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Original Article

Effect of Sulfated Colominic Acid on Enteric Virus (Rotavirus, Poliovirus and Coxsackievirus) Infections In Vitro

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SUMMARY: Sulfated colominic acid exhibited suppressive effects on SA11 (simian rotavirus)- and MO (human rotavirus)-infections, but not on Wa (human rotavirus)-, Sabin 1 (poliovirus 1)-, and Nancy (coxsackie B3 virus)-infections, in vitro. The infection of SA11 was found to be inhibited by mixed treatment and early posttreatment with sulfated colominic acid, but not by pretreatment, by plaque assay and multiple growth assay. The results were confirmed by the infectivity titer, RNA polyacrylamide gel electrophoresis, and electron microscopic analysis. The mechanism of the suppressive effect was suggested to be adsorption inhibition at an early stage of the infection.

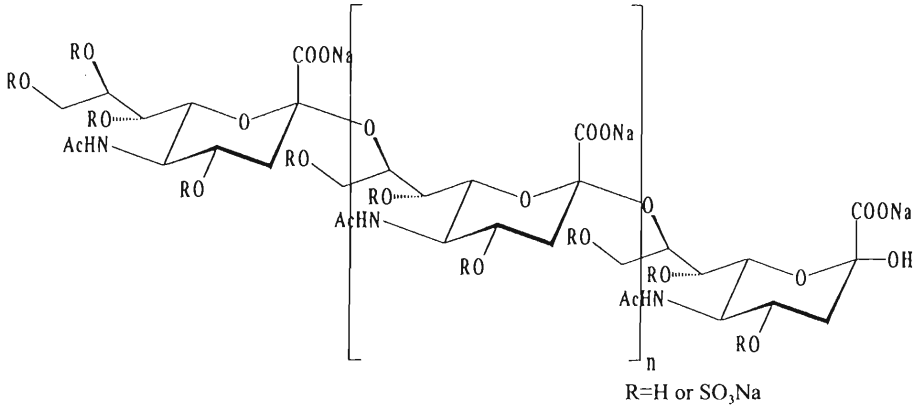
INTRODUCTION

Rotaviruses are recognized as the major etiologic agents of diarrhea in infants and young children. In developing countries, about 800,000 children die annually of rotavirus infection (1,2). To reduce the morbidity and mortality associated with rotavirus infection, intensive efforts made. Rotavirus live vaccine is one of the preventive methods, though a more effective vaccine with fewer side effects still needs to be developed (3). Although various formulations of oral rehydration salt solutions have proved useful, they are insufficient for children with severe and persistent diarrhea, especially for those with rotavirus diarrhea who have complications such as viral encephalitis. An effective anti-rotavirus drug might be beneficial for treatment, especially in immunocompromised patients and malnourished children who are not recommended to receive live rotavirus vaccine or who have persistent diarrhea caused by rotavirus.

Colominic acid is a homopolymer of *N*-acetylneuraminic acid containing α-2,8 ketosidic linkages between the sugar moieties (4). Recently, it has been reported that sulfated colominic acid exhibits anti-HIV activity (5), and suppresses PrP^{Sc} (scrapie prion protein) and HIV-1 gp120-induced neuronal cell death (6). Colominic acid is one of the sugar chains, and reacts as sialic acid. Such sugar chains are present in blood proteins and mammalian breast milk. They are recognized as virus receptors on the cell surface (6). In this study, we examined the suppressive effects of sulfated colominic acid on enteric viruses including rotavirus, poliovirus, and coxsackie B3 virus.

MATERIALS AND METHODS

Chemicals: Sulfated colominic acid was obtained from Marukin Shoyu Co. Ltd. (Kyoto). The sodium salt with an average molecular mass of 24,000 ($n \fallingdotseq 50$) was used (Fig. 1).



Average molecular mass:24,000 ($n \fallingdotseq 50$)

Fig. 1. Structure of sulfated colominic acid.

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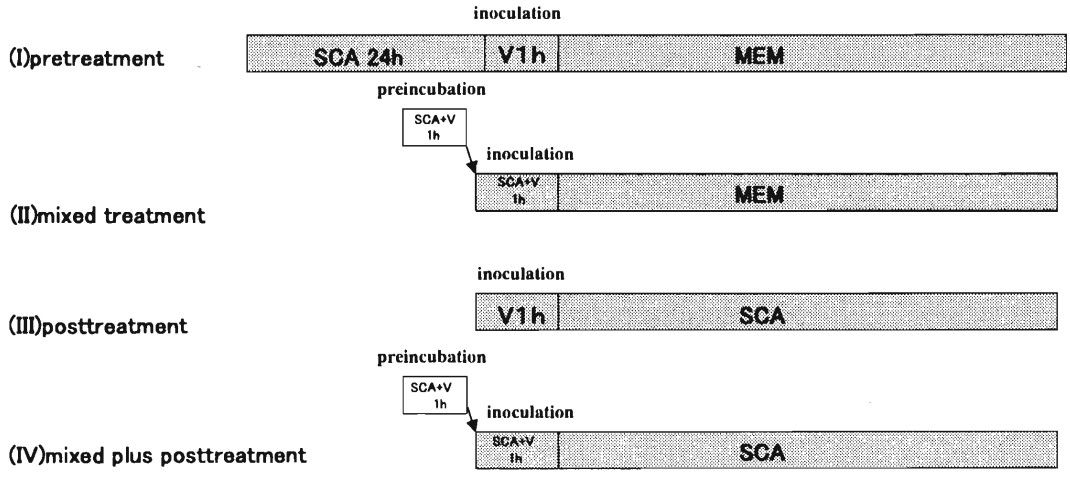

Fig. 2. Schema of four treatments. SCA, V, and MEM represent sulfated colominic acid, virus, and Eagle's minimum essential medium, respectively. ▭ indicates treatment without cells, ▨ indicates treatment on the cells.

The compound was dissolved in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS(-)) containing 10% dimethyl sulfoxide at stock concentrations of 10 mg/ml and stored at –20°C until use.

Sulfated colominic acid, diluted with culture medium at the time of experiment, was added to the medium as follows: 1) at 24 h before inoculation of virus (pretreatment); 2) for 1 h with virus before inoculation (mixed treatment); 3) under continuous incubation after inoculation of virus (posttreatment); or 4) for 1 h with virus before inoculation and under continuous incubation after inoculation (mixed plus posttreatment). The inoculation time of the sulfated colominic acid-treated virus or sulfated colominic acid-untreated virus was 1 h. After the inoculation, the cells were washed with PBS(–) three times for infectivity assay, cell cytopathic assay, and multiple step growth assay. The treatments were conducted at 37°C, and the schema are exhibited in Figure 2.

Cells and viruses: MA104 cells derived from rhesus monkey kidney were cultured and passaged with Eagle's minimum essential medium (MEM) containing 0.15% bicarbonate and 10% fetal bovine serum (FBS). Cultured-rotaviruses (simian rotavirus SA11, and human rotaviruses Wa and MO), -poliovirus serotype 1 (Sabin 1), and -coxsackie B3 virus (Nancy) were inoculated to the cells (7).

Effect of sulfated colominic acid on cell viability: MEM containing sulfated colominic acid was added to MA104 cells at different concentrations (0, 100, 200, 400, 800, or 1500 μg/ml). The cells were cultured for 1 week. After detaching cells with trypsin, the numbers of viable cells were counted by nigrosin dye exclusion method (7). The experiment was conducted in duplicate.

Infectivity assay (direct plaque assay): One hundred plaque-forming units (pfu) of rotaviruses (SA11, Wa), poliovirus 1, and coxsackie B3 virus were adsorbed to cultured cells at 0.2 ml/well on six-well microplates for 1 h. After washing with PBS, the cells were overlaid with agar containing FBS-free MEM supplemented with 0.15% bicarbonate, 0.6% agarose, and 0.002% neutral red. One μg/ml of acetyl trypsin-treated rotaviruses were used in the experiments. Sulfated colominic acid was added to the medium at concentrations of 0, 50, 100, 200, and 400 μg/ml, as indicated. Experiments were performed in duplicate (7).

Cell cytopathic assay: For MO, the plaque assay was not successful because the plaque was not visible. Therefore, the cell cytopathic assay was used for the infectivity assay of MO. To the cultured cells, a 0.2 ml/tube of 100 $TCID_{50}$ of MO was adsorbed for 1 h. Then, FBS-free MEM supplemented with 0.15% bicarbonate and 1 μg/ml of acetyl trypsin was added. Cell cytopathic effect was observed for 10 days.

Multiple step growth assay: One hundred pfu/0.2 ml of SA11 and 100 $TCID_{50}$ /0.2 ml of MO were inoculated into tube cultures of MA104 cells and inoculated for 1 h. After washing the cells with PBS three times, the MEM containing 0.15% bicarbonate and 1 μg/ml of acetyl trypsin was added. When the cytopathic effect was detected in the positive control cells (into which the virus had been inoculated but no sulfated colominic acid had been added), cells and culture medium were separately frozen and thawed three times, then subsequently centrifuged at 6,000×g for 5 min. The supernatant fluid and cells were separately titered by plaque assay for SA11 and cell cytopathic assay for MO (7).

RNA-PAGE (polyacrylamide gel electrophoresis): Rotavirus RNAs were extracted from the supernatant and the cells were separated by using phenol and chloroform. Then each fraction was electrophoresed by Laemmli gel. The RNA segments in the gel were stained with silver (8).

Titration of viral proteins by enzyme immunoassay: The culture supernatants and cells were examined for viral proteins by using commercially available immunoassay kits (Rotaclone, from BioSience, Cambridge, Mass., USA).

Electron microscopic analysis: The cultured viruses were centrifuged at 6,000×g for 20 min (partially purified virus). The supernatant was ultracentrifuged at 100,000×g for 1 h through 1 ml of 35% sucrose in PBS. The pellet was resuspended in PBS and recentrifuged at 100,000×g for 16 h in CsCl. The virus bands were collected and dialyzed in PBS (purified virus). The purified virus particles were mixed with 400 μg/ml or 0 μg/ml of sulfated colominic acid at 35°C for 0, 10, and 60 min, respectively. The treated viruses were negatively stained with 2% uranyl acetate (pH 4.4) and viewed with an electron microscope (model JEOL 100 CX) (8).

RESULTS

Cytotoxicity: It was found that 1,500 μg/ml of sulfated colominic acid reduced the number of viable cells by 50% and 800 μg/ml reduced the number by 30%. No reduction of viable cells was found at concentrations below 400 μg/ml (Table 1).

Table 1. Cytotoxic effect of sulfated colominic acid on MA104 cells for 7 days

Concentration	viable cell
0 μg/ml	100 %
100	100
200	97
400	86
800	72
1500	51

Table 2. Effect of sulfated colominic acid on enteric viruses in plaque assay

virus	method	0	50	100	200	400 μg/ml
SA11	pretreatment	100	88	69	70	75
	mixed treatment	100	57	44	31	27
	posttreatment	100	43	26	29	26
Wa	pretreatment	100	100	100	100	108
	mixed treatment	100	85	125	113	123
	posttreatment	100	100	100	118	92
Sabin 1	pretreatment	100	100	100	100	100
	mixed treatment	100	96	92	98	96
	posttreatment	100	100	94	100	100
Nancy	pretreatment	100	90	95	95	93
	mixed treatment	100	96	97	95	95
	posttreatment	100	100	99	99	100

Data are given as percentages of the number of plaques in the assay containing the compound compared to assays without sulfated colominic acid (set to 100%). One hundred pfu/0.2 ml of virus were inoculated into MA104 cells in each well of a six-well microplate. Treatment with sulfated colominic acid was performed as described in Materials and Methods.

Effects on viral infection by plaque assay and multiple growth assay: The antiviral effect of sulfated colominic acid was examined by inoculation of 100 pfu of SA11 (simian rotavirus), Wa (human rotavirus), MO (human rotavirus), Sabin 1 (poliovirus 1), and Nancy (coxsackie B3 virus), respectively. Pretreatment at 50 to 400 μg/ml reduced the growth of SA11 by 12 to 31%; mixed treatment at 50 to 400 μg/ml reduced it by 43 to 73%; and posttreatment at 50 to 400 μg/ml reduced it by 57 to 74%. These results show that both the mixed and the posttreatment reduced SA11 rotavirus infection remarkably. The pretreatment, mixed treatment, and posttreatment at different concentrations of sulfated colominic acid did not reduce viral titers of Sabin 1, Nancy or Wa (Table 2).

In multiple step growth assay, the pretreatment, mixed treatment, posttreatment, and mixed plus posttreatment reduced plaque numbers of SA11 by 0-, 10-, $10^{3.5}$- and $10^{3.6}$-fold, respectively. The mixed plus posttreatment did not show multiplier effects on SA11. The mixed treatment and posttreatment reduced plaque numbers of MO by $10^{3.2}$- and $10^{3.5}$-fold, respectively (Table 3).

The infectivity titer of SA11 on the infected cells and the culture supernatants: In order to examine the antiviral mechanism of sulfated colominic acid, the virus growth was examined separately in cells and supernatants. By posttreatment, the appearance of cell cytopathic effect and extracellular release of the virus were delayed. The mixed treatment at 400 μg/ml had no effect at 2 days after infection, virus growth was the same as that of the positive control (Table 4).

Enzyme immunoassay and RNA-PAGE: According to the increase of the concentration of sulfated colominic acid

Table 3. Effect of sulfated colominic acid on rotavirus in a multiple step growth assay

virus method	0	50	100	200	400 μg/ml
SA11					
pretreatment	7.1	6.7	7.2	7.0	7.3
mixed treatment	7.2	6.5	6.3	6.3	6.3
posttreatment	7.2	4.6	3.7	3.7	3.7
mixed plus posttreatment	7.1	5.5	3.7	3.7	3.5
MO					
pretreatment	4.7	4.7	4.2	4.2	4.2
mixed treatment	4.4	2.7	2.2	1.4	1.2
posttreatment	4.7	2.7	1.4	1.4	1.2

Data show the virus yields (log of pfu/0.2 ml in SA11 and log of $TCID_{50}$/0.2ml in MO). One hundred pfu of SA11/0.2 ml and 100 $TCID_{50}$ of MO/0.2 ml per tube were inoculated into MA104 cells. Treatment with sulfated colominic acid and assays were performed as described in Materials and Methods.

at posttreatment, virus growth and extracellular virus release were delayed. Mixed treatment at 400 μg/ml seemed to be effective until 2 days after inoculation, and in effective at 3 days (Table 4).

Electron microscopic analysis: The aggregation of SA11 by sulfated colominic acid was examined under an electron microscope. Aggregation was not seen at 0 μg/ml of sulfated colominic acid at 35°C at 0, 10, and 60 min, or at 400 μg/ml at 35°C at 0 min. However, 2 to 3 particle aggregations were noted at 35°C, 10 min, and 5 to 10 particle aggregations were seen at 35°C, 1 hr, under the condition of 400 μg/ml (Fig. 3).

DISCUSSION

Outer capsid protein VP4 is the main rotavirus cell attachment protein, but the cellular receptor remains unknown. Sialic acid on the cell surface has been shown to be required for efficient binding and infectivity of animal rotaviruses including SA11, but not of human rotaviruses including Wa (9-11). MO has not been examined in previous reports. For demonstration of sialic acid-dependent or -independent rotavirus, neuraminidase-treated MA104 cells were used (9-11). In our experiments, sulfated colominic acid, like sialic acid, inhibited animal rotavirus (SA11) and human rotavirus (MO) infections but did not inhibit human rotavirus infection (Wa). This inhibition was shown in response to mixed treatment and posttreatment. These findings indicate that sulfated colominic acid is effective on virus adsorption at the time of and/or after the inoculation of SA11 and MO. Inhibition after inoculation was successful at an early stage of infection; the infection was not inhibited when sulfated colominic acid was added 5 h after the virus inoculation (data not shown). Our results present two problems yet to be resolved. 1) In addition to sialic acid, there may be another receptor for rotavirus, such as gangliosides (12). The presence of a coreceptor may be necessary for some types of rotavirus infection. 2) Inhibition of rotavirus infection by posttreatment may suggest that, in addition to the inhibition of virus adsorption by sulfated colominic acid, other mechanisms may contribute. Given this possibility, we examined intracellular and extracellular double-stranded RNA and virus protein. No meaningful differences were observed. Sulfated colominic acid by posttreatment might block the path of virus infection from infected cells to healthy cells. The results of electron microscopic examination revealed that SA11 reacted directly

Table 4. Effect of sulfated colominic acid on multiplication of SA11 by posttreatment and mixed treatment

method		control		posttreatment				mixed treatment	
concentration		0		50 μg/ml		200 μg/ml		400 μg/ml	
		fluid	cell	fluid	cell	fluid	cell	fluid	cell
1 day	CPE		-		-		-		-
	log pfu/ml	3.4	3.7	2.2	3.6	2.2	2.5	0.7	3.2
	OD	0.03	0.04	0.03	0.04	0.03	0.04	0.03	0.03
	RNA-PAGE	-	-	-	-	-	-	-	-
2 days	CPE		+		-		-		+
	log pfu/ml	5.1	5.6	3.5	4.9	3.5	4.2	6.0	5.4
	OD	0.28	0.78	0.05	0.11	0.04	0.07	0.06	0.19
	RNA-PAGE	+	+	-	+	-	-	-	-
3 days	CPE		++		+		-		++
	log pfu/ml	6.3	6.3	5.5	5.1	3.3	5.0	6.8	6.5
	OD	1.67	1.15	0.83	0.69	0.16	0.13	1.38	1.22
	RNA-PAGE	++	++	+	+	+	+	+	+
4 days	CPE		++		++		+		N.D.
	log pfu/ml	6.4	6.3	6.0	6.3	4.8	5.8	N.D.	N.D.
	OD	1.62	1.50	1.34	1.48	0.30	0.75	N.D.	N.D.
	RNA-PAGE	++	++	++	++	++	+	N.D.	N.D.

One hundred pfu of SA11 was inoculated into MA104 cells. Culture fluids and cells were analysed separately by cell cytopathic effect (CPE), plaque-forming unit (pfu), enzyme immunoassay (OD values), and RNA polyacrylamide gel electrophoresis (RNA-PAGE). -, +, and ++ correspond to negative, visible, and strongly visible, respectively.

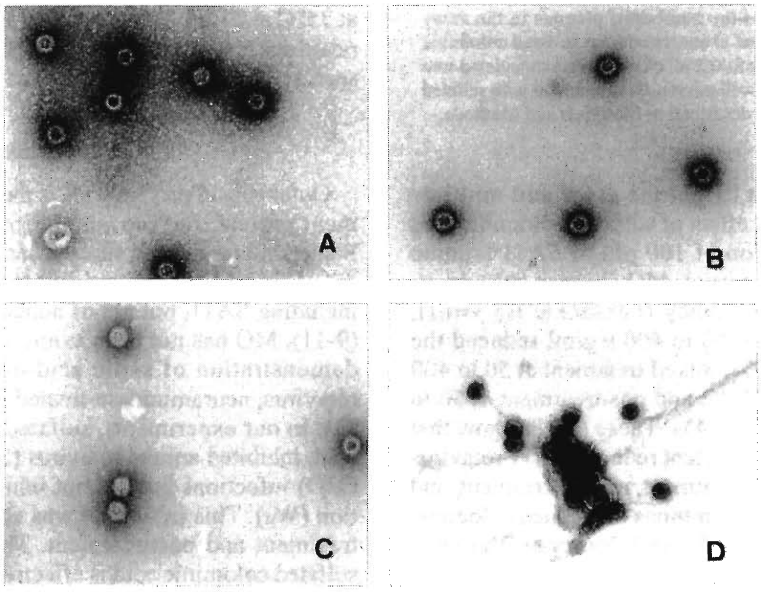

Fig. 3. Electron micrograph of purified SA11 rotavirus after the four respective treatments. The virus was kept without sulfated colominic acid for 1 h at 35°C (A). The virus was treated with 400 μg/ml of sulfated colominic acid at 35°C, 0 min (B); 10 min (C) and 1 h (D).

with sulfated colominic acid. These phenomena indicate that sulfated colominic acid behaves like sialic acid with regard to virus infection.

The receptor of poliovirus is known to belong to an immunoglobulin superfamily (13). The receptors of coxsackie B3 virus are the coxsackievirus and adenovirus receptor (CAR, a member of immunoglobulin superfamily) (14) and the decay-accelerating receptor (DAF) (15). Sulfated colominic acid might not be effective on poliovirus and coxsackievirus because of the difference in cell receptors.

Our results show that sulfated colominic acid may be effective on infection of sialic acid-dependent rotaviruses, which may react to the sialic receptor of host cells, but is not effective on infection of sialic acid-independent rotaviruses, polioviruses, and coxsackieviruses, which do not have a ligand to the sialic receptor of host cells. Further studies are needed to confirm the relationship between virus and cell receptor.

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