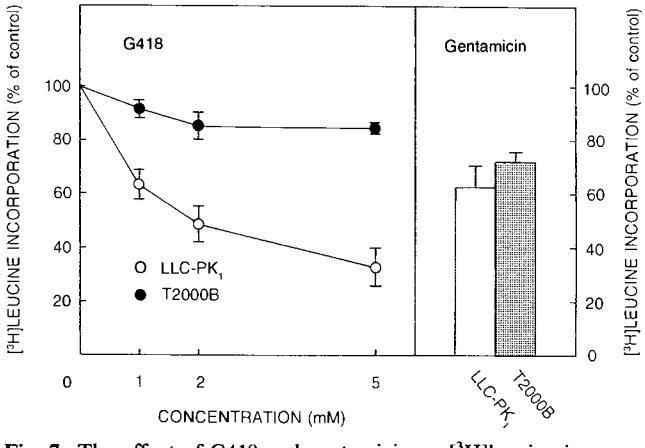


Fig. 7. The effect of G418 and gentamicin on [^{3}H]leucine incorporation in LLC-PK_1 and T2000B cells. LLC-PK_1 and T2000B cells were seeded into six-well cluster plates. At confluence (day 4 or 5), the cells were incubated with 1, 2, or 5 m*M* G418 and 5 m*M* gentamicin for 4 hr at 37°C. Thereafter, the [^{3}H]leucine incorporation over 30 min was measured. Each point or column represents the mean ±SE of three experiments.

their protein synthesis through the failure of initiation and misreading of messenger RNA. Therapeutically available aminoglycosides such as netilmicin and tobramycin inhibit protein synthesis when they are added to the microsome fractions from eukaryotic cells (27–29). Buss and Piatt (30) reported that gentamicin administered *in vivo* reduced protein synthesis in microsomes isolated from rat kidney. Subsequently, Bennett *et al.* (20) demonstrated that [^{3}H]leucine incorporation into renal microsomes isolated from rats injected with gentamicin was significantly reduced, even though the morphology and functions of the kidney were well preserved. However, it is not clear whether the inhibition of protein synthesis represents an early and an essential event for the pathogenesis of aminoglycoside nephrotoxicity. To address this issue, cell culture systems should have some advantages over *in vivo* studies, partly because of the relatively homogeneous cell population. We found that G418 and gentamicin inhibited [^{3}H]leucine incorporation into LLC-PK_1 cells and into microsomes isolated from them. Furthermore, the inhibition of protein synthesis by aminoglycosides preceded the reduction in apical enzyme activity and in cell viability. In T2000B cells, it is likely that the inhibitory effect of G418 on protein synthesis was attenuated due to the metabolism of the drugs, which in turn resulted in the reduced G418 toxicity on apical membrane enzymes and cell viability. Therefore, the inhibition of protein synthesis by G418 is probably the cause of the reduced apical membrane enzyme activity and drug cytotoxicity in LLC-PK_1 cells. Since gentamicin had similar effects to those of G418 on apical membrane enzyme activity and [^{3}H]leucine incorporation into LLC-PK_1 cells and their microsomes, the inhibition of protein synthesis by gentamicin should also be, at least in part, the cause of its cytotoxicity.

In conclusion, the inhibition of protein synthesis by aminoglycoside antibiotics appears to play a role in their reducing cell viability as well as apical membrane enzyme activity in LLC-PK_1 cells. Aminoglycoside-induced nephrotoxicity may thus be explained in part by the inhibitory effect of these antibiotics on protein synthesis. However, the involvement of other mechanisms in aminoglycoside nephrotoxicity *in vivo* cannot be ruled out.

ACKNOWLEDGMENT

This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1. M. A. Sande and G. L. Mandell. The aminoglycosides. In A. G. Gilman, L. S. Goodman, W. R. Theodore, and F. Murad (eds.), *The Pharmacological Basis of Therapeutics: Antimicrobial Agents*, Macmillan, New York, 1985, pp. 1150–1169.
2. H. D. Humes and R. P. O'Connor. Aminoglycoside nephrotoxicity. In R. W. Schrier and C. W. Gottschalk (eds.), *Diseases of the Kidney*, 4th ed., Little, Brown, Boston, MA, 1988, Vol. 2, pp. 1229–1273.
3. G. J. Kaloyanides and E. Pastoriza-Munoz. Aminoglycoside nephrotoxicity. *Kidney Int.* 18:571–582 (1980).
4. R. N. Hull, W. R. Cherry, and W. Weaver. The origin and characteristics of kidney cell strain, LLC-PK_1. *In Vitro* 12:670–677 (1976).
5. K. Amsler and J. S. Cook. Development of Na^+-dependent

hexose transport in a cultured line of porcine kidney cells. *Am. J. Physiol.* 242:C94–C101 (1982).
6. K. Inui, H. Saito, M. Takano, T. Okano, S. Kitazawa, and R. Hori. Enzyme activities and sodium-dependent active D-glucose transport in apical membrane vesicles isolated from kidney epithelial cell line (LLC-PK_1). *Biochim. Biophys. Acta* 769:514–518 (1984).
7. C. A. Rabito and M. V. Karish. Polarized amino acid transport by an epithelial cell line of renal origin (LLC-PK_1): The apical systems. *J. Biol. Chem.* 258:2543–2547 (1983).
8. J. Biber, C. D. A. Brown, and H. Murer. Sodium-dependent transport of phosphate in LLC-PK_1 cells. *Biochim. Biophys. Acta* 735:325–330 (1983).
9. A.-K. Fouda, C. Fauth, and F. Roch-Ramel. Transport of organic cations by kidney epithelial cell line LLC-PK_1. *J. Pharmacol. Exp. Ther.* 252:286–292 (1990).
10. K. Inui, H. Saito, and R. Hori. H^+-gradient-dependent active transport of tetraethylammonium cation in apical-membrane vesicles isolated from kidney epithelial cell line LLC-PK_1. *Biochem. J.* 227:199–203 (1985).
11. H. Saito, M. Yamamoto, K. Inui, and R. Hori. Transcellular transport of organic cation across monolayers of kidney epithelial cell line LLC-PK_1. *Am. J. Physiol.* 262:C59–C66 (1992).
12. M. Takano, M. Kato, A. Takayama, M. Yasuhara, K. Inui, and R. Hori. Transport of procainamide in a kidney epithelial cell line LLC-PK_1. *Biochim. Biophys. Acta* 1108:133–139 (1992).
13. R. Hori, K. Yamamoto, H. Saito, M. Kohno, and K. Inui. Effect of aminoglycoside antibiotics on cellular functions of kidney epithelial cell line (LLC-PK_1): A model system for aminoglycoside nephrotoxicity. *J. Pharmacol. Exp. Ther.* 230:742–748 (1984).
14. R. Hori, M. Okuda, Y. Ohishi, M. Yasuhara, K. Inui, and M. Takano. Decreased cellular toxicity of neomycin in a clonal cell line isolated from LLC-PK_1. *Pharm. Res.* 10:573–576 (1993).
15. K. Inui, H. Saito, T. Iwata, and R. Hori. Aminoglycoside-induced alterations in apical membranes of kidney epithelial cell line (LLC-PK_1). *Am. J. Physiol.* 254:C251–C257 (1988).
16. M. Okuda, M. Takano, M. Yasuhara, and R. Hori. Inhibition of apical membrane enzyme activities and protein synthesis by gentamicin in a kidney epithelial cell line LLC-PK_1. *Chem. Pharm. Bull.* 40:3307–3310 (1992).
17. S. Bar-Nun, Y. Shneyour, and J. S. Beckmann. G-418, an elongation inhibitor of 80 S ribosomes. *Biochim. Biophys. Acta* 741:123–127 (1983).
18. L. G. Davis, M. D. Dibner, and J. F. Battey. *Basic Methods in Molecular Biology*, Elsevier, New York, 1986.
19. M. M. Bradford. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254 (1976).
20. W. M. Bennett, L. M. Mela-Riker, D. C. Houghton, D. N. Gilbert, and W. C. Buss. Microsomal protein synthesis inhibition: An early manifestation of gentamicin nephrotoxicity. *Am. J. Physiol.* 255:F265–F269 (1988).
21. C. A. Rabito, J. I. Kreisberg, and D. Wight. Alkaline phosphatase and γ-glutamyl transpeptidase as polarization markers during the organization of LLC-PK_1 cells into an epithelial membrane. *J. Biol. Chem.* 259:574–582 (1984).
22. H. Saito, K. Inui, and R. Hori. Mechanisms of gentamicin transport in kidney epithelial cell line (LLC-PK_1). *J. Pharmacol. Exp. Ther.* 238:1071–1076 (1986).
23. G. Aubert-Tulkens, F. V. Hoof, and P. Tulkens. Gentamicin-induced lysosomal phospholipidosis in cultured rat fibroblasts. Quantitative ultrastructural and biochemical study. *Lab. Invest.* 40:481–491 (1979).
24. S. Feldman, M.-Y. Wang, and G. J. Kaloyanides. Aminoglycosides induce a phospholipidosis in the renal cortex of the rat: An early manifestation of nephrotoxicity. *J. Pharmacol. Exp. Ther.* 220:514–520 (1982).
25. J. M. Weinberg and H. D. Humes. Mechanisms of gentamicin-induced dysfunction of renal cortical mitochondria. I. Effects on mitochondrial respiration. *Arch. Biochem. Biophys.* 205:222–231 (1980).
26. P. D. Walker and S. V. Shah. Evidence suggesting a role for hydroxyl radical in gentamicin-induced acute renal failure in rats. *J. Clin. Invest.* 81:334–341 (1988).
27. W. C. Buss, M. K. Piatt, and R. Kauten. Inhibition of mammalian microsomal protein synthesis by aminoglycoside antibiotics. *J. Antimicrob. Chemother.* 14:231–241 (1984).
28. M. O. Loveless, S. J. Kohlhepp, and D. N. Gilbert. The influence of aminoglycoside antibiotics on the *in vitro* function of rat liver ribosomes. *J. Lab. Clin. Med.* 103:294–303 (1984).
29. J. M. Wilhelm, S. E. Pettitt, and J. J. Jessop. Aminoglycoside antibiotics and eukaryotic protein synthesis: Structure-function relationships in the stimulation of misreading with a wheat embryo system. *Biochemistry* 17:1143–1149 (1978).
30. W. C. Buss and M. K. Piatt. Gentamicin administered *in vivo* reduces protein synthesis in microsomes subsequently isolated from rat kidneys but not from rat brains. *J. Antimicrob. Chemother.* 15:715–721 (1985).