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Role of *N*-acetylneuraminic acid in rat renal brush-border membrane vesicle aggregation by aminoglycosides

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Interaction between aminoglycosides (AGs) and rat renal brush-border membrane (BBM) vesicles was investigated by the aggregation technique. The order of aggregation was gentamicin > dibekacin ≒ netilmicin > amikacin, and this order corresponds to the strength of the nephrotoxicity of the aminoglycosides in vivo rather than the number of amino groups in the aminoglycosides. BBM vesicles were aggregated through ionic interaction, as evident from the finding that aggregation ceased to occur at alkaline pH. By addition of *N*-acetylneuraminic acid (NANA) to the incubation medium, the vesicle aggregation induced by gentamicin was significantly inhibited. To affect the liberation of the NANA residue from BBM vesicles, the vesicles were treated with neuraminidase, resulting in an about 60% release with a significant decrease in the uptake of gentamicin into the vesicles. The decrease in the degree of vesicle aggregation was in proportion to the amount of NANA liberated. It follows from the findings that the NANA residue may in some way be responsible for the accumulation of aminoglycosides in renal proximal tubular cells.

Introduction

Aminoglycosides have a high potential for combating Gram-negative bacterial infections, while nephrotoxicity is a major limiting factor in their clinical application. Nephrotoxicity due to aminoglycosides is closely related to the concentration of the aminoglycosides in the renal proximal tubular cells after passing through the brush-border membrane (BBM) during the reabsorption process [2,3] and, thus, nephrotoxicity may be initiated by the interaction between aminoglycosides and BBM. Sastrasinh et al. [4] reported the binding sites or receptors for aminoglycosides in BBM to be phosphatidylinositides, such as phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI-*P*) and phosphatidylinositol 4,5-diphosphate (PI-P_2).

Au et al. [5], Yung and Green [6] and Aramaki and Tsuchiya [7] have recently shown on the basis of the liposomal aggregation study that aminoglycosides have affinity toward various acidic phospholipids. Aminoglycosides have the highest affinity for PI-P_2 among acidic phospholipids. Liposomal aggregation is dependent on the number of amino groups in aminoglycosides [6,7], but nephrotoxicity is not necessarily dependent on this parameter.

Aminoglycosides as polycationic drugs possess some affinity toward negatively charged substances such as ATP [9] and mucopolysaccharides [10,11], and aminoglycosides bind to them through ionic interactions.

The electric charge of a cell surface is generally negative, and NANA is considered to be responsible for this negative charge. The present study was thus undertaken to investigate the interaction between aminoglycosides and BBM, by means of the aggregation of BBM vesicles, and an attempt was made to determine the participation of NANA residues in the aggregation of BBM vesicles.

Materials and Methods

Materials. Gentamicin (GM), dibekacin (DKB), netilumicin (NTL) and amikacin (AMK) were supplied from Shionogi Pharmaceutical Co. (Osaka), Meiji Seika kaisha Co. (Tokyo), Sankyo Co. Ltd. (Tokyo) and Banyu Pharmaceutical CO. (Tokyo), respectively. *N*-Acetylneuraminic acid (NANA) and neuraminidase (type VI, from *Clostridium perfringens*) were purchased from Sigma Chemicals (U.S.A.). Other reagents were

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from Wako Pure Chemical Industries (Osaka). Male Wistar rats weighing 200–250 g were purchased from Shizuoka Agricultural Co. (Shizuoka).

Preparation of brush-border membrane vesicles and their aggregation. BBM vesicles were prepared from the rat renal cortex by the magnesium precipitation method according to Booth and Kenny [12], and Malathi et al. [13]. The BBM vesicle alkaline phosphatase activity was enriched up to about 15-times that of the renal cortical homogenate. Contamination of the basolateral membranes was negligible, since the Na^+,K^+-ATPase activity was quite low. The cytochrome-*c* oxidase, *N*-acetyl-β-D-glucosaminidase and glucose-6-phosphatase activities in the BBM vesicle were also lower than those of the homogenate. The BBM vesicle preparation was suspended in 2 mM Tris-HCl buffer (pH 7.1) containing 10 mM mannitol.

BBM vesicles (protein concentration 400–500 μg/ml) and aminoglycosides at various concentrations were mixed, and the degree of BBM vesicle aggregation was determined on the basis of the increase in turbidity, as measured at 400 nm (25° C), according to the method of Yung and Green [6].

Uptake experiment. The uptake of GM by BBM vesicles was assessed by the rapid filtration technique. One milliliter of the BBM vesicle suspension was mixed with 1.0 ml of GM solution (100 μg/ml). Following incubation at 25° C for 30 min, the mixture was filtrated through a Millipore filter (type HA, 0.45 μm), the GM concentration recovered in the filtrate was determined and the amount of GM taken up by BBM vesicles was calculated. The data were corrected by subtracting the non-specific GM binding to the filters.

Removal of N-acetylneuraminic acid. BBM vesicles suspended in 50 mM phosphate buffer (pH 6.0) were incubated with neuraminidase (1.8 U) at 37° C for 60 min. Following incubation, the BBM vesicles were washed twice by centrifugation with 2 mM Tris-HCl buffer (pH 7.1) containing 10 mM mannitol, and suspended in this buffer.

Analytical methods. The electrophoretic mobility of BBM vesicles were determined by rotating prism method of microelectrophoresis (Laser Zee, model 501, Pem Kem, U.S.A.) in 2 mM Tris-HCl buffer (pH 7.1) containing 10 mM mannitol at room temperature. It was converted to Zeta potential (electrokinetic potential) according to Helmholtz-Smoluchowski equation [14]. The GM concentration was determined by bioassay using the *Bacillus subtilis* ATCC 6633 as the test organism [15]. Protein and NANA concentrations were determined by the methods of Lowry et al. [16], and Helen and Edward [17], respectively. The alkaline phosphatase [18], Na^+,K^+-ATPase [19], *N*-acetyl-β-D-glucosaminidase [20], cytochrome-*c* oxidase [21], and glucose-6-phosphatase [22] activities were measured by the reported methods.

Results

Aggregation of brush-border membrane vesicles by aminoglycosides

Interactions between aminoglycosides and BBM vesicles were assessed by the increase in turbidity resulting from the aggregation. As shown in Fig. 1, aminoglycosides caused BBM vesicles to aggregate and the gentamicin > dibekacin ≒ netilmicin > amikacin. This order corresponds to the strength of nephrotoxicity in vivo rather than to the number of amino groups in the aminoglycosides. The number of amino groups in gentamicin, dibekacin, netilmicin and amikacin are 3–4, 5, 3 and 4, respectively. Glucosamine, having only one amino groups in its molecule, was incapable of causing BBM vesicle aggregation. Thus, the aggregation of BBM vesicles requires more than two amino groups.

Effect of the gentamicin concentration on the extent of BBM aggregation is evident from the results illustrated in Fig. 2. Aggregation reached a plateau within

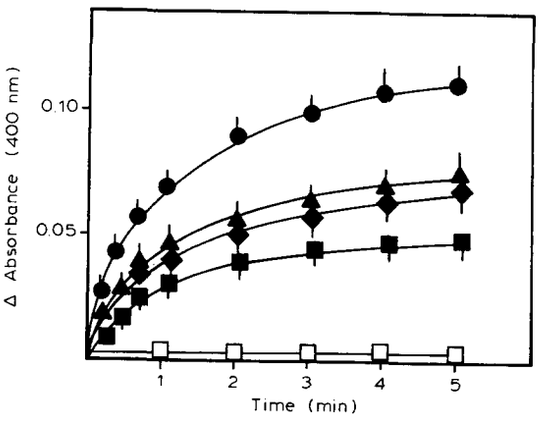

Fig. 1. Aggregation of brush-border membrane vesicles by aminoglycosides. Concentrations of aminoglycosides and glucosamine were $2 \cdot 10^{-3}$ M. Values shown represent means ±S.D. for three experiments. ●, Gentamicin; ▲, dibekacin; ◆, netilmicin; ■, amikacin; □, glucosamine.

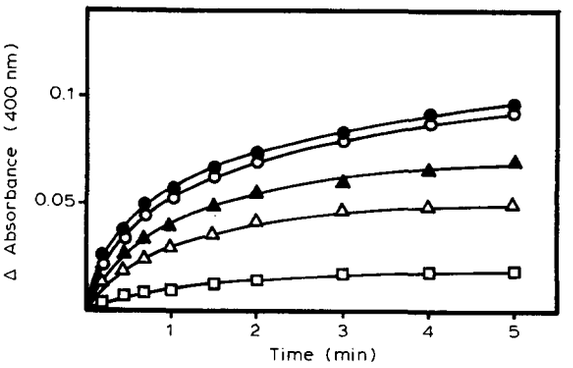

Fig. 2. Effect of gentamicin concentration on brush-border membrane vesicle aggregation. Values shown represent mean for three experiments. ●, $2 \cdot 10^{-3}$; ○, $1 \cdot 10^{-3}$ M; ▲, $5 \cdot 10^{-4}$ M; △, $2.5 \cdot 10^{-4}$ M; □, $1 \cdot 10^{-4}$.

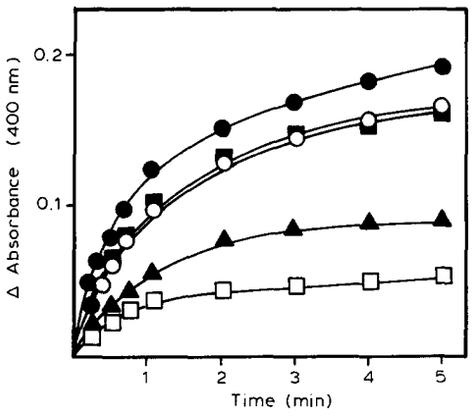

Fig. 3. Effect of pH on brush-border membrane vesicle aggregation induced by gentamicin. Gentamicin concentration was $2 \cdot 10^{-3}$ M. Values shown represent mean for three experiments. ○, pH 5.0; ●, pH 6.0; ■, pH 7.0; ▲, pH 8.0; □, pH 9.0.

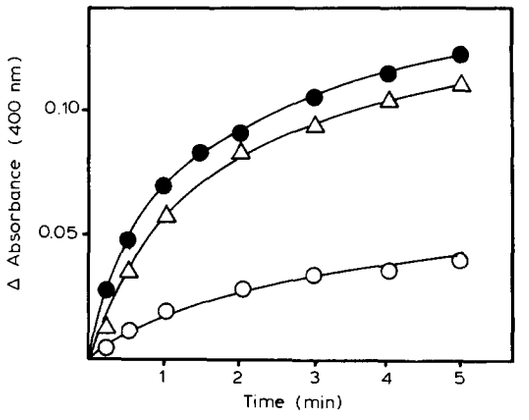

Fig. 5. Effect of *N*-acetylneuraminic acid on the BBM vesicle aggregation induced by gentamicin. Gentamicin concentration was $2 \cdot 10^{-3}$ M. ●, No *N*-acetylneuraminic acid (control); △, $5 \cdot 10^{-4}$ M *N*-acetylneuraminic acid; ○, $1 \cdot 10^{-3}$ M *N*-acetylneuraminic acid.

2–3 min following the addition of gentamicin and was dependent on gentamicin concentration, saturation being observed at $1 \cdot 10^{-3}$ M.

The effect of pH on the BBM vesicle aggregation caused by gentamicin is shown in Fig. 3. Aggregation was influenced by pH, was highest in the acidic pH range, and decreased with the increase in pH.

Fig. 4 shows the effect of Ca^{2+} on the BBM vesicles aggregation caused by gentamicin. Addition of Ca^{2+} to the incubation medium decreased the absorbance, with a 50% (approx.) decline in the absorbance in the presence of $1 \cdot 10^{-2}$ M of $CaCl_2$.

Effect of NANA on brush-border membrane vesicle aggregation

Fig. 5 shows the effect of NANA on the BBM aggregation induced by gentamicin. The aggregation was significantly inhibited by addition of $1 \cdot 10^{-3}$ M NANA.

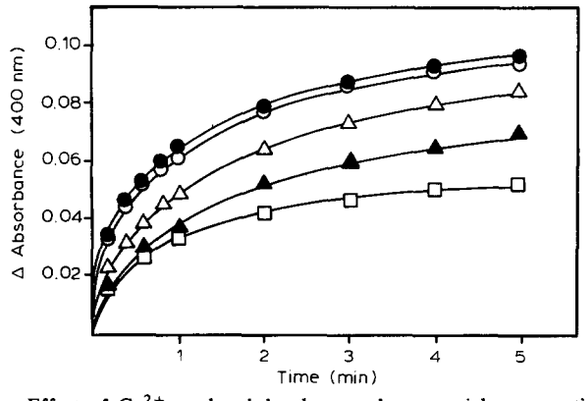

Fig. Effect of Ca^{2+} on brush-border membrane vesicle aggregation induced by gentamicin. Gentamicin concentration was $2 \cdot 10^{-2}$ M. Values shown represent mean for three experiments. $CaCl_2$ concentrations were as follows: ●, control (0 M); ○, $1 \cdot 10^{-4}$ M; △, $1 \cdot 10^{-3}$ M; ▲, $2.5 \cdot 10^{-3}$ M; □, $1 \cdot 10^{-2}$ M.

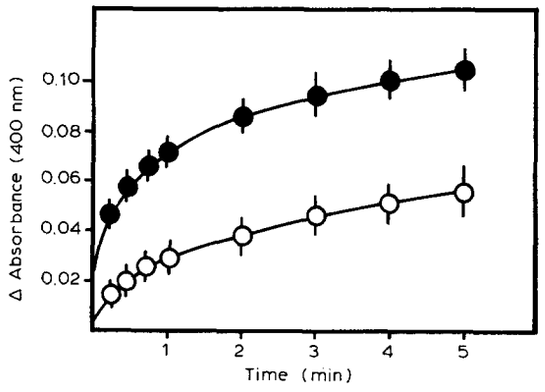

Fig. 6. Effect of neuraminidase treatment on brush-border membrane vesicle aggregation induced by gentamicin. Gentamicin concentration was $2 \cdot 10^{-3}$ M. Values shown represent means ± S.D. for three experiments. ●, control brush-border membrane vesicles; ○, neuraminidase treated brush-border membrane vesicles.

TABLE I

Characterization of BBM vesicles after neuraminidase treatment

Values are means ± S.D. ($n = 3$). * $P < 0.05$, ** $P < 0.01$.

BBM vesicles	Neuraminic acid concn. (μg/mg protein)	Zeta potential (mV)	Gentamicin uptake (μg/mg protein)
Control	31.5 ± 3.0 (100%)	−48.8 ± 2.0	4.8 ± 1.6 (100%)
Neuraminidase-treated	12.3 ± 4.7 * (39%)	−37.6 ± 1.4 **	2.6 ± 1.1 * (54%)

BBM vesicles were treated with neuraminidase, and NANAs on their surface were removed. The features of BBM vesicles following this treatment are listed in Table I. About 60% of the NANA was released from BBM vesicles, and this was accompanied by a reduction in the vesicle charge estimated as zeta potential. The uptake of gentamicin into BBM vesicles assessed by the rapid filtration technique also decreased almost in proportional to the NANA concentration of the BBM vesicles. The degree of aggregation of the neuraminidase-treated BBM vesicles was supressed by about half of the control values throughout the experimental period (Fig. 6).

Discussion

The mechanism of nephrotoxicity of aminoglycosides has been extensively investigated, but still remains inadequately clear. Aminoglycosides accumulate within tubular cells after passing through BBM or basolateral membrane. However, BBM is considered to be a preferential route for the accumulation of aminoglycosides in the proximal tubular cells [3,23], as was reported by Just et al. [24] and Silverblatt and Kuehn [2]. According to Sastrasinh et al. [4], the binding sites for aminoglycosides in BBM are phosphatidylinositides. Interaction between aminoglycosides and phospholipids was examined by means of the liposomal aggregation, and kanamycin B (five amino groups) shows a higher affinity for acidic phospholipids than kanamycin A (four amino groups) [6]. The present authors recently reported the interaction with aminoglycosides of liposomes composed of phospholipids extracted from rat kidney cortex, indicating that the order of intensity was gentamicin > dibekacin ≒ netilmicin > amikacin, which corresponded to the strength of nephrotoxicity of aminoglycosides in vivo rather than the number of amino groups in aminoglycosides [7]. This result is at variance with the report of Yung and Green [6].

Recently, phospholipid asymmetry in the distribution of acidic phospholipids of the membrane has been reported, phosphatidylinositol and phosphatidylserine being the main constituents of the cytoplasmic side of renal BBM [25]. Thus, the possibility that substances other than acidic phospholipids may be involved in the interaction between aminoglycosides and BBM required further consideration. In this report, attention was focused on the NANA residue which is responsible in part for the negative charge of cell surface. The participation of NANA in the aggregation of BBM vesicles caused by aminoglycosides was investigated spectrophotometrically.

The highest aggregation of BBM vesicles was observed by gentamicin (Fig. 1), and it was dependent on the gentamicin concentration (Fig. 2). Upon addition of various amounts of gentamicin to the assay medium, its osmolarity remained constant (16 mosM). Thus, gentamicin could not affect the vesicular volume and the absorbance measured in this assay system. Therefore, the aggregation induced by aminoglycosides may come from the results of the interaction between BBM vesicles and aminoglycosides. The aggregation of BBM vesicles was dependent on pH (Fig. 3). Because the apparent pK_a of gentamycin is considered to be 8.2 and the pK_a of NANA, 2.6, the dissociative groups of both compounds should ionize at pH 5–7. The aggregation of BBM vesicles caused by gentamicin may possibly result from ionic interactions. Further, the BBM vesicle aggregation was decreased by Ca^{2+} (Fig. 4). Forstner and Forstner [26] have reported that Ca^{2+} binds to the carboxyl group of NANA residues of rat intestinal goblet cell mucin through ionic interaction. By addition of NANA in the incubation medium, the vesicle aggregation was also inhibited (Fig. 5). Therefore, NANA residues on the BBM surface may play an important role in the BBM vesicle aggregation induced by gentamicin. Multiple amino groups are a necessary condition for inducing the aggregation, but the intensity of aggregation does not necessarily depend on the number of amino groups in aminoglycosides (Fig. 1). Thus, the steric form of amino group(s) in aminoglycosides may be a more important factor for inducing vesicle aggregation.

After the neuraminidase treatment of BBM vesicles, gentamicin uptake and vesicle aggregation decreased almost in proportion to the removed amount of the NANA residues (Table I and Fig. 6). But, the decrease in zeta potential was not necessarily dependent on the amount of the NANA residues (Table I). There is thus the possibility that other substances such as phospholipids and/or proteins may contribute to the surface charge of BBM vesicles. Niibuchi et al. [27] recently reported the binding of gentamicin to rat intestinal mucin, and this binding to be due to the ionic interaction between the amino groups of gentamicin and the carboxyl group of the NANA residue in mucin. Thus, the NANA residue from glycoproteins and/or glycolipids of BBM strongly contributes to the uptake of gentamicin and the aggregation of BBM vesicles.

As for the development of aminoglycosides nephrotoxicity, the accumulation of aminoglycosides in the renal proximal tubular cells is very important. Lipsky et al. [28] and Josepovitz et al. [29] reported the uptake of gentamicin by rat renal BBM vesicles and by renal cortex, in vitro and in vivo, was inhibited by the co-administration of spermine, a polyamine. Rat renal BBM vesicle aggregation by gentamicin was also inhibited by spermine [30]. From the above facts, it is suggested that a correlation exits between vesicle aggregation by gentamicin and gentamicin uptake by BBM vesicles. For this aggregation, NANA residues appear to be important constituents, since there was a decrease in the

gentamicin uptake and in vesicle aggregation by gentamicin following the neuraminidase treatment (Table I and Fig. 6). The uptake of gentamicin into the BBM vesicles reflects the gentamicin binding to the membrane and the gentamicin incorporation into the intravesicular space. Recently, we investigated the uptake of [^{3}H]dibekacin by rat renal BBM vesicles, and about half of the amount taken up was transported into the intravesicular space of BBM vesicles, and the other half was bound to the vesicle surface [31]. The uptake of gentamicin by neuraminidase-treated BBM vesicles decreased to 55% of that of control BBM vesicles (Table I). But it is still unclear whether the cause of this decrease in gentamicin uptake comes from the decrease in the gentamicin binding, from incorporation into the vesicular space, or from both these phenomena.

In conclusion, the present data demonstrate that gentamicin may bind to NANA residues of BBM vesicles by ionic interaction and may cause vesicles to aggregate. The extent of aggregation induced by aminoglycosides is almost directly proportional to the strength of nephrotoxicity in vivo. For this binding and aggregation, the NANA residue of BBM vesicles appears to play an important role.

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