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Research Article

Interactions Between Aminoglycosides and Phospholipids Using Liposomes: A Possible Mechanism of Nephrotoxicity

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Interactions of aminoglycosides with phospholipids were estimated by the increase in turbidity of liposomes consisting of various phospholipids. The turbidity of liposomes containing negatively charged phospholipids was increased by gentamicin, the highest increase in turbidity being observed for phosphatidylinositol-4,5-diphosphate-containing liposomes. The extent of turbidity was dependent on the concentration of acidic phospholipid in the liposomal membrane as well as the number of amino groups of the aminoglycosides. The release of glucose from glucose-entrapped liposomes depended on the concentration of gentamicin. The turbidity of liposomes containing lipids extracted from rat renal cortex was also increased by aminoglycosides depending on the number of amino groups. From electron microscopic observations, the increase in turbidity of liposome suspensions was caused by liposome fusion.

KEY WORDS: aminoglycoside; nephrotoxicity; phospholipid; liposome; aggregation.

INTRODUCTION

Nephrotoxicity as a serious side effect of aminoglycosides (AG) is closely related to their accumulation of AGs within renal proximal tubular cells (1) after passing through brush border membranes (BBMs) in the reabsorption process (2–4). Therefore the interactions between AGs and BBMs may be involved with the nephrotoxicity.

The amino groups of AGs bind with the phosphate or carboxyl group(s) of acidic phospholipids (5), mucopolysaccharides (6–8), and nucleotides such as ATP (9) through ionic interactions. Sastrasinh *et al.* (5) suggested that the receptors for AGs on BBMs are acidic phospholipids, especially phosphatidylinositides.

Liposomes containing phospholipids and cholesterol are used as a stable model membrane for studying physiological membrane processes. Liposomal aggregation as a result of interactions between phospholipids and drugs can be measured by turbidimetry. Yung and Green (10) examined the interactions between polyamine and phospholipid using liposomes containing various phospholipids, and liposomal aggregation was found to occur and depend on the negative group content in the phospholipid vesicles.

AGs are basic compounds possessing several amino groups. We investigated the interactions between AGs and phospholipids using liposomes containing various phospholipids or lipids extracted from rat kidney cortex.

MATERIALS AND METHODS

Materials. Gentamicin (GM), dibekacin (DKB), and amikacin (AMK) were provided by Shionogi Pharmaceutical Co. (Osaka, Japan), Meiji Seika Kaisha Ltd. (Tokyo), and Banyu Pharmaceutical Co. (Tokyo), respectively. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylinositol (PI), and phosphatidylinositol-4,5-diphosphate ($PI-P_2$) were purchased from Sigma Chemicals (St. Louis, Mo). Cholesterol (Chol) was from Wako Pure Chemicals (Tokyo). All other reagents were of the best available grade. The male Wistar rats were from Shizuoka Agricultural Co. (Shizuoka, Japan).

Preparation of Liposomes and Their Interaction with AGs. Liposomes containing various phospholipids were prepared by sonication method according to Yung and Green (11). Briefly, PC (25 μmol), Chol (12.5 μmol), and various phospholipids (4.1 μmol) were dissolved in a round-bottom flask in 10 ml of organic solvent mixture (chloroform:ethanol, 9:1), and then the solvent was evaporated to make lipid film. To this flask, 10 m*M* Tris–HCl buffer (pH 7.4) containing 0.9% NaCl was added, and liposomes were prepared by sonication for 3 hr (Branson, Model B-221). The liposomal suspension was extruded through polycarbonate membranes (0.4 μm) and multilamella vesicles were obtained. From electron microscopic observations, the diameter of the liposomes was 225 ± 61 nm. The phospholipid concentration was determined from inorganic phosphorus content according to the method of Chen *et al.* (12).

The interactions between AGs and liposomes were estimated from the increase in turbidity as measured at 400 nm, following the method of Yung and Green (11).

Release of Glucose from Liposomes. The glucose-entrapped liposomes consisting of PC:Chol:PA, 6:3:1 (mol), were incubated with various concentrations of GM at 25°C for 15 min and applied to a Sepharose 4B column (1.5 × 16 cm) preequilibrated with 10 m*M* Tris–HCl buffer (pH 7.4)

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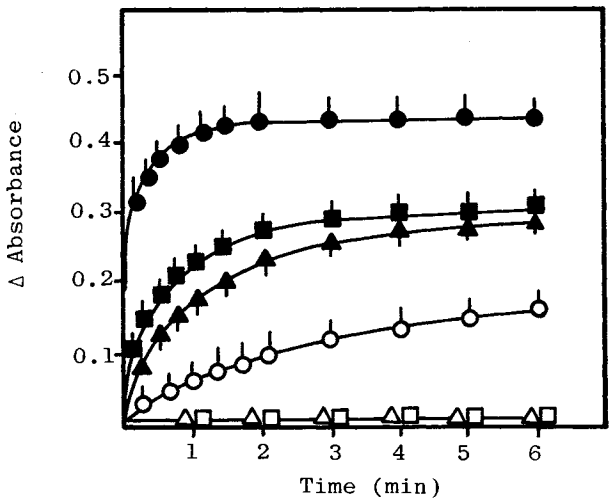

Fig. 1. Increase in turbidity of liposome suspensions by gentamicin. The lipid composition of liposomes, phosphatidylcholine:cholesterol:phospholipids, was 6:3:1 (mol), and the gentamicin concentration was 2×10^{-3} *M*. Values represent the means ± SD of three experiments. (●) Phosphatidylcholine:cholesterol:phosphatidylinositol-4,5-diphosphate; (■) phosphatidylcholine:cholesterol:phosphatidic acid; (▲) phosphatidylcholine:cholesterol:phosphatidylserine; (○) phosphatidylcholine:cholesterol:phosphatidylinositol; (□) phosphatidylcholine:cholesterol:phosphatidylethanolamine; (△) phosphatidylcholine:cholesterol (6:3).

containing 0.9% NaCl to determine the amount of glucose released. Glucose concentration was determined according to Dubois *et al.* (13).

Extraction of Lipids from Rat Renal Cortex. Lipids were extracted from the kidney cortex of Wistar rats by the method of Folch *et al.* (14). The phospholipid concentration in the extracted lipids was estimated as inorganic phosphorus. The liposomes were prepared using these lipids by the same method mentioned above, and the amount of phospholipids was equivalent to that of above liposomes, 29.1 μmol.

Electron Microscopic Observation. Control liposomes or mixtures of liposomes and gentamicin (GM) were placed on a carbon-covered 200-mesh copper grid. After 1 min, excess liposome suspension was removed with the edge of a piece of filter paper. The liposomes on the grid were negatively stained with 2% phosphotungstic acid, pH 7.4, for 1 min. The samples were observed immediately in a JEM-100 CX (JEOL, Japan). Liposomal diameter was determined from electron micrograph prints.

RESULTS

Interaction Between Gentamicin and Liposomes. The interaction between gentamicin (GM) and liposomes was ex-

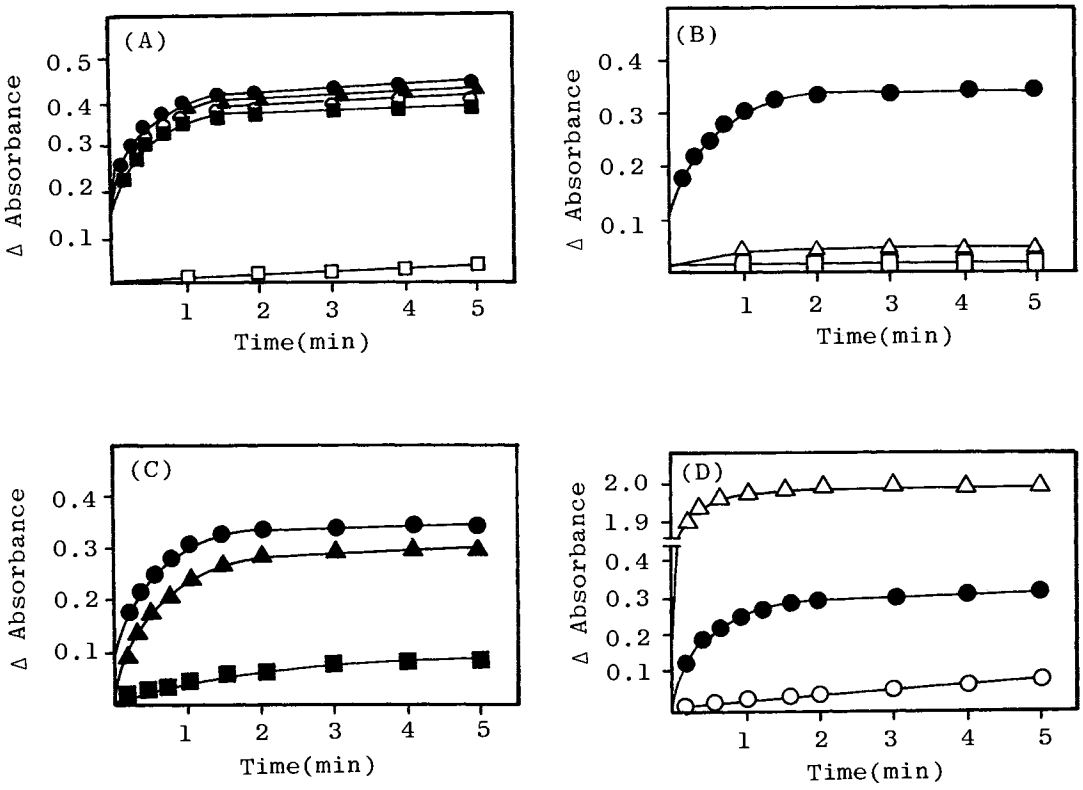

Fig. 2. Effect of pH (A), NaCl (B), gentamicin concentration (C), and phosphatidic acid concentration (D) on extent of turbidity. The lipid composition of liposomes in (A) was phosphatidylcholine:cholesterol:phosphatidic acid, 6:3:1, and the gentamicin concentration was 2×10^{-3} *M*. (●) pH 6.0; (▲) pH 6.5; (○) pH 7.0; (■) pH 7.5; (□) pH 8.0. In B, the lipid composition was phosphatidylcholine:cholesterol:phosphatidic acid, 6:3:1. The gentamicin concentration was 2×10^{-3} *M*. (△) 0.2 *M* NaCl; (□) 0.3 *M* NaCl. In C, the lipid composition was phosphatidylcholine:cholesterol:phosphatidic acid, 6:3:1. Gentamicin concentrations were as follows: (●) 2×10^{-3} *M*; (▲) 1×10^{-3} *M*; and (■) 5×10^{-4} *M*. In D, the gentamicin concentration was 2×10^{-3} *M*. Lipid compositions (phosphatidylcholine:cholesterol:phosphatidic acid) were as follows: (△) 6:3:2, (●) 6:3:1, and (○) 6:3:0.5.

amined using liposomes consisting of various kinds of phospholipids. As shown in Fig. 1, an increase in turbidity occurred with liposomes consisting of acidic phospholipids (PS, PA, PI, and PI-P_2), and the increase in turbidity reached a plateau level within 2–3 min following the addition of GM. The highest increase in turbidity was noted for the liposomes containing PI-P_2. But no increase in turbidity was found in the liposomes containing neutral phospholipids, PC and PE.

The interaction between GM and liposomes was further examined using liposomes consisting of PC:Chol:PA (Fig. 2A). The turbidity increased in an acidic to neutral pH region, while it decreased at alkaline pH. In the high-ionic strength solutions, the turbidity did not increase as shown in Fig. 2B. The extent of turbidity was proportional to the concentrations of GM (Fig. 2C), and at the same GM concentration (2×10^{-3} *M*), it was dependent on the amount of PA contained in liposomes (Fig. 2D).

Extent of Turbidity Induced by Aminoglycosides. The ability of GM to induce turbidity was compared with that of AMK and DKB. The highest turbidity was brought about by DKB containing five amino groups in its molecule (Fig. 3). GM and AMK, each with four amino groups, cause the same degree of turbidity. On the other hand, no increase in turbidity occurred with glucosamine, which has one amino group per molecule.

Effect of Gentamicin on a Characteristic of Liposomal Membranes. The effects of GM for bringing about the release of glucose from glucose-entrapped liposomes were examined. This release increased in proportion to the GM concentration as shown in Table I.

Turbidity of Liposomes Prepared from Rat Kidney Lipids. Changes in turbidity caused by AGs were also examined in liposomes consisting of extracted lipids from the rat kidney cortex. The turbidity increased in the presence of 1×10^{-3} *M* AGs (Fig. 4), and its degree depended on the number of amino groups in the AGs.

Electron Microscopy. As the increase in turbidity can result from liposomal aggregation or fusion, the mixture of liposomes and GM was examined by electron microscopy and compared with that of control liposomes. As shown in Fig. 5, the liposome size was increased and liposome shape was changed markedly by the addition of GM. Thus, liposomal fusion caused the increased turbidity. To confirm this possibility, the effect of neutral salt on the GM-induced turbidity increase of liposomes was investigated. As shown in Fig. 6, turbidity was unaffected by the addition of NaCl, except for the decrease in turbidity as a result of nonspecific dilution. This result confirms the notion that the increase in turbidity is attributable to liposomal fusion.

Table I. Release of Glucose from Glucose-Entrapped Liposomes by Gentamicin[a]

Gentamicin conc. (*M*)	% of Release
2×10^{-2}	33.5 ± 5.5
2×10^{-3}	13.9 ± 4.7
2×10^{-4}	0.3

[a] The lipid composition of liposome was PC:Chol:PA, 6:3:1. Values shown represent the mean of three experiments ± SD.

DISCUSSION

Aminoglycoside-induced nephrotoxicity may be related to its accumulation in the kidney cortex (1). However, clarification of the complex mechanisms involved will require additional study. The interactions between AGs and BBMs (15–17), lysosome (18), ATP (9), acidic mucopolysaccharides (6–8), or phospholipids (5,11) have been suggested to account for nephrotoxicity. During accumulation, AGs pass through the renal proximal tubule following glomerular filtration and accumulate within the proximal tubular cells (4). Thus, interactions between BBMs and AGs may be the first step toward nephrotoxicity. In the interactions of AGs with BBMs, phosphatidylinositides may be essential for the binding of AGs (5). Thus, clarification of the interactions between AGs and phospholipids is of critical importance for determining the mechanism of nephrotoxicity.

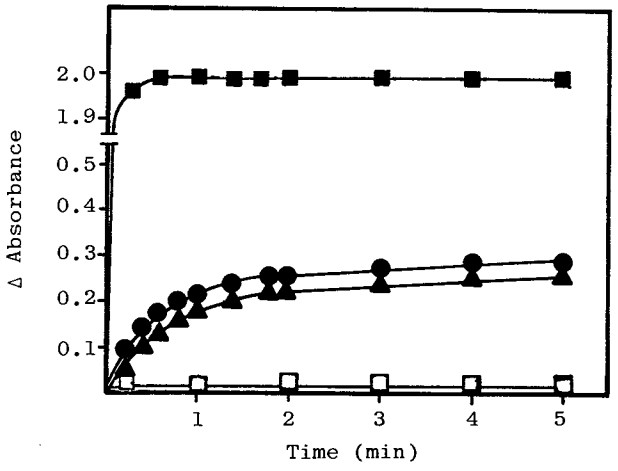

Fig. 3. Effect of three kinds of aminoglycosides on the extent of turbidity. Aminoglycoside concentrations were 2×10^{-3} *M*, and the lipid composition of liposome was phosphatidylcholine:cholesterol:phosphatidic acid, 6:3:1. (■) Gentamicin; (●) dibekacin; (△) amikacin; (□) glucosamine.

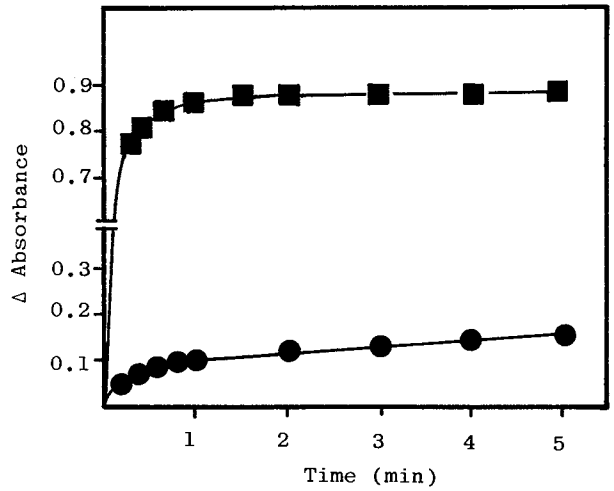

Fig. 4. Increase in turbidity of liposome suspension prepared with lipids extracted from rat kidney cortex. Aminoglycoside concentrations were 1×10^{-3} *M*. (●) Gentamicin; (■) dibekacin.

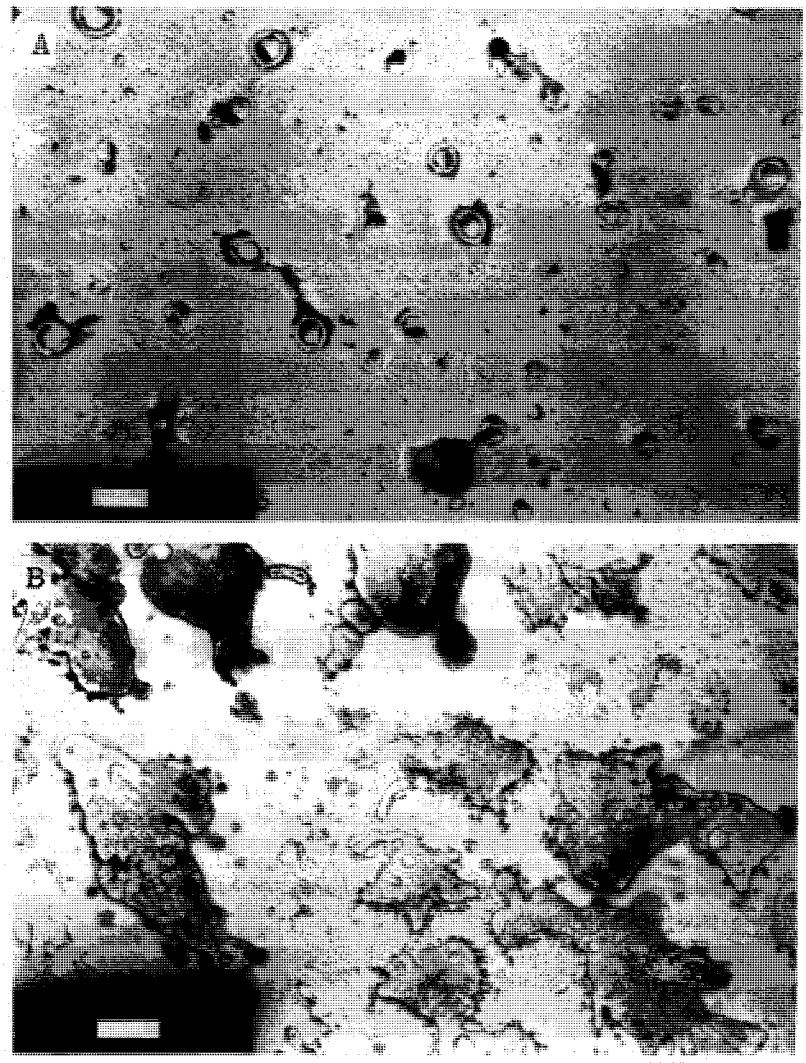

Fig. 5. Electron microscopic observation of liposomes. (A) Control liposomes; (B) mixture of liposomes and gentamicin. White bar indicates 500 nm.

Liposomes are well established as a model membrane, and it is possible to prepare liposomes containing various phospholipids. Liposomes interact with certain drugs to bring about its aggregation. The turbidity of a liposome suspension increases by aggregation, and interactions between phospholipids and drugs can thus be examined spectrophotometrically. Yung and Green (10) reported that liposomal aggregation is useful for studying interactions between phospholipids and polyamines. Thus, in the present study, the interaction between phospholipids and AGs was studied.

In Fig. 1, increased turbidity by the addition of GM was observed when acidic phospholipids, especially PI-P_2, were used as liposomal components, in agreement with the results of Sastrasinh *et al.* (5). These authors demonstrated a decrease in binding to BBMs when the phospholipids of BBM were hydrolysed by phospholipase C, and an increase in the PI content of BBM vesicles caused enhanced GM binding. Thus, acidic phospholipids, especially PI-P_2, are capable of functioning as AGs receptors.

Interactions of AGs with mucopolysaccharides (6–8) and ATP (9) have been documented, and AGs bind to these substances through ionic interactions. Because the turbidity of liposomes was decreased at alkaline pH (Fig. 2A) and the pK_a of GM is 8.2, the ionization of GM may be requisite for interactions between AGs and phospholipids to bring about the increase in turbidity. The extent of turbidity was also found to be dependent on ionic strength, GM concentration, or PA concentration in liposomes (Figs. 2B–D). Consequently, the increase in turbidity may occur by ionic interaction between an amino group(s) of GM and a phosphate group(s) of acidic phospholipids.

The effect of the number of amino groups of AGs on the extent of turbidity was investigated using the PC:Chol:PA (6:3:1) liposomes and those prepared from lipids of rat kidney cortex. GM, AMK, and DKB possess four, four, and five amino groups, respectively. The extent of turbidity by these AGs was dependent on the number of amino groups (Fig. 3). On the other hand, no change in turbidity could be observed by the addition of glucosamine, which has only one amino group in the molecule. Thus, for increasing the turbidity, several amino groups per molecule appear to be essential. The ototoxicity of AGs has been shown to depend on

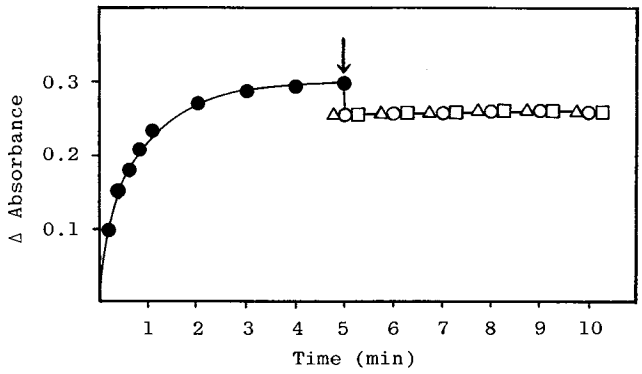

Fig. 6. Effect of NaCl on the extent of turbidity induced by gentamicin. Arrow indicates the addition of NaCl solution into a liposome suspension whose turbidity was increased by gentamicin. (○) Tris–HCl buffer (pH 7.4); (△) final NaCl concentration was 0.3 *M*; (□) final NaCl concentration was 0.45 *M*.

the number of amino groups (11), while nephrotoxicity was independent of this parameter. This relationship is evident from the fact that the degree of nephrotoxicity (19,20) or affinity toward BBMs of AGs is of the order GM > DKB > AMK (21).

Recently, Yung and Green (11) reported liposomal aggregation to occur with kanamycin, the degree of which was dependent on the phospholipid component of liposomes, the order being PI-P_2 > PI > PS. Fluorescence of liposome-entrapped 1-anilino-8-naphthalene sulfonic acid (ANS) was also enhanced by kanamycin. In our previous paper, lysosomal membrane fluidity was increased by AGs and *N*-acetyl-β-D-glucosaminidase, and acid phosphatase was released from the lysosomes (18). Therefore the effect of GM on the liposomal membrane was investigated by measuring the release of glucose from glucose-entrapped liposomes. As shown in Table I, the release of glucose increased in proportion to the GM concentration. This result suggests an increased permeability of the liposomal membrane because of interactions between phospholipids and GM.

Wiessner *et al.* (22) reported that the interaction between small unilamellar liposomes and insulin caused liposomal fusion and aggregation. To clarify the cause of increased turbidity of liposomes by the addition of GM, an electron microscope was used, and the dissociation effect of NaCl on aggregated liposome was studied (Figs. 5 and 6). If the increase in turbidity were caused by aggregation, the turbidity should decrease with the addition of NaCl. The extent of turbidity, however, was unchanged by the addition of NaCl (Fig. 6), suggesting liposomal fusion to occur. Fusion of liposomes would require perturbation of the liposomal bilayers (22). Yung and Green (11) reported that the fluorescence of ANS was enhanced by kanamycin in their liposomal aggregation experiment. However, there was no change in the degree of polarization when 1,6-diphenyl-1,3,5-hexatriene was used as the probe (11). Consequently, further work will be needed to clarify the mechanism of the fusion induced by GM.

During the course of the present study, Yung and Green (10–11) and Au *et al.* (23) reported the occurrence of interactions between AGs and phospholipids, indicating AGs to have a high affinity toward PI-P_2. The role of PI-P_2 has recently come to attract great interest since PI-P_2 mediates the cell response to various physiological stimuli (24). Thus, clarification of the interactions between AGs and phospholipids using liposomes may facilitate an understanding of aminoglycoside toxicity. Liposomal aggregation was dependent on the number of amino groups in the AGs and the concentration of acidic phospholipids in the liposome. These results parallel the ototoxicity of AGs (11,23), while the order of AG nephrotoxicity does not correlate with the number of amino groups of AGs (21). Thus, nephrotoxicity of AGs may be dependent on not only acidic phospholipids but also other substances such as glycoprotein and glycolipids at BBMs.

REFERENCES

1. G. J. Kaloyanides and E. Pastoriza-Munoz. *Kidney Int.* 18:571–582 (1980).
2. F. J. Silverblatt and C. Kuehn. *Kidney Int.* 15:335–345 (1979).
3. R. P. Wedeen, V. Batuman, C. Cheeks, E. Marquet, and H. Sobel. *Lab. Invest.* 48:213–223 (1983).
4. Y. Aramaki, M. Takahashi, A. Inaba, and S. Tsuchiya. *Biochim. Biophys. Acta* 862:111–118 (1986).
5. M. Sastrashinh, T. C. Knauss, J. M. Weinberg, and H. D. Humes. *J. Pharmacol. Exp. Ther.* 222:350–358 (1982).
6. T. Deguchi, A. Ishii, and M. Tanaka. *J. Antibiot.* 31:150–155 (1978).
7. Y. Aramaki, J. Niibuchi, S. Tsuchiya, and J. Hosoda. *Chem. Pharm. Bull.* 35:320–325 (1987).
8. Y. Aramaki, A. Inaba, J. Hosoda, and S. Tsuchiya. *Chem. Pharm. Bull.* 35:756–760 (1987).
9. A. Inaba, Y. Aramaki, M. Takahashi, and S. Tsuchiya. *Life Sci.* 39:1345–1351 (1986).
10. M. W. Yung and C. Green. *Biochem. Pharmacol.* 35:4037–4041 (1986).
11. M. W. Yung and C. Green. *J. Antibiot.* 40:862–867 (1987).
12. P. S. Chen, T. Y. Toribara, and H. Warner. *Anal. Chem.* 28:1756–1758 (1956).
13. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rovers, and F. Smith. *Anal. Chem.* 28:350–356 (1956).
14. J. Folch, M. Lees, and G. H. Sloane-Stanley. *J. Biol. Chem.* 226:497–509 (1957).
15. J. J. Lipsky, L. Cheng, B. Sacktor, and P. S. Lietman. *J. Pharmacol. Exp. Ther.* 215:390–393 (1980).
16. Y. Ishikawa, K. Inui, and R. Hori. *J. Pharmacobio-Dyn.* 8:931–941 (1985).
17. P. D. Williams, H. Hottendorf, and D. B. Bennett. *J. Pharmacol. Exp. Ther.* 237:919–925 (1986).
18. A. Inaba, Y. Aramaki, K. Takeda, and S. Tsuchiya. *Chem. Pharm. Bull.* 32:3173–3178 (1984).
19. G. Viotte, B. Olier, J. P. Morin, J. Hemet, and J. P. Fillastre. *Drugs Exp. Clin. Res.* 9:735–747 (1983).
20. N. Briom, J. Barge, I. Godefroy, F. Dromer, C. Dubois, A. Contrepois, and C. Carbon. *Antimicrob. Agents Chemother.* 25:168–172 (1984).
21. M. Takahashi, Y. Aramaki, A. Inaba, and S. Tsuchiya. *Biochim. Biophys. Acta* 903:31–36 (1987).
22. J. H. Wiessner, H. Mar, D. G. Baskin, and K. J. Hwag. *J. Pharm. Sci.* 75:259–263 (1986).
23. S. Au, N. D. Wener, and J. Schacht. *Biochim. Biophys. Acta* 902:80–86 (1987).
24. M. Berridge. *Biochem. J.* 220:345–360 (1984).