AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 12, Number 15, 1996
Mary Ann Liebert, Inc.

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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MATERIALS AND METHODS

Drugs

Dextran sulfate (approximate MW 8000; DS) was obtained from Sigma (St. Louis, MO). Ca-SP was isolated as described previously,[16] and its purity was confirmed by high-performance liquid chromatography (HPLC) analysis with a Shimadzu LC-6A HPLC system equipped with a refractive index detector (model RID-6A; Shimadzu Corporation, Kyoto, Japan). The removal of calcium from Ca-SP was performed as described previously[16] to give a free acid of spirulan (H-SP). Desulfation of Ca-SP was also done by the method reported previously.[16] The replacement of calcium by sodium, which gives sodium spirulan (Na-SP), was performed as follows: Chelex 100 ion-exchange resin (sodium salt, 100–200 mesh),[17] was washed batchwise with 1 N HCl, H_2O, 1 N NaOH, and H_2O, successively, and then packed into a column. After the column was washed with H_2O, Ca-SP dissolved in H_2O was supplied on the column, which was eluted with the same solvent (H_2O). Each fraction was monitored by the phenol–H_2SO_4 method (UV λ_{max} 480 nm).[18] The eluted fractions were freeze-dried to give colorless Na-SP. The efficiency of replacement of calcium by hydrogen or sodium was confirmed by X-ray microanalysis (Hitachi scanning electron microanalyzer X-650; Hitachi, Ltd., Tokyo, Japan), and no calcium was detected in both Na-SP and H-SP as shown in Fig. 1.

Stock solutions of these compounds were prepared by dissolving in RPMI 1640 medium or Eagle's minimum essential medium (MEM) at the concentration of 20 mg/ml.

Cells and viruses

MT-4,[19] Molt-4 clone No. 8,[20] and Molt-4/HTLV-IIIB[21] cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). HeLa cells were grown in MEM supplemented with 5% FBS and kanamycin (60 μg/ml).

Human immunodeficiency virus type 1 (HIV-1) was obtained from the culture supernatant of persistently infected Molt-4/HTLV-IIIB cells. Herpes simplex virus type 1 (HSV-1) HF strain was grown on HeLa cells.

Titration of virus

HSV-1 was titered by plaque assay as described previously.[22] For the titration of HIV-1, serial 0.5 log dilutions of thawed virus stock were cultured with 3×10^4 MT-4 cells for 5 days. The amount of virus supernatant required to kill 50% of the cells was determined in quadruplicate by calculating with the Reed–Muench method.[23]

Cytotoxicity

For cell growth inhibition studies, 5×10^4 HeLa or MT-4 cells were inoculated in 24-well plates and incubated for 72 hr at 37°C in the presence of increasing amounts of drugs. Viable cells were counted by the trypan blue dye exclusion test. The inhibition data were plotted as dose–response curves, from which the 50% cytotoxic concentration (CC_{50}) was obtained.

HIV-induced syncytium formation

Molt-4 (2.5×10^5/250 μl) and Molt-4/HTLV-IIIB (2.5×10^5/250 μl) cells were mixed with 500 μl of medium containing a drug or control medium. In some experiments, Molt-4 cells were pretreated for 3 hr with a drug followed by cocultivation with Molt-4/HTLV-IIIB cells. Formed syncytia were counted at 20 hr after cocultivation at 37°C. The results were expressed as the percent inhibition of syncytium formation, which was calculated as follows:

$$\text{percent inhibition} = (1 - \text{syncytia formed in the presence of drug/syncytia formed in the absence of drug}) \times 100$$

The 50% inhibitory concentration (IC_{50}) was estimated from graphs of percent inhibition plotted against drug concentration.

Assay for HIV-induced cytopathic effect

Exponentially growing MT-4 cells were pelleted from the medium, infected with HIV-1 at a multiplicity of infection (MOI) of 0.0007 at room temperature for 1 hr with agitation, and then diluted in growth medium to yield 3×10^4 cells/100 μl/well after inoculation. Equal aliquots (100 μl) of the test solutions were added to each well. After 5 days of incubation at 37°C, viable cells were counted by the trypan blue dye exclusion test. From the data, the IC_{50} values for a cytopathology assay were calculated.

Determination of p24 antigen

To determine the activity of a drug against HIV-1, extracellular HIV content was also measured by p24 core antigen production. MT-4 cells were infected with HIV-1 at an MOI of 0.0007. After 5 days of incubation at 37°C, the amounts of p24 antigen in the medium were measured in triplicate by enzyme-linked immunosorbent assay (ELISA) using a p24 assay kit (Abbott Laboratories, Chicago, IL), following the manufacturer instructions. From the data, the IC_{50} values were calculated.

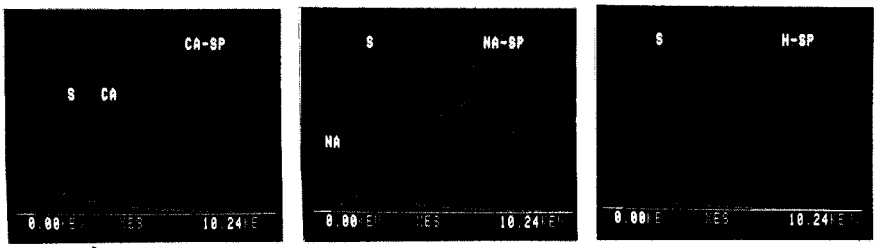

FIG. 1. X-Ray microanalysis of calcium spiluran (Ca-SP), sodium spirulan (Na-SP), and calcium-removed spirulan (H-SP).

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, 26.7 ± 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 1. INHIBITORY EFFECTS OF CALCIUM SPIRULAN AND DEXTRAN SULFATE ON CELL VIABILITY AND ON REPLICATION OF HIV-1 AND HSV-1[a]

	Anti-HIV-1 activity					*Anti-HSV-1 activity*				
		−		+			−		+	
Drug	CC_{50}[b]	IC_{50}[c]	CC_{50}/IC_{50}	IC_{50}	CC_{50}/IC_{50}	CC_{50}[d]	IC_{50}[e]	CC_{50}/IC_{50}	IC_{50}	CC_{50}/IC_{50}
Ca-SP	2770 ± 210[f]	9.3 ± 1.7	304 ± 43	1.8 ± 0.17	1513 ± 71	7533 ± 572	9.7 ± 0.79[g]	776 ± 6.8[h]	0.85 ± 0.079	8847 ± 371
DS	2710 ± 216	9.6 ± 1.4	285 ± 27	2.3 ± 0.21	1165 ± 75	5300 ± 247	173 ± 30	32 ± 4.8	0.92 ± 0.16	5895 ± 835

[a]Drug was absent at the time of infection but added immediately after infection (−), or, present at the time of infection (+), and throughout the incubation.
[b]Concentration (μg/ml) required to reduce the growth of MT-4 cells by 50%.
[c]Concentration (μg/ml) required to reduce HIV-1 replication by 50% when measured by p24 antigen determination.
[d]Concentration (μg/ml) required to reduce the growth of HeLa cells by 50%.
[e]Concentration (μg/ml) required to reduce HSV-1 replication by 50% when measured by plaque yield reduction method.
[f]Each value is the mean ± SD of triplicate assays.
[g]$p < 0.01$ vs. DS (Student's t test).
[h]$p < 0.005$ vs. DS (Student's t test).

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

Time of addition					
3 hr before infection	*During infection*	*0–1 hr postinfection*	*1–2 hr postinfection*	*2–24 hr postinfection*	*Antiviral activity (IC_{50}; μg/ml)*
+[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.

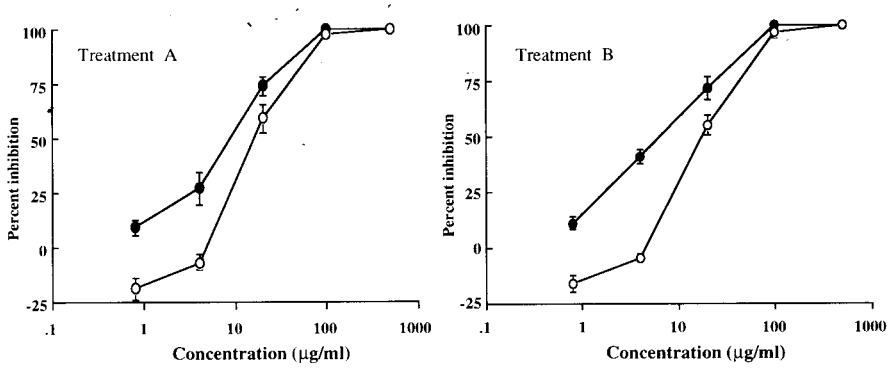

FIG. 2. Effects of Ca-SP (●) and DS (○) on fusion reaction in cocultivation of Molt-4 and Molt-4/HTLV-IIIB cells. Molt-4 cells were incubated with the drugs from 3 hr prior to cocultivation with Molt-4/HTLV-IIIB cells (treatment A) or at the time of cocultivation (treatment B). Each value is the mean ± SD of triplicate assays.

mouse at 2 min to 24 hr thereafter. By 30 min after administration, the serum concentration of Ca-SP reached a peak value of about 1000 μg/ml, and then decreased gradually. The retention time of Ca-SP extracted from sera showed no change in HPLC analysis throughout the 24-hr treatment. However, when sulfur content in the sera at 24 hr was measured by X-ray microanalyzer, it decreased to approximately one-tenth of the content in the starting material (data not shown). When antiviral activities were determined by diluting the serum samples, Ca-SP showed relatively long-lasting effects against both HIV-1 and HSV-1, and inhibitory effects could be detected in the blood even at 24 hr after administration. In parallel experiments in which DS was administered at a dose of 4 mg/mouse, the antiviral activities for both HIV-1 and HSV-1 were much lower than those of Ca-SP, IC_{50} values for HIV-1 and HSV-1 being 55 ± 7 or 51 ± 9 fold dilution of serum, respectively, at 1 hr after intravenous injection. These activities were undetectable by 2 hr after injection (data not shown).

DISCUSSION

Several sulfated polysaccharides have been reported to have a broad-spectrum antiviral activity against enveloped viruses.[4,5,25–28] As an exception, sulfated paramylon is effective against HIV, but is ineffective against HSV and influenza virus.[14] The present study indicates that a sulfated polysaccharide, calcium spirulan (Ca-SP), isolated from *Spirulina platensis*, has an anti-HIV activity almost equal to dextran sulfate, and is a much more potent inhibitor of HSV-1 than dextran sulfate under experimental conditions in which these compounds were added after viral infection. Moreover, Ca-SP is also active against human cytomegalovirus (HCMV) replication.[16] Thus, this compound holds great promise for the treatment not only of HIV-1 but also HSV-1 and HCMV infections, which is particularly advantageous for AIDS patients, who are prone to these life-threatening infections.[29] That is, in AIDS patients the same compound may be used not only to suppress the causative agent (HIV-1) but also to prevent or suppress exacerbations of the opportunistic passengers (HCMV, HSV-1) as pointed by Macher *et al.*[30]

The mechanism by which sulfated polysaccharides inhibit virus replication can be in general explained by the inhibition of virus binding to host cells and the subsequent virus–cell fusion step.[3,4,6–10] Witvrouw *et al.*[31] envisaged that these early inhibitory effects of polysulfates on HIV can be the result of a disruption of ionic interactions between positively charged regions of viral surface glycoproteins and cellular membrane phospholipids. In the present study, the main target for antiviral activity of Ca-SP could be estimated to be the early steps of virus–cell attachment, and virus–cell or cell–cell fusion on the basis of the results of time-of-addition experiments and HIV-induced syncytium assays. However, other factors such as the interference with late steps of replication must be involved

TABLE 4. PROLIFERATIVE EFFECTS OF SULFATED POLYSACCHARIDES ON Molt-4 CELLS[a]

	Concentration (μg/ml)			
Drug	*0.1*	*0.5*	*1*	*10*
DS	179 ± 18[b] (119)	193 ± 22 (128)	194 ± 18 (128)	142 ± 4.0 (94)
Ca-SP	147 ± 4.5 (97)	150 ± 7.6 (99)	147 ± 6.5 (97)	155 ± 9.1 (103)
Na-SP	171 ± 6.6 (113)	180 ± 10 (119)	176 ± 2.2 (117)	148 ± 7.0 (98)
H-SP	174 ± 6.2 (115)	171 ± 7.3 (113)	180 ± 11 (119)	146 ± 4.3 (97)

[a]Molt-4 cells at 8×10^4 cells/ml were cultured in the medium containing different concentrations of drug. Cell viability was measured on day 5. Each value is the mean ± SD of triplicate assays. The value of untreated control cultures was 151 ± 8.6 (100%).

[b]Viable cell count ($\times 10^4$ cells/ml).

TABLE 5. ANTICOAGULANT ACTIVITY OF CALCIUM SPIRULAN AND DEXTRAN SULFATE

Drug	Concentration (μg/ml)	Time (sec)[a] PT[b]	Time (sec)[a] 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.[4,32–34] 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.

proximately 30 min in rabbits[12] and less than 30 min in mice (present study). Abrams *et al.*[36] reported that no evidence of systemic absorption of oral dextran sulfate was available in patients with AIDS and AIDS-related complex. Paramylon sulfate was also shown to disappear rapidly from the plasma when administered intravenously in mice.[14] On the other hand, the inhibitory effect of Ca-SP-administered blood on HIV-1 and HSV-1 was maintained to be effective for a longer time, with an estimated half-life of approximately 150 min. However, the rate of decline of bioactivity exceeded that of the Ca-SP level in the blood. These observations would be explained by time-dependent loss of sulfate groups in Ca-SP as shown by X-ray microanalyzer. Another possibility is, however, that the protein-binding property of Ca-SP might play a role, at least in part, because the 50% inhibitory concentrations of Ca-SP on HSV-1 replication were approximately four- and fivefold higher in the presence of 50 and 75% FBS, respectively, than that with 10% FBS (data not shown).

In conclusion, Ca-SP could be a good candidate antiviral drug because of its low anticoagulant activity, long half-life in the blood, and dose-dependent bioactivity without any showing of stimulation of viral replication at low concentrations.

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