International Journal of Pharmaceutics, 30 (1986) 181–187
Elsevier

IJP 01008

Binding of antibiotics to rat intestinal mucin

Jun-Ji Niibuchi, Yukihiko Aramaki and Seishi Tsuchiya

Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03 (Japan)

(Received July 30th, 1985)
(Modified version received December 5th, 1985)
(Accepted December 26th, 1985)

Key words: intestinal absorption – mucin – β-lactams – aminoglycosides

Summary

The interactions of 13 β-lactam and 3 aminoglycoside antibiotics to rat intestinal mucin were investigated. Intestinal mucus was separated into soluble (S-mucin) and insoluble mucin (I-mucin) fractions. All antibiotics tested were bound to both mucins to various extents, especially cephaloridine (CER) and gentamicin (GM) were significantly bound to I-mucin, but a little to S-mucin. I-mucin had one class of many binding sites for CER and GM, and the binding was dependent on pH and the ionic strength. After the intestinal mucus was washed off, the in vitro absorption of CER and GM was significantly increased, but that of cephradine was not influenced by the surfactant treatment. The binding of GM to neuraminidase-treated I-mucin was markedly decreased, but CER binding could not be affected. The binding sites of GM on I-mucin are considered to be N-acetylneuraminic acid residues of sugar moiety, and those of CER are considered to be protein moiety of the glycoprotein.

Introduction

Mucus adheres to the luminar surface of gastrointestinal tracts as gel or high viscous solution. The physiological role of mucus is considered to protect the underlying delicate epithelial cells from acid and enzyme attack, mechanical damage, and pathogenic organisms (Allen et al., 1984). The mucus acts as a barrier in the process of drug absorption from the alimentary tracts. When a less irritating surface active agent washed off the barrier, the rectal absorption of a drug was significantly increased (Tsuchiya et al., 1983). On the other hand, intestinal mucin makes a nonabsorbable complex with streptomycin (Gibaldi, 1971), and quarternary ammonium compounds (Levine et al., 1955; Levine and Pelikan, 1961). Furthermore, 3′,4′-dideoxykanamycin B binds to bovine submaxillary mucin through ionic interaction, and forms insoluble complexes (Aramaki et al., 1985). Many reports concerning the interaction between mucin and drugs are available, but there are little information about the details of the interaction.

In this study, we investigated the binding of β-lactam or aminoglycoside antibiotics and rat intestinal mucin, and the special emphasis was placed on the binding sites of this glycoprotein.

Materials and Methods

Materials

Cephaloridine (CER), cephalotin (CET), cephalexin (CEX), cephazolin (CEZ), ceftizoxime

Correspondence: Y. Aramaki, Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan.

(CZX), cephradine (CED), cyclacillin (ACPC), gentamicin (GM), dibecacin (DKB) and amikacin (AMK) were supplied. Benzylpenicillin (PCG), carbenicillin (CBPC), ampicillin (ABPC), methicillin (DMPPC), amoxicillin (AMPC), cephalosporin-C, N-acetylneuraminic acid (NANA) and neuraminidase (type VI) were purchased from Sigma (U.S.A.). Mixed glycosidase from *Turbo cornutus* was obtained from Seikagaku Kogyo Co (Japan), and sodium deoxycholate (DOC) was from Difco (U.S.A.). The other reagents were purchased from Wako Pure Chemicals (Japan).

Animals

Male Wistar rats weighing 200–300 g were starved for 24 h before use.

Preparation of mucin

Isolated small intestine was everted and slightly rinsed with chilled saline. The mucosal surface was gently scraped off with a glass slide. A five-fold volume of water was added and stirred at 4°C for 2 h, and then, the suspension was dialyzed against water at 4°C for 48 h. Following the centrifugation at 22,000 × *g* for 20 min, both of the supernatant (S-mucin) and the precipitate (I-mucin) were obtained and lyophilized.

Binding experiment

(1) Equilibrium dialysis. I-mucin or S-mucin was suspended or dissolved with 10 mM phosphate buffer (pH 6.5) at a concentration of 1 mg/ml. One ml of the buffer solution of an antibiotic (100 μg/ml) was added to 1 ml of mucin and dialyzed against the phosphate buffer at 4°C for 24 h. The non-specific binding of antibiotics to the dialysing equipment was negligible.

(2) Equilibrium gel filtration. I-mucin (25 mg) was solubilized with 2 ml of 0.2% sodium deoxycholate (DOC) and centrifuged. The supernatant was thoroughly dialyzed and lyophilized. The phosphate buffer solution of re-lyophilizate was incubated with equivolume of CER solution (50 μg/ml) at 37°C for 60 min. This mixture (1.0 ml) was applied to Sephadex G-25 column (1.3 × 55 cm) pre-equilibrated with the buffer containing CER (25 μg/ml), and 1.35 ml per tube was collected. CER and protein concentrations were determined, and the amount of CER bound to the solubilized fraction of I-mucin was estimated from the area of trough.

Enzyme treatment of I-mucin

I-mucin (16 mg) was suspended in 8 ml of the phosphate buffer containing 1.6 mg neuraminidase, and was incubated at 37°C for 8 h. Then, mixed glycosidase (8 mg) was added and the solution was incubated again for 4 h. The solution was dialyzed against water at 4°C for 48 h to remove the saccharides liberated. Neuraminidase and mixed glycosidase were not removed from the solution, because CER and GM had no affinity to both enzymes (data not shown).

Everted sac experiment

Rats were anesthetized with ethyl carbamate (10 mg/100 g body weight) by intraperitoneal injection. After median incision, the intestine was cannulated and the bile duct was ligated. During the first 30 min, the intestine was perfused with the modified Ringer's solution (Schultz et al., 1966) at a rate of 3.0 ml/min (37°C), and the modified Ringer's solution containing 0.02% (w/v) DOC was perfused for 30–60 min. Then, the modified Ringer's solution was perfused again up to 90 min. As a control experiment, the intestine was perfused with the modified Ringer's solution at 37°C for 90 min. After that, the small intestine was removed at a length of 20 cm from 10 cm below pyrous and everted. The everted intestine was applied to Wiseman's apparatus, which was maintained at 37°C. The mucosal side solution was gassed with 5% CO_2 in oxygen. The solution (2.0 ml) of serosal side was withdrawn at every 10 min up to 60 min, and just after sampling, 2.0 ml of Ringer's solution was added. The glucose transport was estimated as the indication of viability (Wilson, 1962).

Analytical methods

In the equilibrium dialysis and equilibrium gel filtration experiments, the concentration of β-lactams was determined by their absorption maximum wavelength. In the everted sac experiment, the concentration of CER and CED was determined by HPLC method using a Licrosorb

RP-18 column (5 μm, 250 × 4 mm, Merck) and the Uniflow-211 (Japan Spectroscopic Co., Tokyo) equipped with an Uvidec 100-II (ultraviolet detector). The mobile phase was a mixture of acetonitrile–6 mM phosphate buffer (pH 7.5) containing 8 mM tetra-*n*-butylammonium chloride (22 : 77). The flow rate was maintained at 1.4 ml/min. Concentrations of CER and CED were estimated from the peak area of the chromatogram, and CET and CEX were used as the internal standards, respectively. Aminoglycosides, GM, DKB and AMK were determined by the bioassay method with *Bacillus subtilis* ATCC 6633 as test organism. The protein concentration was determined by the method of Lowry et al. (1951). The quantitation of neutral sugar was made by the method of Dubois et al. (1956) with glucose as the standard. The NANA concentration was assayed according to the method of Helen and Edward (1964).

Results and Discussion

Binding of antibiotics of mucin

All of the antibiotics used in this experiment had an affinity to I-mucin and S-mucin after investigation by the equilibrium dialysis method (Table 1). CER especially had a high affinity to I-mucin, and 53 μg of CER bound to 1 mg of I-mucin. But the other β-lactams had a low affinity to I-mucin or S-mucin, and their binding fell in about the range from 10 to 20 μg/mg. In the case of aminoglycosides, the amount from 20 to 30 μg of GM, DKB, and AMK interacted with 1 mg of I-mucin, and these also had an affinity to the S-mucin fraction, but were only half of that to I-mucin fraction. It is reported that aminoglycoside antibiotics bind to acidic mucopolysaccharide, chondroitin sulfate A, and the binding of GM is greater than that of DKB (Deguchi et al. 1978). During the preparation of I-mucin and S-mucin in present experiment, soluble intestinal mucin or acidic mucopolysaccharides would be contained in S-mucin fraction. Thus, the affinities of aminoglycosides obtained in this experiment of S-mucin were consistent with the results obtained by Deguchi et al. (1978).

TABLE 1

BINDING OF ANTIBIOTICS TO I-MUCIN OR S-MUCIN

Antibiotics	Binding of antibiotics (μg/mg mucin) *	
	I-mucin	S-mucin
β-lactams		
Benzylpenicillin (PCG)	22.6 ± 5.2	
Carbenicillin (CBPC)	16.9 ± 0.1	
Methicillin (DMPPC)	10.1 ± 5.6	
Ampicillin (ABPC)	17.5 ± 5.6	
Amoxillin (AMPC)	12.3 ± 1.0	1.1 ± 2.0
Cyclacillin (ACPC)	13.2 ± 0.1	16.1 ± 4.6
Cephaloridin (CER)	53.0 ± 5.6	9.6 ± 1.0
Cepholotin (CET)	16.7 ± 2.2	
Cephazolin (CEZ)	13.0 ± 4.3	
Ceftizoxime (CZX)	11.7 ± 1.6	
Cephalosporin-C	13.3 ± 2.2	
Cephalexin (CEX)	9.5 ± 0.4	5.9 ± 1.9
Cephradin (CED)	9.0 ± 0.6	6.0 ± 2.9
Aminoglycosides		
Gentamicin (GM)	30.9 ± 1.5	22.3 ± 1.6
amikacin (AMK)	21.1 ± 7.1	9.0 ± 7.6
Dibecacin (DKB)	27.6 ± 10.1	11.3 ± 2.1

* Mean ± S.D. (n = 3).

The binding of CER or GM on I-mucin (1 mg) was saturated at 250 and 150 μg of CER and GM, respectively (Fig. 1). The Scatchard plot of each antibiotic shows a linear regression line over the concentration range tested, and the K values of 6.39×10^4 for CER and 3.58×10^4 M^{-1} for GM were obtained. I-mucin had a slightly greater number of binding sites for CER.

Fig. 2 shows the effect of pH on the binding of CER or GM to I-mucin. Both of the drugs show almost the same pH profile, and the binding was drastically decreased on acidic and alkaline pH ranges. CER has a pK_a of 1.67 (Yamana and Tsuji, 1976) and is stable at the examined pH range. As shown in Table 2, the protein moiety of I-mucin may participate in the binding of CER, because the conformational change of the protein moiety may cause a very low binding of CER to I-mucin under these unphysiological conditions. The binding of GM to I-mucin was markedly reduced at pH 8.5 compared with that at pH 5.5. The pK_a of GM is 8.2 (Horioka and Fukumuro, 1982), so the GM binding to I-mucin may be

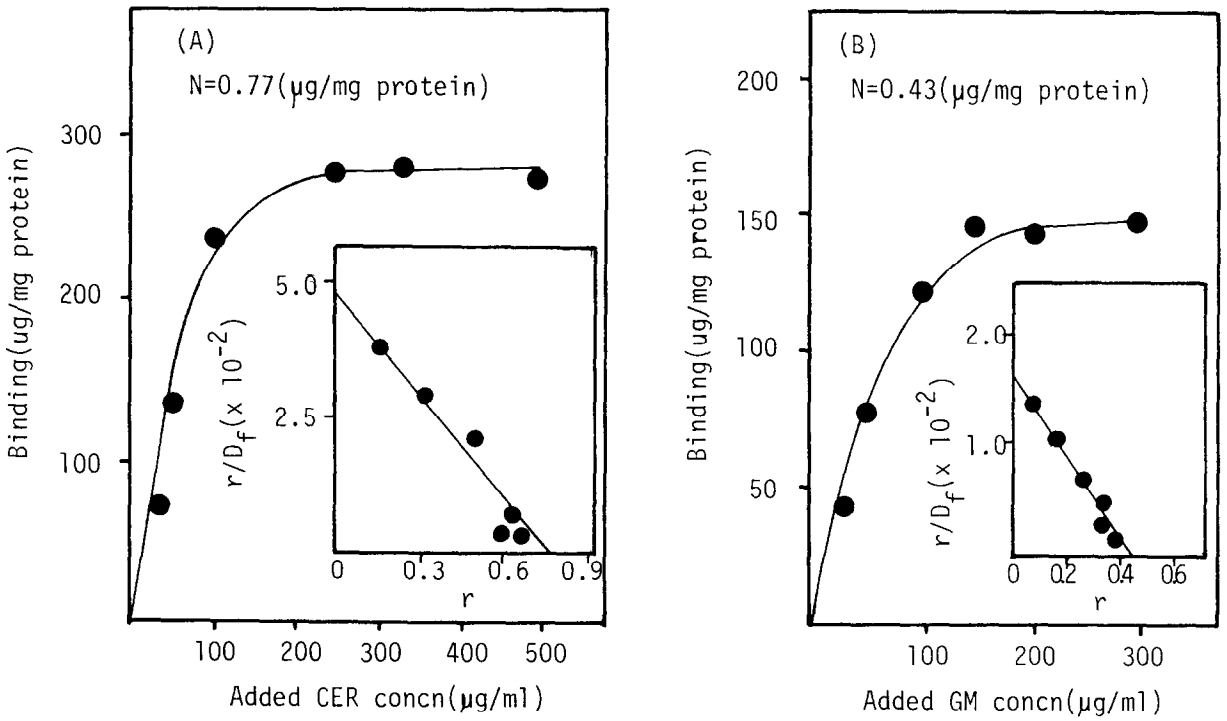

Fig. 1. Cephaloridin (A) and gentamicin (B) binding to I-mucin as function of concentration. Insets indicate the Scatchard plots of cephaloridin (A) and gentamicin (B) binding to I-mucin. Regression lines were obtained by the method of least-squares. N = Binding sites (μg/mg of protein)

dependent on the ionization of GM. Fig. 3 shows the effect of the NaCl concentration in the binding of CER of GM to I-mucin. The bindings of both antibiotics were decreased with increasing NaCl concentration. These results suggest that the ionic interaction may participate in the binding of both drugs to I-mucin.

The binding of CER to I-mucin was demonstrated by the equilibrium gel filtration method. As described in Methods, solubilized I-mucin was

TABLE 2

EFFECT OF ENZYME TREATMENTS ON THE BINDING OF GM AND CER TO I-MUCIN

Enzyme	Reaction time (h)	Components of I-mucin (% of control			Bound antibiotic (% of control)	
		Protein	neutral sugar	NANA	GM	CER
		(μg/mg of I-mucin)			(μg/mg of I-mucin)	
Control	8	169.3(100.0)	24.9(100.0)	20.6(100.0)	32.6(100.0)	23.8(100.0)
Neuraminidase	8	172.3(101.8)	19.9 (80.4)	8.1 (39.6) **	10.0 (30.8) **	21.2(90.0)
Neuraminidase + Mixed glycosidase	8 + 4	165.4 (97.7)	16.0 (63.9) *	6.4 (31.3) **	15.6 (48.1) **	25.2(105.9)

Mean value of three experiments was represented.
* $P < 0.05$, ** $P < 0.01$.

applied to a pre-equilibrated Sephadex column. Fig. 4 shows the elution profiles of CER and protein, and 36.4 μg of CER was bound to 1 mg of the solubilized fraction of I-mucin. This was about 70% of the value obtained from the equilibrium dialysis method. This result suggests that most of the binding substance in I-mucin was solubilized by DOC but not completely.

Effects of mucin on drug absorption in vitro

Vasseur et al. (1978, 1979) and Tsuchiya et al. (1983) have reported that the surface-active agents wash off the mucin from the intestinal or rectal surface and promote the drug absorption from the treated region. The effects of DOC perfusion on the CER or GM absorption from the intestine were studied by the everted sac method (Fig. 5A and B). The absorption of CER or GM from DOC treated intestine was increased significantly, and the saturation was observed.

On the other hand, the CED transfer across the everted intestine was sufficient, and the same degree of absorption was obtained from the DOC-treated or the non-treated intestine (Fig. 5C). It is well known that CER and GM are hardly absorbed, but CED is easily absorbed from the intestine (Tsuji et al., 1981).

These results suggest that the mucin interacts with certain kinds of drugs, and obstructs their absorption from the intestine.

Binding site of I-mucin

The binding sites of CER and GM on I-mucin were investigated by the treatment of neuramini-

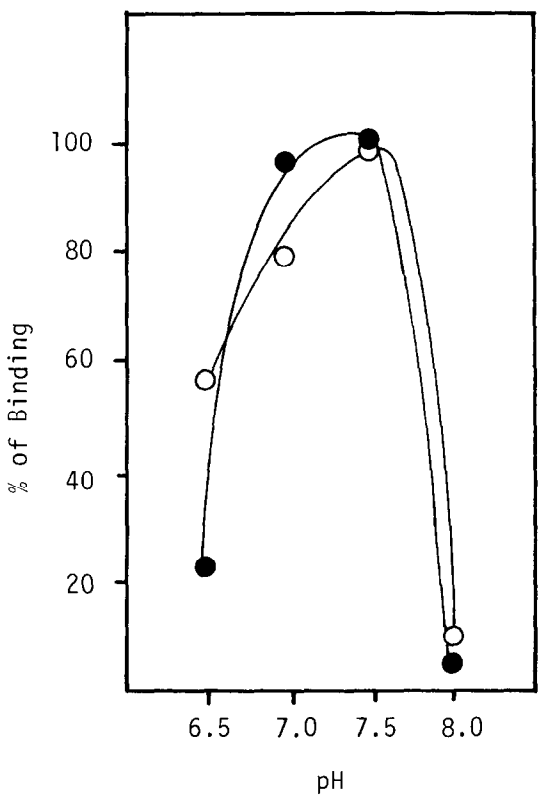

Fig. 2. Effect of pH on the cephaloridin and gentamicin binding to I-mucin. Each pH of 10 mM phosphate buffer was used in this experiment. Binding of both antibiotics at pH 7.5 was defined as 100%, and compared with the binding at various pH. ●———●, cephaloridin; ○———○, gentamicin.

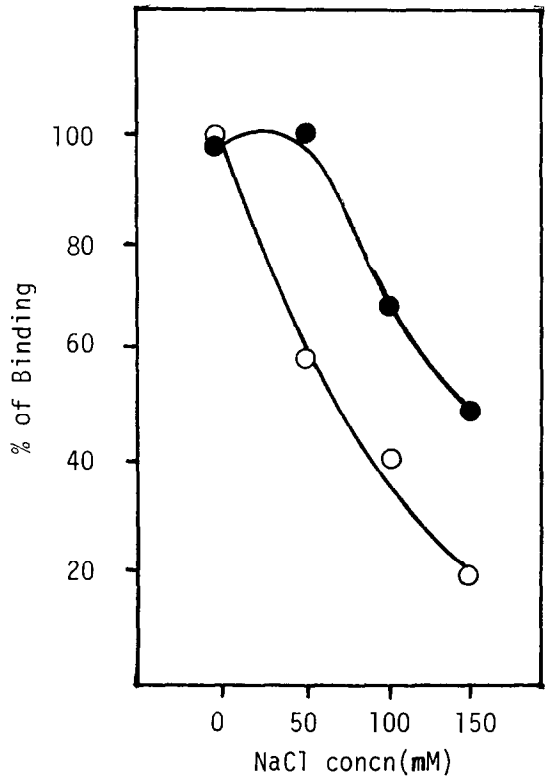

Fig. 3. Effect of NaCl concentration on the binding of cephaloridin and gentamicin to I-mucin. Phosphate buffer (10 mM, pH 6.5) containing various concentrations of NaCl was used. Binding of CER at 50 mM of NaCl was defined as 100%, and in the case of GM, binding at 0 mM NaCl was defined as 100%. ●———●, cephaloridin; ○———○, gentamicin.

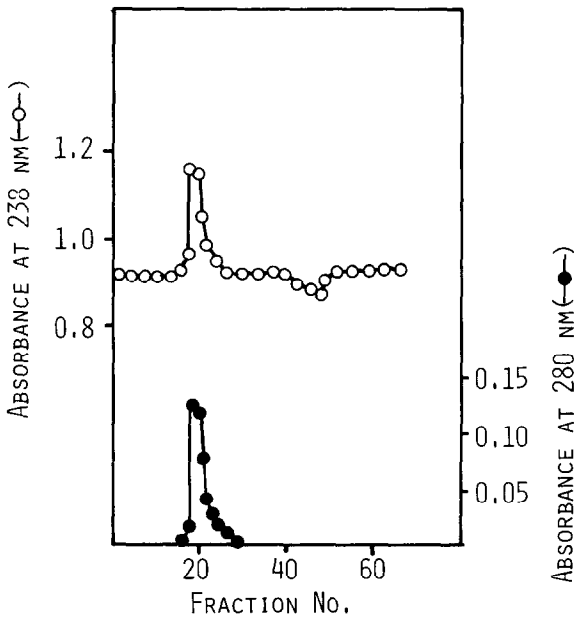

Fig. 4. Elution profile of the binding of cephaloridin to solubilized I-mucin fraction. Sephadex G-25 was used in this experiment. Cephaloridin and protein were determined by the absorption at 238 nm (○———○) and 280 nm (●———●), respectively.

dase and/or mixed glycosidase. As shown in Table 2, about 60% of NANA was liberated from I-mucin by neuraminidase treatment, and the binding of GM was decreased to about 30% of control. When I-mucin was incubated with mixed glycosidase after treatment of neuraminidase, 36% of neutral sugar and 69% of NANA were liberated. When the neutral sugar content of I-mucin was decreased, the binding of GM to the glycoproteins was not decreased. These results suggest that the terminal NANA of sugar chain participates in the binding of GM to I-mucin. It has been reported that the aminoglycosides interact with submaxillary mucin (Aramaki et al., 1985), phospholipids (Sastrasinh et al., 1982) and acidic mucopolysaccharides through the ionic interaction (Deguchi et al., 1978). Taking the above facts and the results of Figs. 2 and 3 into account, it is considered that the ionic interaction take part in the binding of GM to I-mucin.

On the other hand, no difference in CER binding was obserbed between I-mucin and enzyme-treated I-mucin. As the sugar moiety of I-mucin had no connection with CER, the protein moiety must have strongly participated in the CER binding.

Recently, Nakashima et al. (1984), Iseki et al. (1984) and Kimura et al. (1982) also reported the existence of the carrier-mediated system in the absorption of these β-lactams. Miyazaki et al. (1982a and b) reported that β-lactams, such as CED and CEX, strongly bound to the soluble

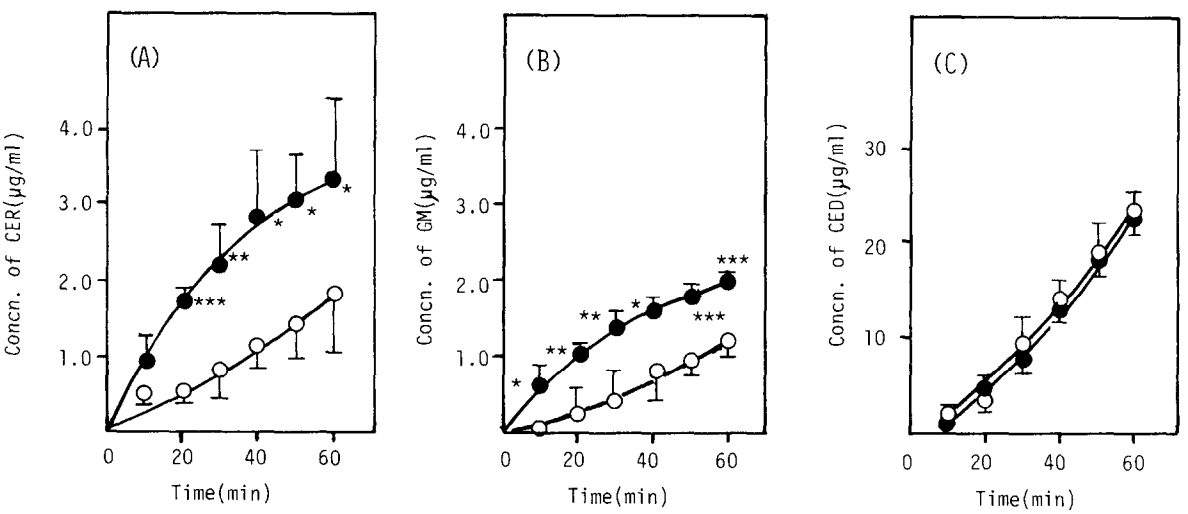

Fig. 5. Cephaloridin (A), gentamicin (B) and cephradin (C) across everted small intestine. Data are expressed as mean ± S.D. (n = 3). Statistically significant differences (P) by t-test are indicated as follows; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ●———●, intestine was perfused with 0.02% of DOC; ○———○, intestine was perfused without DOC.

components obtained from rat intestinal mucosa by ultracentrifugation. But the binding substance(s) for CER or GM in I- and S-mucin may be presented in mucus of intestine, because in DOC perfusion experiment, absorptions of CER and GM by isolated intestine were increased by the washing off the mucus (Fig. 5). In this experiment, I- and S-mucin had a very low affinity to CED and CEX (Table 1). Thus, both of the mucin preparations may differ from the soluble protein, obtained by Miyazaki et al. (1982a and b) and binds to CED or CEX.

From above findings, it may be concluded that GM, which are poorly absorbed from gastrointestinal tracts, bind to the sugar moiety, especially N-acetylneuraminic acid residues, of mucin. In the case of CER, its low absorbability from intestine was contributed to the binding of CER to the protein moiety of mucin, but the cause of low absorbability of CET of CEZ is now investigated.

References

Allen, A., Hutton, D.A., Person, J.P. and Sellers, L.A., Mucus glycoprotein structure, gel formation and gastrointestinal mucus function. In Mucus and Mucosa (Ciba Foundation Symposium 109), Pitman Press, London, 1984, pp. 137–156.

Aramaki, Y., Niibuchi, J., Tsuchiya, S. and Hosoda, J., to be published.

Deguchi, T., Ishii, A. and Tanaka. M., Binding of aminoglycoside antibiotics to acidic mucopolysaccharides J. Antibiot., 31 (1978) 150–155.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rovers, P.A. and Smith, F., Colorimetric method for determination of sugars and related substances. Anal. Chem., 28 (1956) 350–356.

Gibaldi, M., Introduction of Biopharmaceutics, Lee and Febiger, Philadelphia, 1971, p. 16.

Helen, H.H. and Edward, R., Fluorometric assay of sialic acid in brain, J. Biol. Chem., 239 (1973) 3215–3220.

Horioka, M. and Fukumuro, K., Guide to Newly Developed Drugs, 2nd Edn., Yakugyo-Jiho, 1982, p. 809.

Iseki, K., Iemura, A., Sato, H., Sunada, K., Miyazaki, K. and Arita, T., Intestinal absorption of several β-lactam antibiotics. V. Effect of amino β-lactam analogous and dipeptides on the absorption of amino β-lactam antibiotics. J. Pharm. Dyn., 7 (1984) 768–775.

Kimura, T., Yamamoto, T., Mizuno, M., Suga, Y., Kitade, S. and Sezaki, H., Characterization of aminocephalosporin transport across rat small intestine. J. Pharm. Dyn., 6 (1983) 246–253.

Levine, R.M., Blair, M.R. and Clark, B.B., Factors influencing the intestinal absorption of certain monoquaternary anticholinergic compounds with special reference to benzometamine. J. Pharm. Exp. Ther., 114 (1955) 78–86.

Levine, H.R. and Pelikan, E.W., The influence of experimental procedures and dose on the intestinal absorption of an oniumcompound, benzomethamine. J. Pharm. Exp. Ther., 131 (1961) 319–327.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193 (1951) 265–275.

Miyazaki, K., Ohtani, K., Umeniwa, K. and Arita, T., Intestinal absorption of several β-lactam antibiotics. III. Competitive inhibition behavior among zwitterionic β-lactam antibiotics in the rat intestinal absorption. J. Pharm. Dyn., 5 (1982a) 555–563.

Miyazaki, K., Iseki, K. and Arita, T., Intestinal absorption of several β-lactam antibiotics. IV. Binding to the various components in the intestinal mucosa of rat and role in absorption process. J. Pharm. Dyn., 5 (1982b) 593–602.

Nakashima, E., Tsuji, A., Kagatani, S and Yamana, T., Intestinal absorption mechanism of amino-β-lactam antibiotics. III. Kinetics of carrier-mediated transport across the rat small intestine in situ. J. Pharm. Dyn., 7 (1984) 452–464.

Sastrasinh, M., Knauss, T.C., Weinberg, J.M. and Humes, H.D., Identification of the aminoglycoside binding site in rat renal brush border membranes. J. Pharmacol. Exp. Ther., 222 (1982) 350–358.

Schultz, S.G., Fuisz, R.E. and Curran, P.F., Amino acid and sugar transport in rabbit ileum. I. Gen. Physiol., 49 (1966) 849–866.

Tsuchiya, S., Aramaki, Y., Ozawa, S. and Matsumaru, H., Effect of polyoxyethylene cetyl ether on the absorption of 3', 4'-dideoxykanamycin B from rat rectum. Int. J. Pharm., 14 (1983) 279–290.

Tsuji, A., Nakashima, E., Izumi, K. and Yamana, T., Intestinal absorption mechanism of amophoteric β-lactam antibiotics I: Comparative absorption and evidence for saturable transport of amino-β-lactam antibiotics by in situ rat small intestine. J. Pharm. Sci., 70 (1981) 768–777.

Vasseur, M., Ferand G. and Pousse, A., Rat intestinal brush border enzymes release by deoxycholate in vivo. Pflügers Arch. Ges. Physiol., 373 (1978) 133–138.

Vasseur, M., Ferand, G. and Pousse, A., Do low dose of deoxycholate modify the release of rat jejunal brush border hydrolases? Pflügers Arch. Ges. Physiol., 379 (1979) 297–299.

Wilson, T.H., Intestinal absorption, W.B. Saunders, Philadelphia, 1962, pp. 69–109.

Yamana, T. and Tsuji, A., Comparative stability of cepharosporins in aqueous solution: Kinetics and mechanisms of degradation. J. Pharm. Sci., 65 (1976) 1563–1574.