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Sulfated sialic acid-polymers inhibit the cytotoxic action of bee and snake venom

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Colominic acid is an α2,8-linked sialic acid polymer produced by *Escherichia coli.* We found that synthetic sulfated-colominic acids (SC) remarkably inhibited the cytotoxicity of bee and snake venom toward mouse fibroblast cells, but colominic acids showed no inhibition themselves, indicating the important role of sulfate groups in the inhibitory activity of SC. Other sulfated carbohydrates such as chondroitin sulfates, heparin and heparan sulfate showed no inhibition. SC also exhibited potent inhibition of melittin, a highly basic peptide, which is a major cytotoxic component of bee venom. SC did not inhibit phospholipase A_2 activity in bee venom. This suggests that the inhibition of bee and snake venom by SC is due to inhibition of melittin and cardiotoxin, which is a cytolytic peptide in snake venom, respectively. SC with a higher sulfur content and a larger molecular mass showed more potent activity. The interaction between SC and melittin basically seems an ionic one, however, the conformation of SC is also likely important. For the binding of SC to melittin leading loss of its cytotoxic activity, the sulfate groups of SC must be properly arranged to interact with lysine and arginine residues of melittin molecules, which play an important role in the cytolytic activity. A higher molecular mass of SC substituted with more sulfate groups is required for more obvious inhibition of the cytotoxic activity.

Keywords: **sialic acid, colominic acid, sulfation, bee venom, melittin, cytotoxicity.**

Abbreviations: **SC, sulfated colominic acid; WST-1,2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium-Na; PMS, 1-methoxy-5-methylphenazium methylsulfate; PBS, phosphate-buffered saline.**

Introduction

Sulfated carbohydrates have been found in microorganisms, plants and animals. Sulfate groups have been reported to be involved in the functions of several carbohydrates. GlyCAM-1, a ligand on endothelial cells for L-selectin, is a mucin-like glycoprotein having 6′-O-sulfated sialyl Lewis^x capping groups [1], which are involved in leucocyte-endothelium cell adhesion [2]. Lutropin and thyrotropin hormones bearing sulfated oligosaccharides are rapidly removed from the circulation by a receptor on hepatic endothelial cells which recognizes the terminal sulfated carbohydrate structure [3,4]. Some *Rhizobium* species produce sulfated lipo-oligosaccharides that play a role in the interaction with legume plants. Sulfation of the oligosaccharides is closely related to the host specificity as to infection and nodulation of the fungi [5,6]. These observations suggest that the sulfation of carbohydrates affords compounds having new biological activities. Recently, some sulfated polysaccharides have been examined as antiviral drugs [7] and sialidase inhibitors [8].

Colominic acid is a homogeneous polymer composed of N-acetylneuraminic acid linked through α (2–8) glycosidic linkages. It is produced by several strains of bacteria, such as *Escherichia coli* and *Neiseria meningitidis* [9], and a pathogenic determinant of these bacteria [10]. Colominic acid was reported to be sulfated by using sulfur trioxide pyridine complex [11]. In this study, we prepared sulfated colominic acids (SC) containing various amounts of sulfate, and examined their inhibitory effects on the cytotoxicity of bee and snake venom toward mouse fibroblast cells.

Materials and methods

Materials

Bee venom (*Apis mellifera*), snake venom (*Naja mossambica mossambica*), melittin, heparin (porcine intestinal mu-

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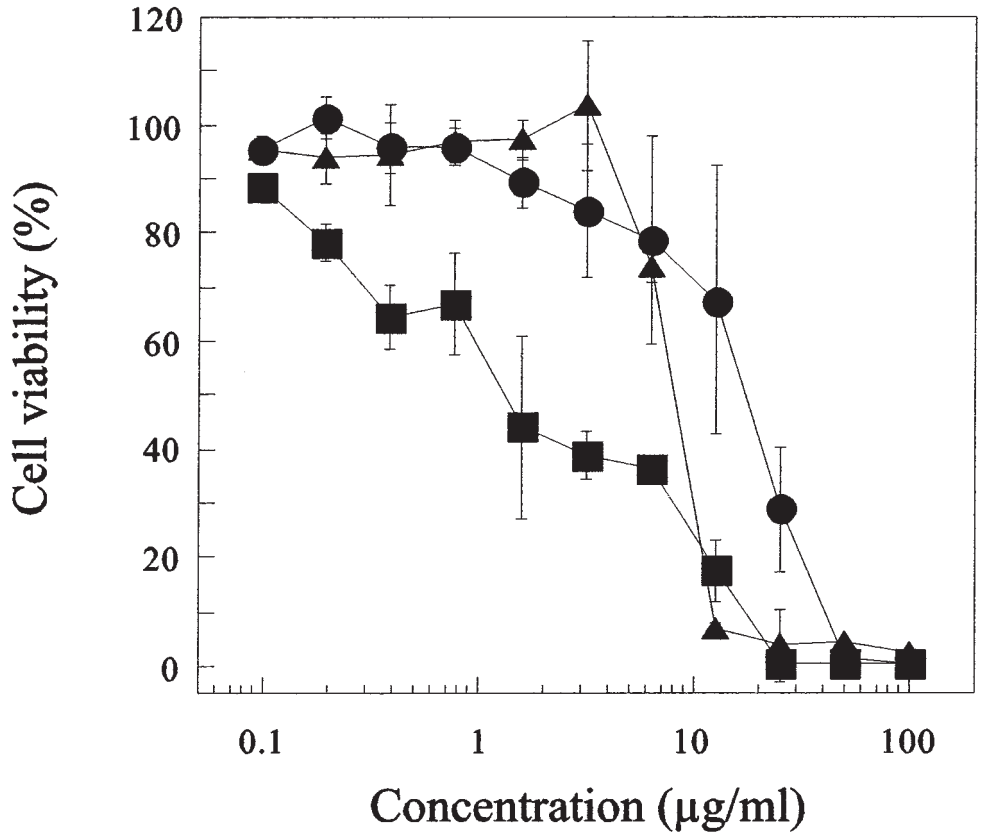

Figure 1. Cytotoxic effects of bee venom, snake venom and melittin on mouse fibroblast cells. Mouse fibroblast cells were incubated with cytotoxic substances (▲, bee venom; ●, snake venom; ■, melittin) in a 96-well culture plate for 4 h, and the cell viability in each well was determined by the modified MTT method as described under Materials and methods. Values are means ± standard deviations of triplicate determinations.

cosa), and N-acetylneuraminic acid were purchased from Sigma Co. (St. Louis, U.S.A.). Chondroitin 4-sulfate (whale cartilage), chondroitin 6-sulfate (shark cartilage) and heparan sulfate (bovine kidney) were products of Seikagaku Kogyo (Tokyo, Japan). The sulfur contents and molecular masses (Mr) of these glycosaminoglycans were as follows: chondroitin-4-sulfate, S content, 6.2–6.6%, Mr, 25–50 kDa; chondroitin-6-sulfate, S content, 6.4–6.8%, Mr, 40–80 kDa; heparan sulfate, S content, 5–6%, Mr, 15 kDa; heparin, S content, 9–10%, Mr, 15 kDa. Colominic acid (*Escherichia coli*), of which the mean Mr is 17 kDa, was purchased from Nacalai Tesque (Kyoto, Japan). 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetra zolium-Na (WST-1) and 1-methoxy-5-methylphenazium methylsufate (PMS) were purchased from Wako (Osaka, Japan). Phosphatidylcholine, L-α-1-palmitoyl-2-linoleoyl-(linoleoyl-1-^{14}C) (2 GBq/mmol), was a product of American Radiolabeled Chemicals Inc. (U.S.A.).

Sulfated colominic acids (SC)

Colominic acid (Nakarai Tesque) was fractionated by gel filtration on Sephadex G-50 and 100 (Pharmacia, Uppsala, Sweden). Elution was performed with water and the sugar fractions were monitored by the resorcinol-HCl procedure [12]. The molecular masses of the fractions were determined using standard samples of dextran having mean Mr of 10, 15–20, 35–45 and 60–90 kDa (Sigma Co.). Three fractions with mean Mr of approximately 6.8, 14 and 69 kDa were obtained.

The three colominic acid preparations obtained and the original colominic acid were sulfated with sulfur trioxide pyridine complex (Aldrich, St Louis, MO) and 4-dimethylaminopyridine (Wako, Osaka, Japan) in dimethylformamide [11]. Reactions with various amounts of the sulfating reagent were carried out at −20 and +30 °C for 3–24 h to obtain various degrees of sulfation. The reaction mixture was neutralized with 1M sodium hydrogen carbonate and then dialyzed against distilled water. The dialyzed solution was passed through Amberlite IR-120B (Na^+ form). The eluent and washings with distilled water were combined and lyophilized. The total sulfur contents of the sulfated compounds were determined [13]. Sulfated colominic acid with a sulfur content of 5.3% and a mean Mr of 17 kDa was used as sulfated colominic acid unless otherwise indicated.

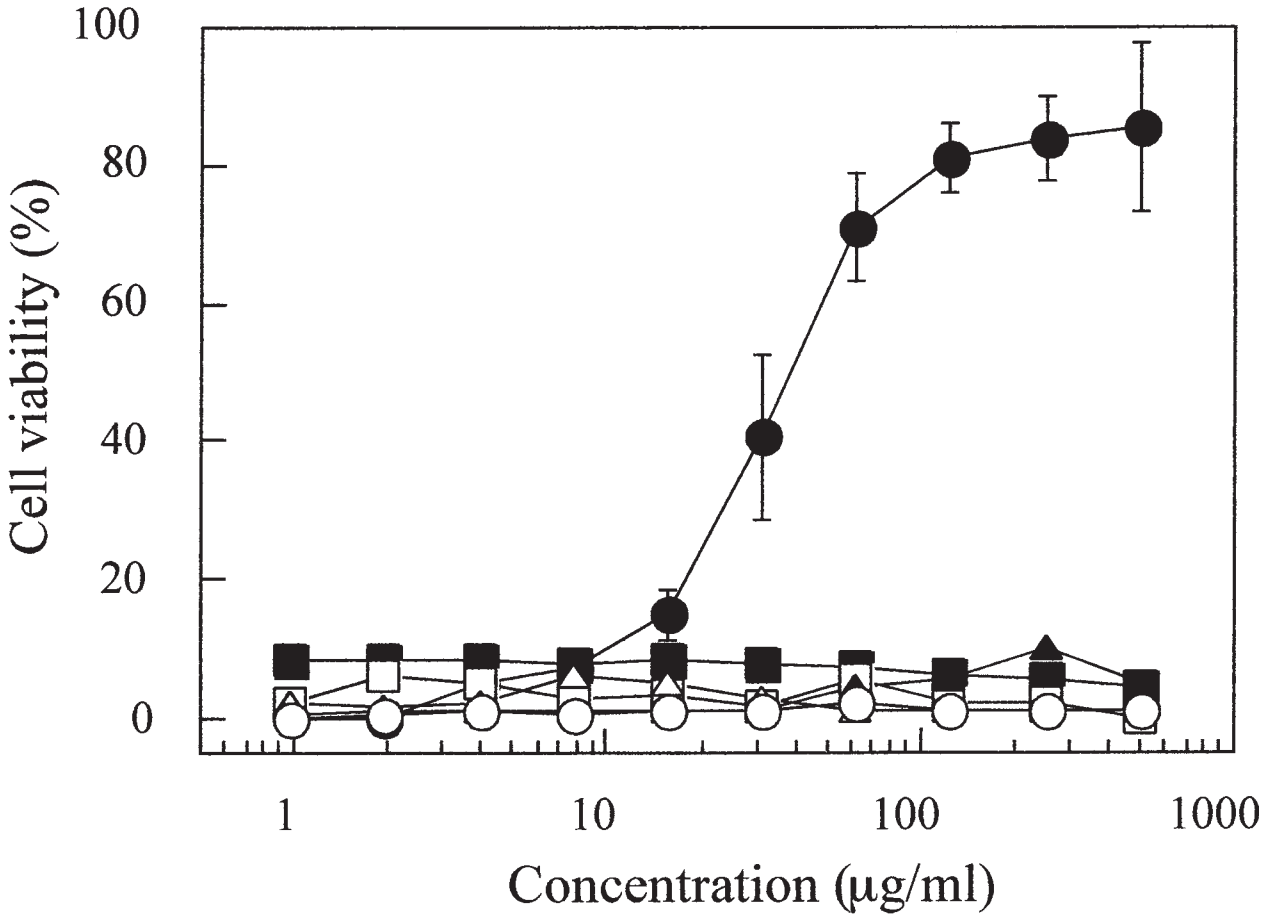

Figure 2. Inhibitory effects of sulfated colominic acid and acidic polysaccharides on bee venom. ●, SC; ■, colominic acid; ▲, heparin; ○, heparin sulfate; □, chondroitin 4-sulfate; △, chondroitin 6-sulfate. Bee venom was used at 25 μg/ml. Values are means ± standard deviations of triplicate determinations.

Cell culture

Dorsal skin was obtained from newborn ddY mice (Shimizu Jikken Doubutsu, Kyoto, Japan). The skin was cut into 3 to 5 mm square pieces, which were floated on 10 ml of 0.025% trypsin/0.002% EDTA in PBS at 37 °C for 20 min under 5% CO_2 in air. 10 ml of newborn calf serum (Gibco BRL, Rockville, U.S.A.) was added and the cells were suspended by pipetting. The large tissue pieces were removed with a cell stainer (10 μm; Falcon, New Jersey, U.S.A.), and the cells were harvested by centrifugation. The cells were suspended in Dulbecco's modified Eagle medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% newborn calf serum and kanamaycin (60 μg/ml; Meiji Seika, Tokyo, Japan), and then maintained at 37 °C under a humidified atmosphere of 5% CO_2 in air, and used between passages 3–7.

Inhibition assay of cytotoxicity

Mouse fibroblasts were seeded into a 96-well culture plate at the density of 5,000 cells per well. After incubation at 37 °C for 16 h under 5% CO_2 in air, the culture medium was removed and 200 μl of the culture medium containing cytotoxic material was added to each well. After 4 h incubation, cell viability was examined by means of the modified MTT assay [14]. Briefly, 5 μl of WST/PMS (5 mM WST and 0.2 mM PMS in 20 mM HEPES, pH 7.4) was added to each well and then the plate was placed in an incubator for 2 h. The optical density of the medium was measured at 450 nm. For inhibition experiments, cells were incubated in 200 μl of culture medium containing a cytotoxic substance and a test sample for 4 h, and then cell viability was determined as described above. Bee venom, snake venom and melittin were used at the final concentrations of 25 μg/ml, 50 μg/ml and 12.5 μg/ml, respectively. The viability of the cells exposed to toxic compounds in the presence of a test sample was expressed as a percentage based on the decrease in absorbance compared to that observed for the intact cells.

Assay of phospholipase activity

A mixture in 50 mM Tris/HCl (pH 7.4, 100 μl) containing bee venom (2 ng), SC and ^{14}C-phosphatidylcholine (3.7 ×

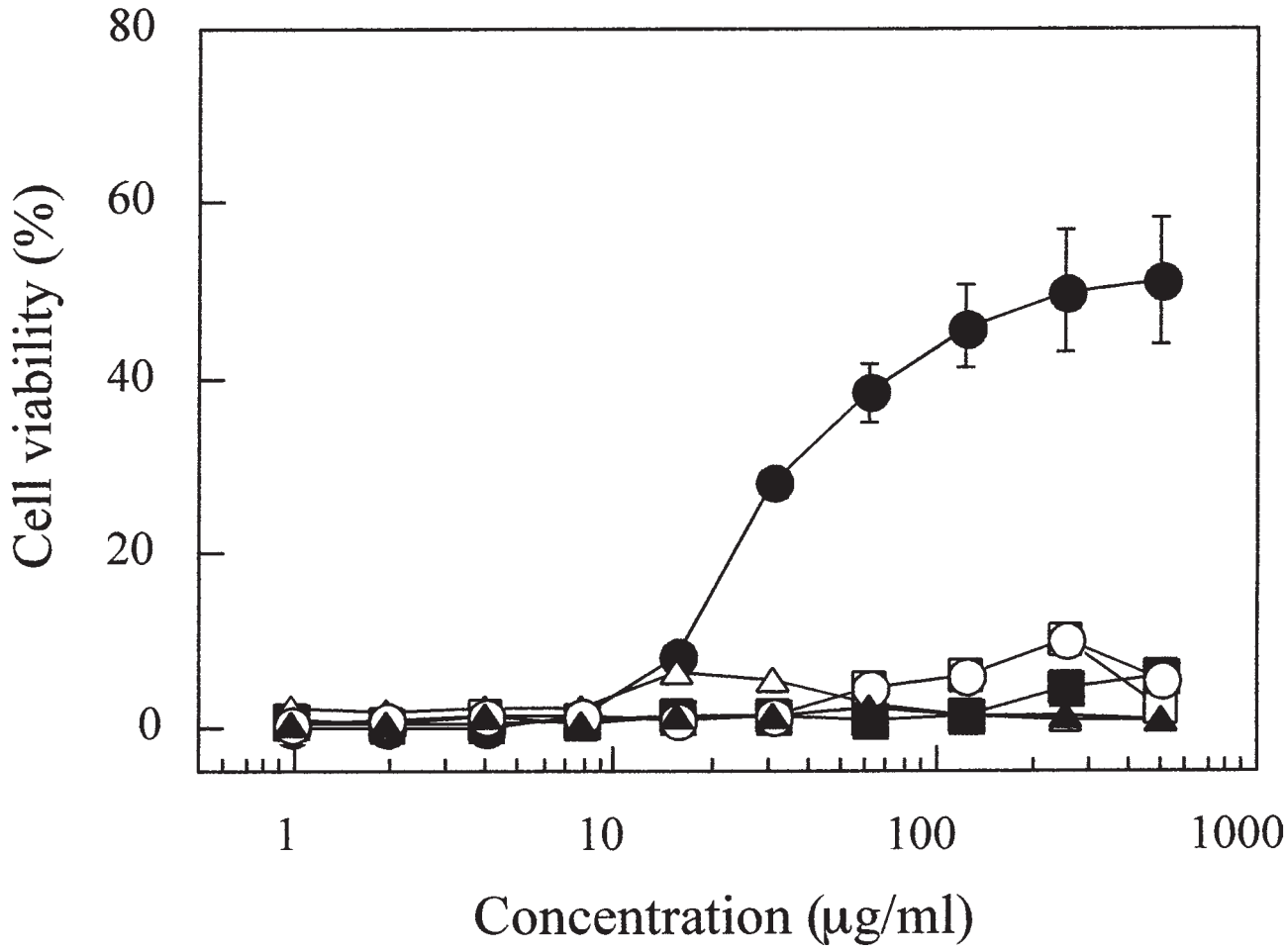

Figure 3. Inhibitory effects of sulfated colominic acid and acidic polysaccharides on snake venom. Snake venom was used at 50 μg/ml. Values are means ± standard deviations of triplicate determinations. The symbols are the same as in Figure 2.

10^3 Bq) was incubated at 37 °C for 15 min. The reaction was stopped by the addition of 100 μl of butanol and the organic layer was separated by centrifugation. An aliquot (20 μl) of the butanol layer was subjected to thin layer chromatography on a Silica Gel 60 plate (Merck) with a solvent system of chloroform/methanol/acetic acid (65 : 35 : 2). The spots of fatty acids and phosphatidylcholine were detected with iodine vapor and then scrapped off, and the radioactivity was determined with a liquid scintillation counter.

Results

Bee and snake venom showed cytotoxicity toward mouse fibroblast cells. Melittin, a major component of bee venom [15], also showed obvious toxicity. The LD_{50} values were 8 μg/ml, 20 μg/ml and 0.9 μg/ml for bee venom, snake venom and melittin, respectively, as shown in Figure 1. In the presence of bee venom at the concentration of 25 μg/ml, the cultured cells lysed immediately.

We examined the inhibitory effects of some acidic polysaccharides on the cytotoxic activity of bee venom at 25 μg/ml, with which fibroblast cells can no longer survive, as shown in Figure 1. The results are shown in Figure 2. SC remarkably inhibited the cytotoxic action of bee venom. Cytolysis was completely inhibited with 50 μg/ml of SC. On the contrary, colominic acid and other sulfated polysaccharides, such as chondroitin 4- and 6-sulfates, heparin and heparan sulfate, showed little or no inhibition even at 500 μg/ml.

Snake venom, which contains cardiotoxic substances, showed similar cytotoxic activity. The cultured cells were completely lysed with 50 μg/ml of snake venom. In the presence of snake venom at this concentration, SC effectively inhibited the cytotoxic activity of snake venom, as shown in Figure 3. However, other polysaccharides did not show any inhibition.

A major component of bee venom, melittin, showed similar cytotoxic effect on the cultured cells, and lysed all the cells at 12.5 μg/ml. SC showed a similar inhibitory effect to that observed for bee venom, as shown in Figure 4 (see also

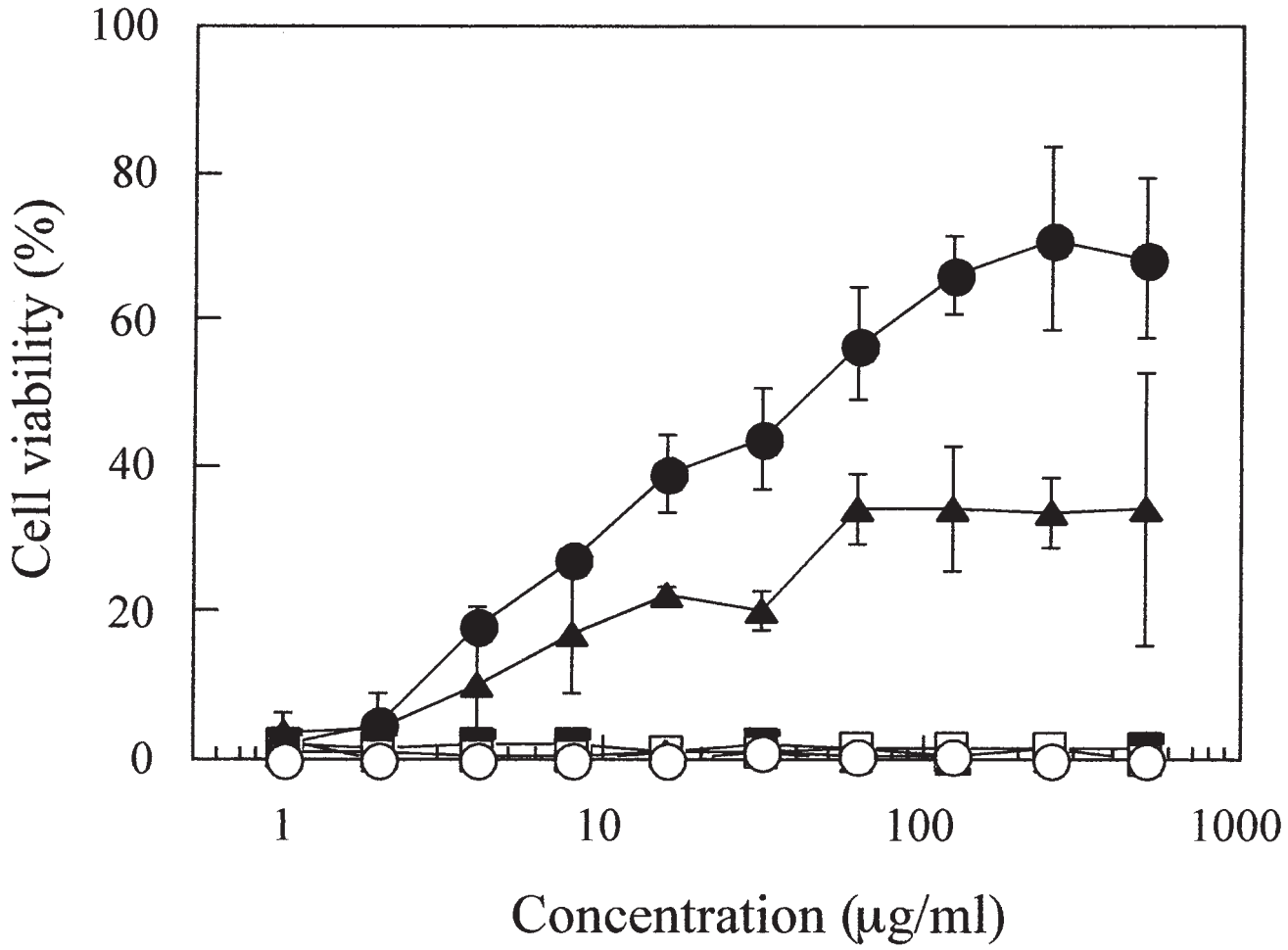

Figure 4. Inhibitory effect of sulfated colominic acid and acidic polysaccharides on melittin. Melittin was used at 12.5 μg/ml. Values are means ± standard deviations of triplicate determinations. The symbols are the same as in Figure 2.

Fig. 1). With higher concentrations than 300 μg/ml of SC, the pH of the culture became lower and the cells died. Interestingly, heparin did not inhibit the cytolysis caused by bee and snake venom, but exhibited a distinct inhibitory effect.

Bee venom contains phospholipase A_2, which is another important component for the expression of cytotoxicity. SC at a concentration from 1 to 200 μg/ml, however, did not inhibit the activity with phosphatidylcholine as the substrate (data not shown).

The inhibitory effect on the cytotoxicity of bee venom was examined using some SC preparations with different molecular masses and containing various amounts of sulfate groups. The results are shown in Figure 5. Among SC with a mean Mr of 17 kDa, SC with a higher content of sulfur showed more potent inhibition, and the preparation containing 11.5% sulfur completely inhibited the cytotoxicity at 60 μg/ml. SC containing 2.5% sulfur showed 40% inhibition even at 125 μg/ml. On comparison of the inhibitory activities of SC preparations containing more than 9.0% sulfur, the order of the inhibitory potency was found to be in accord with their molecular masses, 69,000 > 14,000 > 6,800 Da.

Discussion

Melittin, a basic peptide of 26 amino acid residues, is the major component which comprises about 50 weight % of dried venom. Melittin interacts with the negatively-charged groups of lipids and disturbs lipid bilayers in cell membranes, thereby causing cytolysis [16,17]. The positively-charged region near the C-terminal of the melittin molecule, which is composed of two arginine and two lysine residues, plays an important role in the binding process [18,19]. However, the detailed mechanism underlying the direct cytotoxic property is not fully understood although many studies on the interactions between melittin and cell membranes or lipids have been performed.

Phospholipase A_2 is another important cytotoxic component in bee venom, which hydrolyzes phospholipids to form cytolytic compounds, lysophospholipids [15]. Most of the

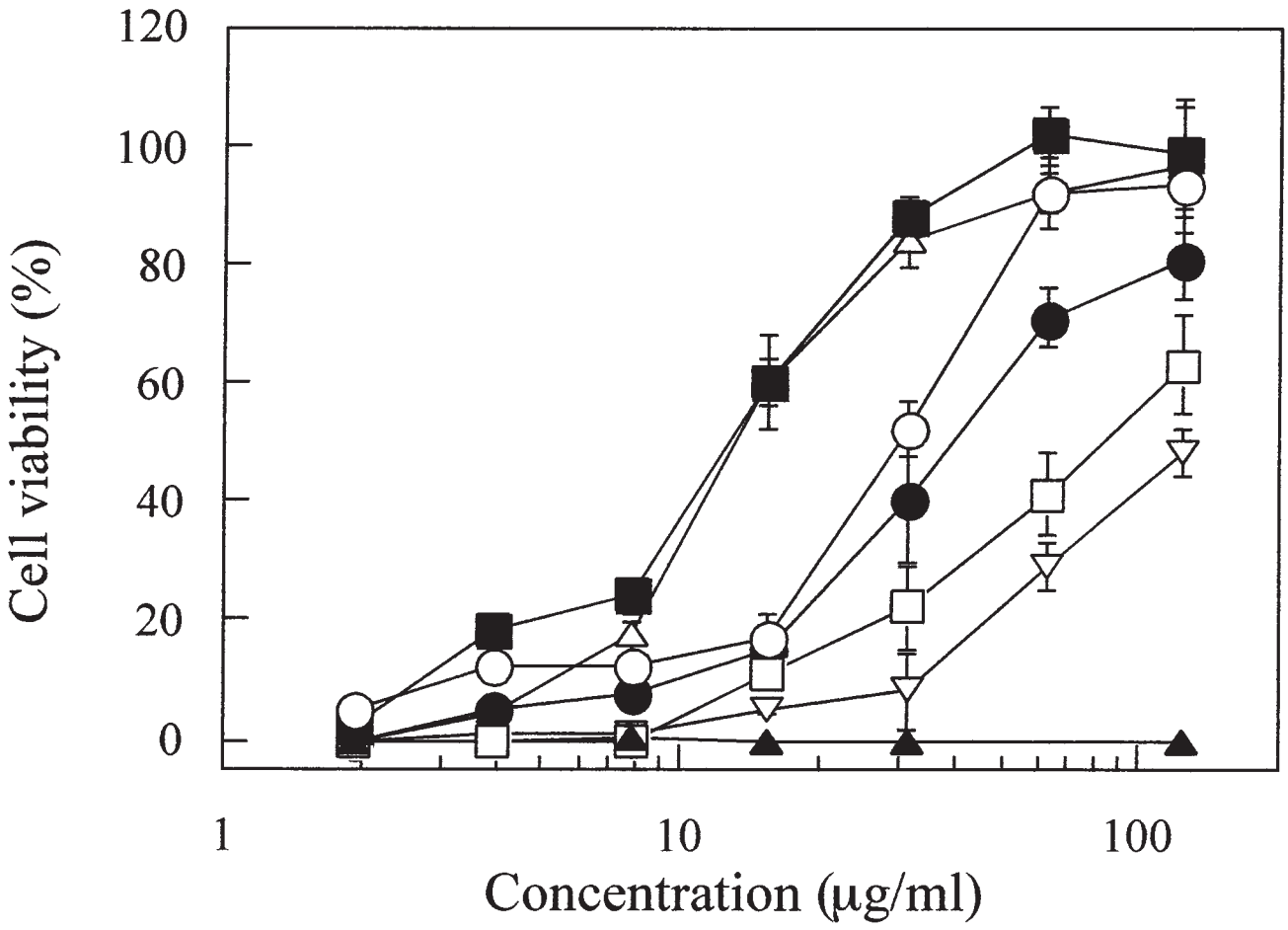

Figure 5. Inhibitory effects of various sulfated colominic acid preparations on bee venom. Bee venom was used at 25 μg/ml. The sulfur contents and molecular masses of the preparations were as follows: ▽ 2.5%, 17,000 Da; ●, 5.3%, 17,000 Da; ▲, 7.7%, 1,800 Da; ■, 9.5%, 69,000 Da; △, 10.1%, 14,000 Da; □, 10.3%, 6,800 Da; ○, 11.5%, 17,000 Da.

cytolytic action of bee venom are ascribed to melittin and phospholipase A_2, although bee venom contains various bioactive substances such as histamine, dopamine and hyaluronidase [15]. Melittin showed a higher cytotoxic effect toward mouse fibroblast cells than that of bee venom. SC strongly inhibited the cytotoxic activity of melittin, but not the phospholipase A_2 activity in bee venom. These findings indicate that the inhibition of the cytotoxicity of bee venom by SC is mainly due to inhibition of melittin. Snake venoms also contain cardiotoxins, that are highly basic and cytolytic peptides like melittin [20,21]. Hence, the inhibition of snake venom by SC may be due to the inhibition of cardiotoxins as well as melittin in bee venom. Patel et al. reported that heparin and heparan sulfate bound to cardiotoxin, but not chondroitin sulfate and dermatan sulfate under physiological conditions, as found in affinity chromatography, circular dichroism and fluorescence spectrometric studies [22].

In our study, heparin showed substantial inhibition of melittin, however, it did not inhibit bee or snake venoms. This suggests that a cardiotoxin or melittin still maintains its cytotoxic activity in the bound state with heparin. However, the reason for the weak inhibitory activity of heparin toward bee and snake venoms is not clear.

SC showed the different inhibiting patterns on bee and snake venoms (Figs. 2 and 3). Snake venom (*Naja mossambica mossambica*) contains four cardiotoxins (cytotoxin V^{II} 1~4) [23]. Although cardiotoxins and melittin are basic cytotoxic peptides, they are different in chemical structure. Cardiotoxins consist of 60 amino acid residues, of which 12 are lysine and arginine residues. Molecular masses of snake toxins are two-times larger than that of melittin. The different inhibitory effects of SC to bee and snake venoms are probably due to differences in molecular masses of melittin and cardiotoxins. Cytolysis is a result of various actions of many factors in the venoms, although it is mainly ascribed to the actions of melittin or cardiotoxin and phosholipase A_2 as described above. Phospholipase A_2 is also known to increase cytotoxic action of melittin and cardiotoxin [24,25]. Phospholipase A_2 was not inhibited by SC, there-

fore, cytotoxic action of the venom in the presence of SC seems to be mainly dependent on the action of phospholipase A_2. This is another reason why different inhibitory effects of SC to bee and snake venoms were observed. However, further work is necessary to clarify which component in each venom is responsible for the different inhibitory effects of SC to the venoms.

Colominic acid is a mixture of homo-polymers of α2,8-linked N-acetylneuraminic acid [26]. Theoretically, three sulfate residues can be substituted in one molecule of the linked N-acetylneuraminic acid residue, of which the surfur content is about 17.5%. The highest sulfur content of SC obtained in this study was 11.5%, because it was difficult to obtain a higher sulfated-product probably due to the steric effect of negative charges of sulfate groups. SC with a lower sulfur content (2.5%) and a large molecular mass of 17 kDa showed only weak inhibition to bee venom like SC with a high sulfur content (10.3%) and a small molecular mass of 6,800 Da. For expression of full activity, SC requires both a high sulfur content and a large molecular mass. Chondroitin 4- and 6-sulfate are composed of repeating D-glucuronic acid→N-acetyl-D-glucosamine-4-sulfate and D-glucuronic acid→N-acetyl-D-glucosamine-6-sulfate disaccharides, respectively. The sulfur contents of the chondroitin sulfates used are more than 6% and their molecular masses are larger than 25 kDa. Although chondroitin sulfates were expected to show inhibitory effects based on the consideration regarding the activity of SC mentioned above, they exhibited no inhibition even at 125 μg/ml. Besides sulfate groups, carboxyl groups of SC may be involved to some extent in the interaction with melittin. The interaction between SC and melittin seems to be basically an ionic one, but the conformation of SC is also important. For the binding of SC to melittin leading loss of its cytotoxic activity, the sulfate groups of SC must be properly arranged to interact with lysine or arginine residues of melittin, which play an important role in cytolysis, as mentioned above. SC with a large molecular mass and a high sulfate content interacts with melittin and changes the melittin conformation required for expression of its cytolytic activity. Melittin preparation obtained from the commercial source still contains various contaminants. We tried to isolate melittin in pure state for examining the contribution of lysine and arginine residues of melittin on the interaction with SC. However, it was a challenging problem and precise studies on the mechanism will be discribed elswhere.

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