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Release of Enzymes from the Lysosomes of Rat Kidney Cortex by Aminoglycoside Antibiotics

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The effects of aminoglycosides, 3',4'-dideoxykanamycin B (DKB), gentamicin, and amikacin, on rat kidney cortical lysosomes were investigated. The binding of ^3H -DKB to the lysosomal fraction was significantly larger than that to any other subcellular fraction examined. In the presence of 10^{-2} M aminoglycosides, about 70% of the *N*-acetyl- β -D-glucosaminidase was released. However, the release of this enzyme fell below the control level at 10^{-5} M DKB. In the case of acid phosphatase, the release increased gradually with aminoglycosides concentration. The higher the concentration of aminoglycosides became, the greater was the fluidity of the lysosomal membrane.

These results suggest that aminoglycoside binds to the lysosomal membrane and increases the membrane fluidity, leading to the release of *N*-acetyl- β -D-glucosaminidase and acid phosphatase from the lysosomes.

Keywords—aminoglycoside; 3',4'-dideoxykanamycin B; gentamicin; amikacin; lysosomal enzyme; *N*-acetyl- β -D-glucosaminidase; acid phosphatase; membrane fluidity; 1,6-diphenyl-1,3,5-hexatriene

Aminoglycoside antibiotics are widely used to treat gram-negative bacterial infections, but they have well-known nephro- and ototoxicity. The first step in the pathogenesis of nephrotoxicity is the transport of the aminoglycoside into proximal tubular cells, where it affects one or more intracellular metabolic processes.¹⁾ The biochemical mechanism of nephrotoxicity is still unclear. It has been reported that aminoglycoside antibiotics which are transported into proximal tubular cells are concentrated within the lysosomes.¹⁾ Furthermore, aminoglycosides affect the urinary excretion of lysosomal acid hydrolases²⁾ and alter the intracellular distribution of the lysosomal enzyme, *N*-acetyl- β -D-glucosaminidase.³⁾ In a previous paper, we reported that lysosomal enzymes obtained from the homogenate of rat kidney cortex bind to a 3',4'-dideoxykanamycin B(DKB)-conjugated Sepharose 4B column, and the *N*-acetyl- β -D-glucosaminidase and lysozyme activities were changed by the presence of aminoglycosides.⁴⁾

Based on these findings, it is considered that the first step in the nephrotoxicity of aminoglycosides is the interaction of aminoglycosides with the lysosomes of proximal tubular cells. In this report, the binding of aminoglycosides to the lysosomal fraction obtained from rat kidney cortex was examined, and the enzyme activities of *N*-acetyl- β -D-glucosaminidase and acid phosphatase released from lysosomes were assayed.

Materials and Methods

Materials—3',4'-Dideoxykanamycin B (DKB) and ^3H -DKB (66 $\mu\text{Ci}/\text{mg}$) were kindly provided by Meiji Seika Kaisha Ltd. (Tokyo). Amikacin and gentamicin were obtained from Banyu Pharmaceutical Co., Ltd. (Tokyo) and Shionogi Pharmaceutical Co., Ltd. (Osaka), respectively. The other reagents were of the highest purity commercially

available. Male Wistar rats (200–250 g) were purchased from Tokyo Jikken Dobutsu (Tokyo).

Cell Fractionation of Rat Kidney Cortex—Cell fractionation of rat kidney cortex was carried out according to the procedure of Meisner.³⁾ Kidney cortices, obtained from rats which had been fasted for 24 h, were minced and a 10% (w/v) homogenate was prepared with 0.45 M sucrose containing 1 mM Na-ethylenediaminetetra acetic acid (EDTA) in a Potter-Elvehjem homogenizer (clearance 0.18 mm). The homogenate was centrifuged at $400 \times g$ with a Hitachi SCR 18 B centrifuge at 2 °C for 10 min, and yielded a pellet (N fraction) and supernatant fraction. The supernatant was centrifuged again at $6400 \times g$ for 10 min. The resulting pellet consisted of two layers, each of which was separated carefully by washing off with a pipet. The upper layer was the mitochondrial (Mt) fraction and the bottom layer, the lysosomal (L) fraction. The supernatant was further centrifuged at $12000 \times g$ for 20 min to yield a microsomal (Mc) fraction and a supernatant (S) fraction. Each fraction, except the S fraction, was suspended in a 0.25 M sucrose solution at a concentration of 10% (w/v). Recovery from the original homogenate was from 92–108%.

Identification of Subcellular Fractions—To identify the subcellular fractions, cytochrome c oxidase (Mt fr.), acid phosphatase and *N*-acetyl- β -D-glucosaminidase (L fr.), glucose-6-phosphatase (Mc fr.), and lactic dehydrogenase (S fr.) activities were measured. Cytochrome c oxidase activity was assayed by the method of Wharton and Tzagoloff.⁵⁾ Glucose-6-phosphatase activity was assayed by the method of Aronson and Touster⁶⁾ using sodium glucose-6-phosphate as the substrate. Acid phosphatase activity was determined by the Bessey–Lowry method, using *p*-nitrophenylphosphate as the substrate.⁷⁾ Lactic dehydrogenase activity was determined by the method of Wroblewski and LaDue.⁸⁾ *N*-Acetyl- β -D-glucosaminidase was determined as described by Loomis.⁹⁾ The lysosomal enzyme activity of each subcellular fraction was measured in the presence of 1.0% Triton X-100. Protein concentration was measured by the method of Lowry *et al.*¹⁰⁾

Binding of ^3H -DKB to Subcellular Fractions—The binding of ^3H -DKB to each subcellular fraction of the kidney cortex was examined as follows. Two hundred microliters of each fraction (protein concentration, 1.0 mg/ml) and 100 μl of ^3H -DKB (10 μg of ^3H -DKB) were mixed and incubated at 37 °C for 30 min. After incubation, the mixture was ultrafiltered with a Mini-Module (Funakoshi Pharmaceutical Co., Ltd.). The filter was dried and the radioactivity trapped on the filter was counted with a liquid scintillation counter (Packard model 3330) using a toluene cocktail composed of Omnifluor (New England Nuclear) 4 g/toluene 538 ml–Triton X-100 462 ml.

Release of *N*-Acetyl- β -D-glucosaminidase and Acid Phosphatase from Lysosomes—The lysosomal fraction suspended in 0.25 M sucrose (0.75 ml) was mixed with aminoglycoside solution (0.75 ml) of various concentrations and the mixtures were incubated at 37 °C for 60 min. A control incubation without aminoglycosides was run at the same time. After the incubation, the mixture was centrifuged at $100000 \times g$ for 60 min (4 °C) in a Hitachi RP-65 centrifuge. Enzyme activity in the supernatant and pellet was assayed in the presence of Triton X-100 at 1% final concentration. The activity of enzymes was not influenced by the addition of aminoglycoside under these conditions.

Fluidity of Lysosomal Membrane—The fluidity of the lysosomal membrane was measured by the method of Shinitzky and Inbar¹¹⁾ using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a fluorescent probe. The lysosomal fraction (1.5 ml, 1.0 mg protein) was mixed with 1 mM DPH (1.5 ml) dissolved in 0.25 M sucrose and the mixture was left to stand for 60 min at 25 °C so that the DPH would become incorporated into the lysosomal membrane. The mixture was then centrifuged at $36000 \times g$ for 20 min to remove any excess DPH. The lysosome labeled with DPH were resuspended in 0.04 M Tris-HCl buffer (pH 7.4) containing 0.18 M sucrose and incubated both with and without aminoglycoside at 37 °C for 30 min. The mixture was centrifuged at $36000 \times g$ for 20 min to remove any excess aminoglycosides, the lysosomal pellet was resuspended in 0.25 M sucrose solution, and the fluorescence polarization was measured with fluorescence spectrophotometer (Hitachi MPF-4). The polarization (*P*) value was calculated from Eq. 1,

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities observed through a polarizer oriented parallel and perpendicular to the plane of polarization of the exciting beam, respectively.

Results and Discussion

Binding of ^3H -DKB to the Subcellular Fractions of Rat Kidney Cortex

To investigate the binding of the aminoglycoside antibiotic DKB to the rat kidney cortex, subcellular fractionation was carried out. The subcellular fractions were identified by measurement of marker enzymes, and the results are shown in Fig. 1. These results indicate that subcellular fractionation was sufficiently effective.

The binding of ^3H -DKB to the above subcellular fractions was investigated as described in Materials and Methods. As shown in Fig. 2, ^3H -DKB has a high affinity for the lysosomal

Fig. 1. Subcellular Fractionation of Rat Kidney Cortex

Identification of each fraction was carried out by measuring the above four marker enzymes.

- A: *N*-Acetyl-β-D-glucosaminidase (lysosomal fr.) is expressed as mol *p*-nitrophenol released per min per mg protein at 37°C.
 B: Acid phosphatase (lysosomal fr.) is expressed as nmol phenol released per min per mg protein at 37°C.
 C: Cytochrome c oxidase (mitochondrial fr.) is expressed as mol cytochrome c oxidized per min per mg protein at 25°C.
 D: Glucose-6-phosphatase (microsomal fr.) is expressed as mol Pi released per min per mg protein at 37°C.

Fig. 2. The Binding of ³H-DKB to the Subcellular Fraction of Rat Kidney Cortex

N, nuclear fr; L, lysosomal fr; Mt, mitochondrial fr; Mc, microsomal fr; S, supernatant fr. Each value represents the mean of three experiments ± S.D.

fraction and ³H-DKB was bound to this fraction to a greater extent than to any other fraction.

Aminoglycoside antibiotics have been reported to have affinity for lysosomes, and aminoglycosides accumulate in lysosomes *in vivo*.²⁾ In our *in vitro* experiment, the binding activity of DKB was the highest in lysosomes. Mitochondria and microsomes also contained considerable amounts of DKB, but the specific activity was only about a half of that of

lysosomes. The microsomal fraction contains brush border membrane, which was also reported to bind with aminoglycoside antibiotics.¹⁶⁾ However, it may be considered that lysosomes constitute the key subcellular fraction for aminoglycoside nephrotoxicity.

Lysosomes are acidic subcellular particles having a pH of 4–5 because of the sialic acid residues of the glycoprotein or glycolipid of their membranes.^{12,13)} Indeed, the binding of DKB to submaxillary mucin occurs through an interaction between the sialic acid residues of mucin and the amino groups of DKB (data to be published elsewhere).¹⁴⁾ Deguchi *et al.* reported that aminoglycosides bind to acid mucopolysaccharide, heparin or chondroitin sulfate A, through an ionic interaction between the amino group(s) of aminoglycoside and carboxy or sulfate groups of acid mucopolysaccharide.¹⁵⁾ Furthermore, the binding of aminoglycosides and phospholipids, particularly phosphatidylinositol, has been well investigated by Sastrasinh *et al.*¹⁶⁾ On the basis of the above findings, aminoglycosides may bind to sialic acid residues or the phospholipids of lysosomal membranes and accumulate there. The blood concentration of gentamicin after the therapeutic dose in humans is about 10^{-5} M and the level accumulated in the kidney will be higher than that.¹⁷⁾ In rats given 100 mg/kg of gentamicin for 4 d, the concentration in the kidney cortex reaches 5×10^{-3} M.¹⁸⁾ Nephrotoxicity may occur as a result of the breakdown of lysosomal functions. The effects of aminoglycosides on the release of lysosomal enzymes and the fluidity of the lysosomal membrane was thus investigated in order to clarify the effects of aminoglycosides on the lysosomal membrane.

Release of Lysosomal Enzymes

The effects of DKB on the release of lysosomal enzymes, *N*-acetyl- β -D-glucosaminidase and acid phosphatase, were investigated. As shown in Fig. 3, the release of *N*-acetyl- β -D-glucosaminidase increased remarkably at 10^{-2} and 10^{-3} M DKB, but fell below the control value (by 4%) at a low concentration of DKB (10^{-5} M). Powell and Reidenberg reported that low concentrations of gentamicin inhibit the release of lysosomal enzymes, and lysosomal membranes are stabilized. This stabilization may be the reason for the accumulation of aminoglycoside within the lysosomes.¹⁹⁾ Our results show that the release of *N*-acetyl- β -D-glucosaminidase was inhibited at a low concentration of DKB and amikacin, just as in the

Fig. 3. The Releases of *N*-Acetyl- β -D-glucosaminidase, Acid Phosphatase, and Phosphorus from the Lysosomal Fraction in the Presence of Various Concentrations of DKB

—○—: Acid phosphatase.
—●—: *N*-Acetyl- β -D-glucosaminidase.
—△—: Phosphorus.

Each value represents the mean of three experiments $\pm$ S.D.

Control values of both enzymes and phosphorus are indicated as follows.

▒ : Acid phosphatase.
▨ : *N*-acetyl- β -D-glucosaminidase.
▩ : Phosphorus.

TABLE I. Release of Lysosomal Enzymes by Treatment with DKB, Gentamicin, and Amikacin

Drug concn. (M)		% release	
		Glucosaminidase	Acid phosphatase
Control		28.4 ± 0.5	32.9 ± 0.7
DKB	10 ⁻²	74.2 ± 0.4	44.8 ± 0.7
	10 ⁻⁵	3.9 ± 0.3	36.2 ± 0.9
Gentamicin	10 ⁻²	83.7 ± 0.6	64.3 ± 1.4
	10 ⁻⁵	3.8 ± 0.3	38.8 ± 0.1
Amikacin	10 ⁻²	71.3 ± 0.5	44.0 ± 1.4
	10 ⁻⁵	4.3 ± 0.1	34.1 ± 1.3

Mean ± S.D. (*n* = 3).

Significant differences among three aminoglycoside (10⁻² M) were as follows.

N-Acetyl-β-D-glucosaminidase (glucosaminidase): gentamicin-DKB, *p* < 0.001; DKB-amikacin, *p* < 0.01.

Acid phosphatase: gentamicin-DKB, *p* < 0.001; DKB-amikacin, *p* < 0.5.

TABLE II. Effect of Aminoglycoside on the Fluorescence Polarization of Lysosomal Fraction

Concn. (M)	Value of polarization		
	DKB	Amikacin	Gentamicin
0 (control)	0.240 ± 0.010	0.240 ± 0.010	0.240 ± 0.010
10 ⁻²	0.226 ± 0.004 ^{a)}	0.221 ± 0.008 ^{a)}	0.219 ± 0.007 ^{a)}
10 ⁻⁵	0.233 ± 0.004	0.232 ± 0.005	0.244 ± 0.008
10 ⁻⁷	0.238 ± 0.003	0.232 ± 0.004	0.244 ± 0.007

Results are each the mean ± S.D. of at least six experiments.

^{a)} *p* < 0.01.

case of gentamicin. However, the release of the enzyme is facilitated at high concentrations of aminoglycosides.¹⁸⁾ The release of *N*-acetyl-β-D-glucosaminidase may cause the lysosomal enzymuria in aminoglycoside nephrotoxicity. In the case of acid phosphatase, the release slowly increased with increasing DKB concentration, in contrast to the case of *N*-acetyl-β-D-glucosaminidase. These results suggest different localizations of these two enzymes within the lysosome.

Furthermore, the effects of amikacin and gentamicin on the release of lysosomal enzymes were investigated. As shown in Table I, the release patterns of *N*-acetyl-β-D-glucosaminidase and acid phosphatase were similar to those observed with DKB.

In general, the severity of the nephrotoxicity of aminoglycosides is considered to be in the order, gentamicin > DKB > amikacin.²⁰⁾ The percentage releases of lysosomal enzymes, *N*-acetyl-β-D-glucosaminidase and acid phosphatase at comparable concentrations of aminoglycosides are consistent with that order.

Fluidity of Lysosomal Membranes

The effects of aminoglycosides on the fluidity of lysosomal membranes were investigated by using DPH as a fluorescent probe. The polarization (*P*) values are considered to be inversely proportional to membrane fluidity. As shown in Table II, the *P* value decreased with increasing concentration of aminoglycosides.

Furthermore, to determine whether destruction of lysosomes occurs after incubation with aminoglycoside, the release of phospholipids was investigated. The extraction of phospholipids was carried out by the method of Shukla *et al.*,²¹⁾ and phospholipid concentration was determined by measuring the phosphorus concentration according to Chen *et al.*²²⁾ No release of phospholipids into the incubation medium could be observed, as shown in Fig. 3. Thus, it is considered that there was no destruction of lysosomal membranes.

From the results of the above experiments, the increase in the release of enzymes from lysosomes upon addition of DKB may result from an increase in membrane fluidity, but not the destruction of membrane structures, *i.e.* the permeability of lysosomal membranes increases and the enzymes are released. One possible cause of aminoglycoside nephrotoxicity may be this enzyme loss arising from the change in membrane fluidity. However, although the release of acid phosphatase from lysosomes is consistent with the increase in membrane fluidity, the release of *N*-acetyl- β -D-glucosaminidase at low concentrations (10^{-5} M) of DKB is not. Thus, more than one mechanism may be involved in the release of lysosomal enzymes.

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