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Interaction of 3′,4′-Dideoxykanamycin B with a Lysosomal Enzyme, *N*-Acetyl-β-D-glucosaminidase

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The binding of 3′,4′-dideoxykanamycin B with a lysosomal enzyme, *N*-acetyl-β-D-glucosaminidase, obtained from rat kidney was examined by affinity chromatography of the enzyme on 3′,4′-dideoxykanamycin B-conjugated Sepharose 4B. *N*-Acetyl-β-D-glucosaminidase could be adsorbed on the affinity column. Furthermore, the enzyme activity was significantly increased by the addition of 3′,4′-dideoxykanamycin B. These findings indicate that 3′,4′-dideoxykanamycin B does interact with *N*-acetyl-β-D-glucosaminidase.

Keywords——aminoglycoside antibiotic; lysosomal enzyme; *N*-acetyl-β-D-glucosaminidase; 3′,4′-dideoxykanamycin B.

Aminoglycoside antibiotics are widely used for the therapy of various gram-negative bacterial infections. However, one of the disadvantages of these antibiotics is that they are accumulated in the kidneys and show nephrotoxicity. It has been reported that aminoglycoside antibiotics, such as gentamicin, kanamycin, and amikacin, accumulate in the subcellular lysosomal fraction of cultured rat fibroblast cell.[1] Furthermore, Hostetler and Hall[2] indicated that the activities of lysosomal phospholipase were inhibited in the presence of aminoglycoside *in vitro,* and Feldman *et al.*[3,4] suggested that the accumulation of phospholipids in the kidneys associated with aminoglycoside nephrotoxicity was due to the inhibition of phospholipase activities. Recently, Meisner[5] reported that the renal subcellular distribution of *N*-acetyl-β-D-glucosaminidase activity was shifted towards the less dense fraction by treatment with gentamicin. These findings indicate that the nephrotoxicity of aminoglycoside antibiotics may involve the lysosomes of the kidney cortex.

The purpose of this investigation was to determine whether 3′,4′-dideoxykanamycin B binds with a lysosomal enzyme, *N*-acetyl-β-D-glucosaminidase, by the use of an affinity chromatography technique, as a first step in clarifying the mechanisms of nephrotoxicity.

Materials and Methods

Materials——3′,4′-Dideoxykanamycin B (DKB) and 3′,4′-(^{3}H)-DKB (66 μCi/mg) were kindly donated by Meiji Seika Kaisha (Tokyo). Sephadex G-100 and Sepharose 4B were purchased from Pharmacia, and bovine serum albumin (Fraction V) was from Sigma. The other reagents were of the best available grade. Male Wistar rats (200—250 g) were purchased from Tokyo Jikken Dobutsu (Tokyo).

Preparation of *N*-Acetyl-β-D-glucosaminidase——Kidney cortex, obtained from 20 h fasted rats, was homogenized, and 20% (w/v) homogenate was prepared in 10 mM phosphate buffer (pH 6.5). The homogenate was ultrasonicated at 50 W for 5 min, and then the enzyme solution was obtained by ultracentrifugation at 100000 ×*g* for 30 min. With *p*-nitrophenyl-β-D-*N*-acetylglucosaminide as the substrate, the hexosaminidase activity was determined according to the method of Loomis.[6] The protein concentrations were determined by the method of Lowry *et al.*[7] and by measurement of the absorbance at 280 nm, using bovine serum albumin as the standard protein.

Preparation of Affinity Column——DKB-conjugated Sepharose 4B was prepared by the method of Sera *et al.*[8] The CNBr-activated Sepharose 4B (gel volume 20 ml) was washed with 1 mM HCl on a glass filter, and then washed with 500 mM NaCl–100 mM $NaHCO_3$ buffer (pH 8.3). The washed gel was suspended in the same buffer (150 ml) containing DKB (500 mg), and incubated at 37°C for 2 h. The reaction mixture was washed with the buffer, and then 1.0 M glycine (30 ml) was added to the gel. This suspension was incubated at 37°C for 2 h, and the resulting gel was washed with NaCl–$NaHCO_3$ buffer, then 500 mM NaCl–100 mM acetate buffer (pH 4.0), and 10 mM phosphate buffer (pH 6.5), in this order. The amount of DKB bound to this affinity gel

was measured by the method of Wagner[9] after hydrolysis of the gel with 1 N HCl at 100°C for 4 h and was found to be 1.37 μmol of DKB bound to 1 mg of the gel.

The radioactivity was determined by the method of Petterson and Green.[10] Fifty μl of each fraction of the Sephadex G-100 gel filtrate and 7.0 ml of toluene cocktail were mixed, and then the radioactivity was counted with a Packard Model 3300 liquid scintillation counter.

Results and Discussion

The affinity column of DKB-conjugated Sepharose 4B was employed to investigate the binding of DKB with *N*-acetyl-β-D-glucosaminidase. When the supernatant of the homogenate of rat kidney cortex was applied to the affinity column of DKB-conjugated Sepharose 4B and eluted with 10 mM phosphate buffer (pH 6.5), no enzyme activity could be detected in the eluate, although proteins were detected. When the elution buffer was changed from 10 mM phosphate buffer to 10 mM phosphate buffer containing 1.0 M NaCl, the enzyme was eluted from the column as shown in Fig. 1. When the supernatant of the homogenate of kidney cortex was applied to a Sepharose 4B column and eluted with 10 mM phosphate buffer (pH 6.5) or 10 mM phosphate buffer (pH 6.5) containing 1.0 M NaCl, the enzyme was not adsorbed (data not shown). This result suggests that the adsorption of *N*-acetyl-β-D-glucosaminidase on the affinity column occurred as a result of the binding of DKB with *N*-acetyl-β-D-glucosaminidase molecules.

We next investigated, by means of gel filtration with Sephadex G-100, whether *N*-acetyl-β-D-glucosaminidase binds to DKB. The enzyme active fractions obtained by the affinity chromatography were pooled, dialyzed against 10 mM phosphate buffer (pH 6.5) to remove NaCl,

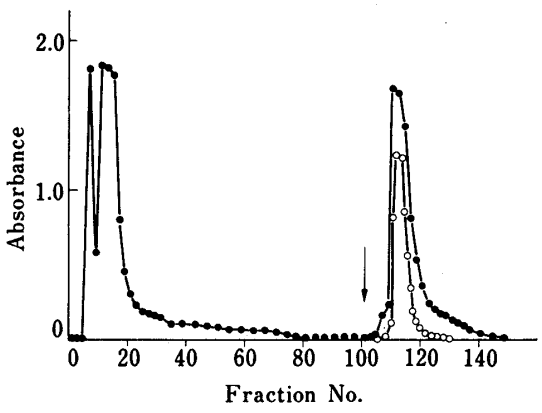

Fig. 1. Affinity Chromatography of the Enzyme Solution obtained from Rat Kidney Cortex

The column size was 1.3×15 cm, and each fraction was 1.2 ml. The arrow indicates the change of the elution buffer from 10 mM phosphate buffer (pH 6.5) to 10 mM phosphate buffer containing 1.0 M NaCl.

●——●; protein (absorbance at 280 nm).
○——○; *N*-acetyl-β-D-glucosaminidase activity (absorbance at 400 nm).

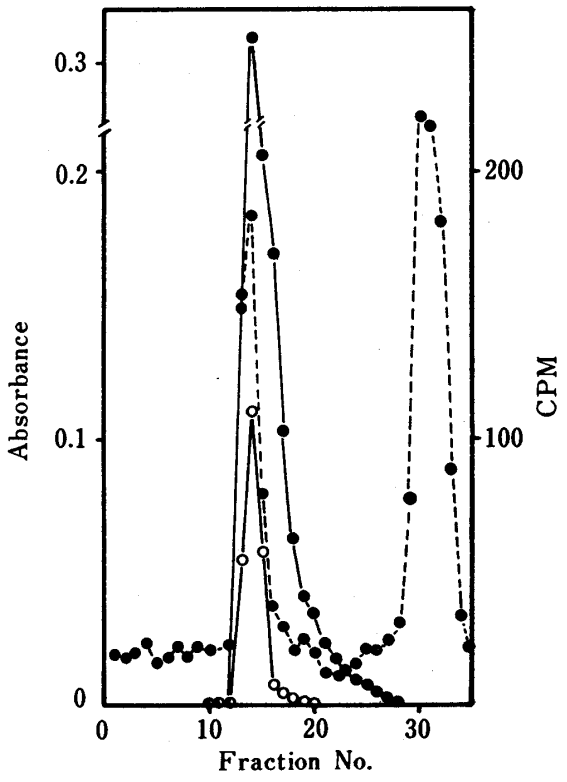

Fig. 2. Sephadex G-100 Column Chromatography of the Reaction Mixture of the Affinity-bound Fraction and ^{3}H-DKB

The column size was 1.3×66 cm, and the mixture was eluted with 10 mM phosphate buffer (pH 6.5).

○——○; *N*-acetyl-β-D-glucosaminidase activity.
●——●; protein (Lowry method).
●------●; radioactivity (cpm).

and then concentrated by ultrafiltration using an Amicon PM-10 membrane. This enzyme solution was incubated with ^{3}H-DKB at 37°C for 20 min, and then applied to a Sephadex G-100 column.

As shown in Fig. 2, the elution position of *N*-acetyl-β-D-glucosaminidase and the radioactivity of ^{3}H-DKB overlapped at the void volume. These results indicate that *N*-acetyl-β-D-glucosaminidase, which is one of the lysosomal enzymes, and DKB may bind to each other, because a shift of the elution position of DKB from low molecular weight field to high molecular weight field was observed. Meisner[5] reported that the intracellular distribution of *N*-acetyl-β-D-glucosaminidase activity in rat kidney cortex was changed by the administration of an aminoglycoside antibiotic, gentamicin. In view of the results of the present investigation, the change in the distribution of the enzyme may be due to the binding of *N*-acetyl-β-D-glucosaminidase molecules with the aminoglycoside.

A lysosome is considered to be an acidic subcellular particle having a pH of 4—5.[1] The negative charge of lysosomes is considered to be due to sialic acid residues.[11,12] Allen and Reagen[13] reported lysosomal acidic hydrolases are glycoprotein in nature, and *N*-acetyl-β-D-glucosaminidase is considered to be a glycoprotein containing sialic acid.[14] In general, it is considered that *N*-acetyl-β-D-glucosaminidase is localized at the lysosomal membrane.[15] Thus, the negative charge of a lysosome may be at least partly attributed to the sialic acid residues of *N*-acetyl-β-D-glucosaminidase molecules. Orme and Cutler[16] previously reported that kanamycin could not bind to serum proteins, and in our equilibrium dialysis experiment, the binding of DKB with bovine serum albumin could not be observed (data not shown). In addition, it was reported that aminoglycosides bound to substances having negative charge, such as heparin and chondroitin sulfate A, and their binding sites were considered to be carboxyl groups or sulfate groups.[17] As described above, *N*-acetyl-β-D-glucosaminidase is a glycoprotein whose binding site may be located in the sugar moiety, not the protein moiety. Thus, we consider that the binding of DKB with *N*-acetyl-β-D-glucosaminidase occurs as a result of the ionic interaction of the amino group of the antibiotic with the sugar moiety — probably a sialic acid residue — of this enzyme.

The effect of DKB on the enzyme activity was also examined. The enzyme solution obtained by affinity chromatography was preincubated with DKB solution at 37°C for 10 min, and after the addition of the substrate solution, the mixture was incubated at 37°C for 30 min. When 1 mM DKB was added, the enzyme activity increased by 12.3% (Table I). The same concentration of glucose as that of DKB was used to investigate the effect of the osmotic pressure; it had no effect on the enzyme activity. Thus, the increase of the enzyme activity on the addition of DKB may be attributed to some conformational change of the enzyme caused by the binding of DKB to the enzyme molecule.

TABLE I. Effects of DKB and Glucose on the Enzyme Activity

Concentration (M)	Enzyme activity (units/mg of protein)	
	DKB (%)	Glucose (%)
0 (Control)	7.419±0.008(100.0)	7.419±0.008(100.0)
10^{-6}	7.590±0.053(102.3)[a]	7.383±0.103(99.5)[d]
10^{-5}	7.660±0.080(103.2)[a]	7.410±0.255(99.9)[d]
10^{-4}	7.918±0.108(106.7)[a]	7.503±0.108(101.2)[d]
10^{-3}	8.331±0.078(112.3)[c]	7.436±0.089(100.2)[d]
10^{-2}	8.073±0.029(108.8)[b]	7.574±0.158(102.1)[d]

One unit of the enzyme activity was defined as the amount of the enzyme that released 1 nmol of *p*-nitrophenol per min at 37°C.
Significant differences between means: *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$. No significant difference between means: *d*).

The nephrotoxicity which is induced by aminoglycoside antibiotics is the result of the accumulation of aminoglycosides within the kidney proximal tubular cells, especially the lysosomal fraction.[3,4] From our findings, it is considered that one of the accumulation mechanisms of DKB in rat kidney lysosomes is the binding of DKB to a lysosomal enzyme, *N*-acetyl-β-D-glucosaminidase, which is localized at the lysosomal membrane,[15] and that this binding causes a change of the enzyme activity. However, the total amount of proteins bound to the DKB-conjugated Sepharose 4B column is so large that other DKB-binding substances may also be adsorbed on the affinity column.

We are carrying out further studies of the interaction of *N*-acetyl-β-D-glucosaminidase and DKB, the cause of the nephrotoxicity of DKB and the intracellular activities of lysosomal enzymes.

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