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Synthesis of an Inhibitor of Human Immunodeficiency Virus Infection

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Anti-HIV effects of lentinan sulfate were investigated by using an HTLV-I-carrying cell line, MT-4, *in vitro*. Lentinan, a fungal branched (1→3)-β-D-glucan, was sulfated to various degrees by means of two kinds of procedures using piperidine N-sulfonic acid in dimethyl sulfoxide or chlorosulfonic acid in pyridine. Lentinan sulfate with a sulfur content of more than 13.9% effectively prevented HIV-induced cytopathic effects (CPE) at concentrations of more than 3.3 μg/ml. However, low-substituted lentinan sulfate did not prevent HIV-induced CPE at any concentration tested. When the counter-cation was 50% Na^+ and 50% pyridinium ion, the inhibitory capacity was low. Anticoagulant activity of the lentinan sulfate was also assessed.

Key words: Anti-HIV effect — Sulfated polysaccharides — Lentinan sulfate — Anticoagulant activity

It is well-known that AIDS (acquired immune deficiency syndrome) in a contagious disease which shows high mortality and is caused by human immunodeficiency virus (HIV).[1)] Its prevention and medical treatment are important and urgent problems.

HIV binds to a T4 lymphocyte receptor (CD4) using a glycoprotein encoded by an *env* gene of HIV, and penetrates into the cell.[2,3)] Then, virus RNA is transcribed to DNA by reverse transcriptase and is integrated into the cell's DNA.

Concerning treatment, AZT (3′-azido-2′,3′-dideoxythymidine) is effective against HIV,[4)] and is undergoing clinical trials.[5,6)] Recently, it has been reported that heparin, dextran sulfate, and other sulfated polysaccharides are potent anti-HIV substances *in vitro*.[7,8)] It has also been shown that sulfated polysaccharides inhibit reverse transcriptase of HIV without interfering with cell growth *in vitro*.[8,9)] Furthermore, lentinan, which is a branched (1→3)-β-D-glucan having antitumor activity, was sulfated and the obtained lentinan sulfate completely prevented HIV-induced cytopathic effects (CPE) at low concentration.[10)]

In this study, the relationship of the structure of lentinan sulfate to the anti-HIV effect was investigated *in vitro*. The anticoagulant activity of lentinan sulfate was also studied.

The sulfation of lentinan ($\overline{M}_n = 5 \times 10^5$) was carried out with piperidine N-sulfonic acid in DMSO according to the method of Nagasawa *et al.*,[11)] or with chlorosulfonic acid in pyridine according to a modification of the method of Wolfrom and Shen Han.[12)] The results are shown in Table I.

Sulfation of lentinan with piperidine N-sulfonic acid at 70°C (LS-1) gave a high-molecular-weight polysaccharide with low sulfur content. The number of sulfate groups per glucose unit in LS-1 was 0.50, based on the elemental analysis data. LS-1 was relatively difficult to dissolve in water. When the reaction temperature was 80°C (LS-2, LS-3, LS-4), the obtained polysaccharides were of comparatively low molecular weight, were more substituted, and were much more soluble in water. In the case of LS-2, the reaction temperature was slightly lower than 80°C, resulting in the formation of a high-molecular-weight polymer. So, this sulfation reaction is very sensitive to reaction conditions. From data on sulfur content and $\overline{M}_n$ of sulfated polysaccharides, it was speculated that the sulfation of the hydroxyl groups and the main chain scission occurred simultaneously. When the reaction temperature was below 70°C, the resulting polysaccharide did not show the metachromasia reaction with toluidine blue, indicating the absence of organic sulfate groups.

Sulfation of lentinan with chlorosulfonic acid in pyridine gave a lentinan sulfate in which two of the three hydroxyls were sulfated (LS-5). The ^1H and ^{13}C NMR spectra of LS-5 in D_2O have absorptions in the aromatic region (8.0–8.7 ppm in PMR and 130, 144, and 150 ppm in CMR) which were not found in the spectra of other lentinan sulfates (LS-1–4). Those aromatic absorptions can be assigned to pyridinium cation. Thus, LS-5 contains two kinds of sulfate groups, one of which is present as the pyridinium salt and the other, as the sodium salt.

Anti-HIV activity of the sulfated lentinan was assayed in terms of the inhibition of HIV-induced CPE and the

Table I. Sulfation of Lentinan[a]

No.	Lentinan (g)	Piperidine-N-sulfonic acid (g)	DMSO (ml)	Temp. (°C)	Yield (g)	$[\alpha]_D^{25}$[b] (deg)	$\overline{M}_n$[c] ($\times 10^4$)	Sulfur content (%)
LS-1	0.5	5.0	140	70	0.59	−5.8	>10	6.83
LS-2	0.3	3.0	50	80	0.32	+3.9	4.8	13.91
LS-3	0.3	3.0	50	80	0.44	+3.2	1.8	16.19
LS-4	0.3	3.0	50	80	0.55	+2.2	1.9	16.35
LS-5	0.5	17.5[d]	100[e]	90	1.42	+1.1	2.3	14.50

a) Reaction time 1 h.
b) Measured in water (c 1).
c) Determined by GPC.
d) Sulfating reagent, chlorosulfonic acid.
e) Solvent, pyridine.

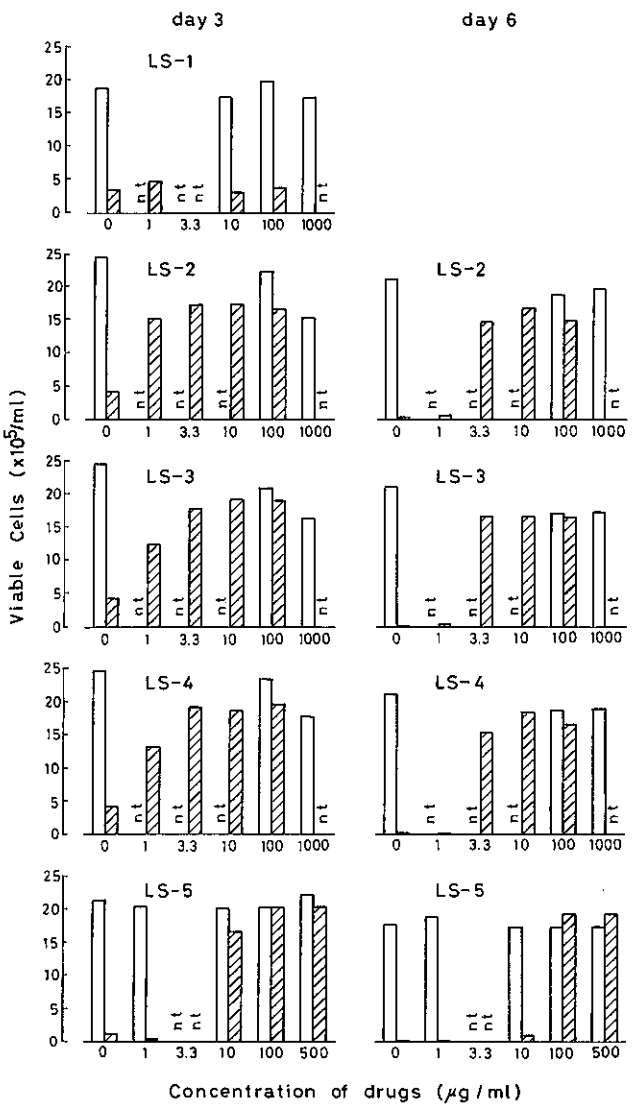

Fig. 1. Effects of lentinan sulfates synthesized by different procedures on cell growth and HIV-induced CPE. MT-4 cells (open bars) and MT-4 cells infected with HIV at a multiplicity of infection of 0.002 (slash bars) were adjusted to 3×10^5 cells/ml and cultured in the presence of various concentrations of the compounds. On the 3rd day after infection, half of the medium was changed. The effects of the compounds were monitored by counting the viable cells (identified by the trypan blue dye exclusion method) 3 and 6 days after infection. nt, Not tested.

expression of virus-specific antigens in MT-4 cells, a human CD4-positive cell line carrying HTLV-I. Details of the method were reported previously.[8, 10] The growth-inhibitory effect of lentinan sulfate on MT-4 cells and the protective effect against HIV-induced CPE were assessed on the 3rd and 6th days after HIV infection.

At concentrations less than 1000 μg/ml (500 μg/ml for LS-5), no sulfated polysaccharide inhibited the growth of the MT-4 cells. It is noteworthy that LS-2, which has a number-average molecular weight ($\overline{M}_n$) of 4.8×10^4 and a sulfur content of 13.91%, did not inhibit the cell growth at 1000 μg/ml (Fig. 1, open bars), in contrast to the inhibition of the cell growth by dextran sulfate ($\overline{M}_n$ 3.4×10^4) at 1000 μg/ml.[8] When MT-4 cells were infected with HIV, the number of viable cells decreased due to the HIV-induced CPE (Fig. 1, slash bars). At concentrations above 3.3 μg/ml, LS-2, LS-3, and LS-4 effectively protected MT-4 cells from destruction by HIV infection. The drug concentration of 3.3 μg/ml is quite low, showing that lentinan sulfate with more than 1.7 sulfate groups per glucose unit is one of the best anti-HIV compounds among the sulfated polysaccharides. It is remarkable that these lentinan sulfates (LS-2, LS-3, LS-4) mostly inhibited the HIV infection at concentrations 300-fold lower than those inhibiting the growth of cells without HIV-infection.

Table II. Biological Activities of Lentinan Sulfate

No.	Sulfur content (%)	$\overline{M}_n$ ($\times 10^4$)	Number of sulfate groups per glucose unit [a]	Anticoagulant activity [b] (unit/mg)	Concentration for protection of HIV-induced CPE (μg/ml)
LS-1	6.83	>10	0.50	0	>100
LS-2	13.91	4.8	1.69	54	3.3
LS-3	16.19	1.8	1.95	21	3.3
LS-4	16.35	1.9	2.07	21	3.3
LS-5	15.31	2.3	ca. 2	26	100

a) Calculated from elemental analysis data.
b) Determined by use of bovine serum according to a modification of the United States Pharmacopoeia method.[13]

Table III. Expression of Viral Antigen in HIV-infected MT-4 Cells[a]

Concentration (μg/ml)	IF-positive cells (%)				
	LS-1	LS-2	LS-3	LS-4	LS-5
1000	nt[b]	0	0	0	0[c]
100	32[d]	0	0	0	0
10	nt	0	0	0	>50
3.3	nt	0	0	0	nt
1	nt	100	100	100	100
0.33	nt	100	100	100	100
0	100	100	100	100	100

a) Measured by the indirect immunofluorescence method on 6th day after infection.
b) Not tested.
c) Concentration, 500 μg/ml.
d) The 3rd day after infection.

The lentinan sulfate, LS-1, which has a small number of sulfate groups per glucose unit did not protect MT-4 cells from HIV-induced CPE.

When approximately half of the sodium ions was replaced with pyridinium ions as counter cations (LS-5), the anti-HIV effect was low. At the concentration of 10 μg/ml, LS-5 did not protect MT-4 cells from HIV-induced CPE. In this case, the concentration of 100 μg/ml was necessary for effective protection of MT-4 cells from HIV-induced CPE. At present, this result must be regarded as tentative, since the different conditions used for the preparation of LS-5 may have resulted in a structurally different product, which would have different activity.

Inhibition of HIV-induced CPE by lentinan sulfate depended on the counter cation of the sulfate group and the degree of sulfation, and might not be so much affected by the molecular weight, whereas the anticoagulant activity (AA) of lentinan sulfate depended on the degree of sulfation and the molecular weight. The AA of LS-5 with $\overline{M}_n$ of 2.3×10^4 was 26 units/mg, being similar to those of LS-3 and LS-4 with $\overline{M}_n$ of 1.8×10^4 and 1.9×10^4, respectively. On the other hand, AA of LS-2 with $\overline{M}_n$ of 4.8×10^4 was 54 units/mg, being much higher than those of LS-3, LS-4, and LS-5. LS-1 did not show the anticoagulant activity at all.

The inhibitory effect of lentinan sulfate was also investigated in an assay of the HIV-specific antigens. MT-4 cells infected with HIV at a multiplicity of infection of 0.002 were cultured in the presence of various concentrations of lentinan sulfate. The inhibitory effect of lentinan sulfate on the expression of HIV-specific antigen was determined by the indirect immunofluorescence (IF) method, using a seropositive anti-HIV human serum and an anti-human IgG conjugated with a fluorescent substance. More than 500 cells were counted under a fluorescence microscope and the percentage of IF-positive cells was calculated 3 and 6 days after infection. LS-2, LS-3, and LS-4 strongly inhibited viral antigen formation at a concentration of 3.3 μg/ml (Table III).

In order to prevent the development of AIDS in asymptomatic carriers by treatment with sulfated poly-

saccharide, long-term administration may be necessary. In this case, lentinan sulfate (LS) is superior to dextran sulfate (DS) in some respects, as follows. 1. LS showed high anti-HIV activity and even relatively high-molecular-weight LS (LS-2) caused no growth inhibition of cells. On the other hand, high-molecular-weight DS inhibited cell growth at the concentration of 1000 μg/ml and the anti-HIV effect of low-molecular-weight DS was relatively weak.[8] 2. Antibodies are formed against dextran,[14] whereas lentinan is not antigenic. 3. Since DS and LS are α- and β-glucopyranans, respectively, a longer lifetime of LS in human blood, which contains a lot of α-glucosidase, is expected.[15, 16]

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