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A Natural Sulfated Polysaccharide, Calcium Spirulan, Isolated from *Spirulina platensis: In Vitro* and *ex Vivo* Evaluation of Anti-Herpes Simplex Virus and Anti-Human Immunodeficiency Virus Activities

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ABSTRACT

A sulfated polysaccharide named calcium spirulan (Ca-SP) has been isolated from a sea alga, *Spirulina platensis*, as an antiviral component. The anti-human immunodeficiency virus type 1 (HIV-1) and anti-herpes simplex virus type 1 (HSV-1) activities of Ca-SP were compared with those of dextran sulfate (DS) as a representative sulfated polysaccharide. Anti-HIV-1 activities of these agents were measured by three different assays: viability of acutely infected CD4-positive cells, or a cytopathology assay; determination of HIV-1 p24 antigen released into culture supernatants; and inhibition of HIV-induced syncytium formation. Anti-HSV-1 activity was assessed by plaque yield reduction. In addition, their effects on the blood coagulation processes and stability in the blood were evaluated. These data indicate that Ca-SP is a potent antiviral agent against both HIV-1 and HSV-1. Furthermore, Ca-SP is quite promising as an anti-HIV agent because even at low concentrations of Ca-SP an enhancement of virus-induced syncytium formation was not observed, as was observed in DS-treated cultures, Ca-SP had very low anticoagulant activity, and showed a much longer half-life in the blood of mice when compared with that of DS. Thus, Ca-SP can be a candidate agent for an anti-HIV therapeutic drug that might overcome the disadvantages observed in many sulfated polysaccharides. When the role of chelation of calcium ion with sulfate groups was examined by removing calcium or its replacement by sodium, the presence of calcium ion in the molecule was shown to be essential for the dose-dependent inhibition of cytopathic effect and syncytium formation induced by HIV-1.

INTRODUCTION

SULFATED POLYSACCHARIDES, such as dextran sulfate (DS) and heparin, have proved to be potent inhibitors of human immunodeficiency virus type 1 (HIV-1) *in vitro*.[1–5] As to their mechanism of action, these agents have been shown to inhibit the binding of the virions to CD4 molecules on target cells[3,4,6–8] and virus-induced syncytium formation.[3,9,10] However, their effectiveness *in vivo* has not been established as yet because of their poor absorbability, short half-life time in the body, and unfavorable anticoagulant activity in blood.[11–14]

We have reported that the water-soluble extract of *Spirulina platensis*, a blue-green alga, exhibited an inhibitory effect against herpes simplex virus type 1 (HSV-1) replication.[15] From the extract, we have isolated a new sulfated polysaccharide, named calcium spirulan (Ca-SP), as an antiviral component.[16] Ca-SP was found to be active against not only HSV-1 but also HIV-1. To acquire clues that may lead to the development of related anti-HIV components that are more likely to be active *in vivo*, we compared the antiviral activity of Ca-SP with that of DS. Through *in vitro* and *ex vivo* experiments, this natural compound was shown to be superior to DS or other sulfated polysaccharides as a therapeutic agent for AIDS. Furthermore, the importance of retention of molecular conformation by chelation of calcium ion with sulfate groups was also investigated in the context of antiviral activities.

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Anti-HSV-1 activity

The method for anti-HSV-1 assay has been described previously.[22] HeLa cell monolayers were infected with virus at an MOI of 0.2 for 1 hr at room temperature and refed with maintenance medium (MEM plus 2% FBS). In the time-of-addition experiments, HeLa cells were infected with HSV-1 at an MOI of 10, and treated with Ca-SP at the times indicated. Virus yields were determined by plaque assay at 1-day incubation.

Anticoagulant activity

After blood was collected from a normal subject, it was spun down at 3000 rpm for 15 min. The plasma was collected and distributed into 10-ml tubes. Drugs dissolved in the plasma were then added to achieve a concentration of 50, 100, or 200 μg/ml. The samples were subjected to prothrombin time (PT) assay and to activated partial thromboplasmin time (APTT) assay by using two coagulation reagents, Sysmex PT II and Sysmex APTT II (TOA Medical Electronics, Kobe, Japan) and measuring with a CA-5000 automatic coagulation timer (TOA Medical Electronics).

Quantification of Ca-SP in animal serum and ex vivo antiviral assay

Female ddY mice (6 weeks, 36.7 ± 0.57 g) obtained from Sankyo Labo Service Co. (Shizuoka, Japan) were given 4 mg of Ca-SP or DS intravenously. The blood samples were taken individually from carotid vein under ether anesthesia at 2 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr, and 24 hr after injection. To measure the amounts of Ca-SP in these samples, an aliquot (0.1 ml) of the serum was diluted with H_2O (0.8 ml) and 100% (w/v) trichloroacetic acid (0.1 ml) was added. After centrifugation, the supernatant was dialyzed against H_2O by using seamless cellulose tubing (small size 18; Wako Pure Chemical Industries, Ltd., Osaka, Japan). The nondialyzable part was lyophylized to give a colorless residue. The residue was dissolved in 0.1 ml of H_2O and 5 μl of each sample was used for HPLC analysis, using a YMC Pack Diol-300 column (500 × 8.0 mm I.D.), H_2O as mobile phase, and a flow rate of 1 ml/min at ambient temperature.

For the evaluation of antiviral activities, an aliquot (0.1 ml) of the serum was serially diluted with FBS-free medium, and added to the medium at the time of virus infection and throughout the incubation. Anti-HIV and -HSV activities were assessed under *in vitro* conditions by a cytopathology assay or plaque yield reduction assay, respectively.

RESULTS

Antiviral activity of Ca-SP in in vitro *experiments*

Calcium spirulan and DS were examined for their inhibitory effects on the replication of HIV-1 and HSV-1 (Table 1). When anti-HIV-1 activity was assessed using a p24 antigen assay, the concentration of Ca-SP or DS required for 50% inhibition (IC_{50}) was 9.3 and 9.6 μg/ml, respectively, under the conditions in which the drug was added after infection. In a cytopathology assay, similar results were obtained as in the p24 antigen assay: the IC_{50} values for Ca-SP and DS were 7.2 and 8.3 μg/ml, respectively (data not shown). The activity was five- or four-fold higher in the cultures treated during infection with Ca-SP or DS, respectively, when compared with that in the cultures treated with drug after infection. As the cytotoxicity of Ca-SP was similar to that of DS, the resulting selectivity indices (CC_{50}/IC_{50}) for the two drugs showed no significant difference.

When anti-HSV-1 activity was evaluated in the cultures treated with drug after infection, the selectivity index for Ca-SP was 24-fold higher than that for DS ($p < 0.005$ by Student's t test). In the cultures treated with drug during infection, however, there was no significant difference in antiviral effect between Ca-SP and DS. The inhibitory effects of both drugs on HSV-1 replication were markedly potentiated by adding at the time of infection.

These results suggest that Ca-SP may interfere with a very early stage of viral replication such as virus binding and penetration. To delineate the drug-sensitive phase, time-of-addition experiments were carried out with HeLa cells infected at a high MOI of 10 (Table 2). The compound suppressed HSV-1 replication efficiently when it was present during infection, while no or reduced effect was seen when it was absent during infection.

Effect of Ca-SP on HIV-induced syncytium formation

To assess the possible inhibitory effect of Ca-SP on the cell-to-cell transmission of HIV-1, a syncytium formation assay was performed under conditions in which the drug was added at the time of cocultivation of Molt-4 cells with Molt-4/HTLV-IIIB cells (Table 3). Calcium spirulan showed almost complete inhibition of syncytium formation at higher concentrations than 25 μg/ml, while DS did not block completely the fusion reaction even at 100 μg/ml. The IC_{50} values of Ca-SP and DS were 7.3 and 14.2 μg/ml, respectively. Whereas Ca-SP showed dose-dependent inhibition in the concentration range tested, DS stimulated the cell fusion at lower concentrations than 1 μg/ml. In the other experiments, the effects of pretreatment of uninfected Molt-4 cells with Ca-SP or DS were determined on syncytium formation (Fig. 2). In treatment A, Molt-4 cells were exposed to the compound from 3 hr prior to cocultivation with the counterpart. In treatment B, the compound was added at the time of cocultivation as in Table 3. The inhibitory effects of both Ca-SP and DS on cell fusion were not markedly different from each other between the two treatments at higher concentrations than 20 μg/ml. In both treatments, however, the stimulation of syncytium formation by lower concentrations of DS was again observed.

Comparison of anti-HIV effects of Ca-SP with Na-SP, H-SP, and desulfated SP

To determine the significance of calcium ion in the bioactivity of Ca-SP, Ca-SP, Na-SP, and H-SP were subjected to syncytium formation and cytopathology assays. As shown in Table 3, H-SP showed no marked inhibition of syncytium formation at the concentrations tested, and exhibited the stimulating effect at lower concentrations than 5 μg/ml. Sodium spirulan exerted an inhibitory effect comparable to that of Ca-SP at higher concentrations than 5 μg/ml, with an IC_{50} of 10.5 μg/ml, while the compound stimulated cell fusion at lower concentrations than 1 μg/ml. When desulfated spirulan was also evaluated for

TABLE 2. EFFECT OF TIME OF ADDITION OF CALCIUM SPIRULAN ON HSV-1 REPLICATION

Time of addition					Antiviral activity (IC_{50}; μg/ml)
3 hr before infection	During infection	0–1 hr postinfection	1–2 hr postinfection	2–24 hr postinfection	
+[a]	−	−	−	−	>200[b]
−	+	−	−	−	0.97 ± 0.10
−	−	+	−	−	>200
−	−	−	+	−	>200
−	−	−	−	+	24.5 ± 4.6
−	−	−	−	−	0.92 ± 0.20
+	+	−	−	−	1.2 ± 0.15
−	+	+	−	−	>200
−	−	+	+	+	0.95 ± 0.19
+	+	+	+	+	0.83 ± 0.12
−	+	+	+	+	13.2 ± 2.2
−	−	+	+	+	

[a]HeLa cells were treated in the absence (−) or presence (+) of different concentrations of Ca-SP during the period indicated.
[b]Each value is the mean ± SD of triplicate assays.

its effect against syncytium formation, no marked inhibition was observed at concentrations up to 25 μg/ml, and the stimulation of cell fusion was seen at 5 μg/ml or less. When anti-HIV-1 activities of these compounds were assessed by a cytopathology assay using acutely infected MT-4 cells, the IC_{50} values for Na-SP, H-SP, and desulfated SP were 8.2 ± 1.0, >100 and >50 μg/ml, respectively (data not shown).

Lympholiferative activity of sulfated polysaccharides

Previously, Anand *et al.*[24] have reported that virus enhancement at low concentrations of a sulfated polysaccharide appeared to be linked to its lymphoproliferative effect. We determined whether such an effect might also be observed at low concentrations of the compounds tested. When uninfected Molt-4 cells were exposed to 0.1–1 μg of each compound per milliliter for 5 days, DS, Na-SP, and H-SP stimulated cell growth by 19–28, 13–19, and 13–19%, respectively, compared with the control cultures (Table 4). On the other hand, there was no proliferative effect in Ca-SP-treated cells at the same concentrations.

Anticoagulant activity of Ca-SP

The anticoagulant activity of Ca-SP was evaluated by PT and APTT (Table 5). The PT value of the untreated blood was 11.1 sec. Treatment of the blood with Ca-SP at lower concentrations than 100 μg/ml did not remarkably exceed the control value, while the treatment at the higher concentration of 200 μg/ml resulted in a considerably increased PT value. On the other hand, treatment with DS remarkably increased the PT values at 50–200 μg/ml. The APTT values of blood treated with Ca-SP also showed no remarkable change at lower concentrations than 100 μg/ml. The concentrations of Ca-SP and DS required to obtain twofold APTT were 112 μg/ml and less than 50 μg/ml, respectively. Thus, the anticoagulant activity of Ca-SP was very weak when compared with that of DS.

Evaluation of ex vivo *antiviral activity*

In this study, we measured directly the Ca-SP level in mouse serum and also its antiviral activity (Table 6). After mice were treated intravenously with Ca-SP, sera were obtained from each

TABLE 3. EFFECTS OF Ca-SP, Na-SP, H-SP, AND DESULFATED SP ON SYNCYTIUM FORMATION BY COCULTIVATION OF Molt-4 AND Molt-4/HTLV-IIIB[a]

Drug	Percent inhibition of syncytium formation					50% inhibitory concentration (μg/ml)
	0.2 μg/ml	1 μg/ml	5 μg/ml	25 μg/ml	100 μg/ml	
DS	−13 ± 5.4	−8.6 ± 1.2	3.8 ± 0.64	74 ± 9.9	98 ± 1.6	14.2
Ca-SP	6.3 ± 1.2	14 ± 3.9	27 ± 6.7	99 ± 1.2	100	7.3
Na-SP	−8 ± 5.0	−7.3 ± 2.9	10 ± 2.2	95 ± 2.9	99 ± 1.2	10.5
H-SP	−8.7 ± 1.7	−9.7 ± 6.0	−1.3 ± 0.45	13 ± 3.4	27 ± 8.1	>100
Desulfated SP	−12 ± 4.9	−13 ± 5.4	−12 ± 3.5	9.3 ± 4.0	ND[b]	>25

[a]Drug was added at the time of cocultivation of Molt-4 cells with the counterpart. Each value is the mean ± SD of triplicate assays.
[b]ND, Not determined because of its high cytotoxicity.

TABLE 5. ANTICOAGULANT ACTIVITY OF CALCIUM SPIRULAN AND DEXTRAN SULFATE

Drug	Concentration (μg/ml)	Time (sec)[a] PT[b]	APTT[c]
Control (saline)	—	11.1 ± 0.65	29.1 ± 2.0
Ca-SP	50	12.3 ± 0.53	34.3 ± 3.3
	100	12.3 ± 1.3	48.1 ± 3.0
	200	13.6 ± 1.1	124 ± 18
DS	50	13.2 ± 1.5	201 ± 28
	100	14.1 ± 1.4	>240
	200	16.2 ± 1.5	>240

[a]Each value is the mean ± SD of triplicate assays.
[b]PT, Prothrombin time.
[c]APTT, Activated partial thromboplastin time.

as well because, unlike dextran sulfate, Ca-SP showed considerably higher activity against HSV-1 even when added after infection.

Sulfation of substances such as polysaccharides[4,32–34] and gangliosides[35] enhances their antiviral activities. The anti-HIV-1 activity of dextran sulfate is reported to be highly dependent on its sulfur content.[7] At present, it is not clear why sulfation of the compounds results in the generation of such activities. In this study, desulfation of Ca-SP resulted in the disappearance of its anti-HIV activity. Similar results have been obtained in an anti-HSV-1 assay,[16] wherein the desulfated compound showed a remarkable reduction of its antiviral activity when compared with Ca-SP. These observations reconfirmed the important role of sulfate groups in exhibiting the antiviral activity of sulfated polysaccharides.[1,32–33] However, in spite of the presence of sulfated groups in calcium-free spirulan (H-SP), an inhibitory effect on HIV replication was not maintained in this compound. Thus, it is suggested that the molecular conformation of Ca-SP by chelation of calcium ion with sulfate groups might be essential for its biological activity.

Anand *et al.*[24] observed the enhancement of HIV replication in the presence of sodium pentosan polysaccharide and dextran sulfate at lower concentrations. Lentinan sulfate, curdlan galactose sulfate, and curdlan arabinose sulfate were also found to stimulate syncytium formation in cocultures of Molt-4 and Molt-4/HTLV-IIIB cells at low concentrations.[34] Because many sulfated polysaccharides are only poorly absorbed from the gastrointestinal tract or rapidly metabolized in the body, systemic concentrations of these compounds after administration could be at a virus-enhancing low level. Thus, it is particularly important to check for such a deleterious effect of virus enhancement at lower concentrations in the course of the evaluation of sulfated polyanions as therapeutic agents. In the present study, Ca-SP was confirmed to show dose-dependent inhibition of HIV replication without the stimulation of virus-induced syncytium formation at lower concentrations, while dextran sulfate showed repeatedly such a deleterious effect. Calcium spirulan contains calcium ion in its molecular structure, while many other sulfated polysaccharides including dextran sulfate are sodium salts. When the calcium ion of Ca-SP was replaced by sodium ion, the resulting sodium spirulan (Na-SP) showed no more dose-dependent inhibition of syncytium formation but stimulated this event at low concentrations as observed for dextran sulfate. A proliferative effect on Molt-4 cells, which was observed when the cells were treated with low concentrations of dextran sulfate and Na-SP, might explain at least in part their virus-enhancing effects.

In spite of the fact that sulfated polysaccharides are promising candidates for the treatment of AIDS because of their potent *in vitro* antiviral activities and low toxicities for CD4-positive cells, the anticoagulant activity has often hampered their usefulness as anti-AIDS therapeutics. Among these compounds, however, dextran sulfates of low molecular weight (MW 5000, 8000) have been known not to be markedly inhibitory to the coagulation process.[4] Calcium spirulan showed much lower anticoagulant activity than dextran sulfate. Thus, anti-HIV activity of Ca-SP can be attained at a concentration without showing any anticoagulant activity.

From the viewpoint of therapy, the conservation of potent antiviral activity *in vivo* and the bioavailability of the agent after administration are the most important aspects to be focused on in the development of antiviral agents. The half-life of dextran sulfate in the blood, however, was very short, being ap-

TABLE 6. CONCENTRATION OF DRUG AND ANTIVIRAL ACTIVITY IN SERA OBTAINED FROM MICE TREATED INTRAVENOUSLY WITH CALCIUM SPIRULAN[a]

Time after administration	Concentration in serum (μg/ml)	Anti-HSV-1 activity[b] (IC_{50}; fold dilution of serum)	Anti-HIV-1 activity[b] (IC_{50}; fold dilution of serum)
2 min	696 ± 54	2460 ± 54	ND[c]
30 min	1076 ± 85	2423 ± 26	957 ± 46
1 hr	910 ± 276	1650 ± 360	803 ± 58
2 hr	571 ± 89	1430 ± 187	667 ± 52
4 hr	572 ± 113	613 ± 62	335 ± 61
8 hr	510 ± 36	349 ± 25	185 ± 34
24 hr	416 ± 62	61 ± 15	44 ± 4.3
Untreated control	0	<20	<40

[a]Each value is the mean ± SD from three mice.
[b]Antiviral assays were performed by adding the serially diluted serum samples to the medium at the time of infection.
[c]ND, Not determined.

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