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RAPID COMMUNICATION

SULFATION OF THE IMMUNOMODULATING POLYSACCHARIDE LENTINAN: A NOVEL STRATEGY FOR ANTIVIRALS TO HUMAN IMMUNODEFICIENCY VIRUS (HIV)

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It has been clearly established that human immunodeficiency virus (HIV) is a causative agent of the acquired immunodeficiency syndrome (AIDS). The virus preferentially infects and kills CD-4-positive (helper/inducer) T lymphocytes resulting in severe T4 lymphopenia as well as indirect suppression of multiple immune functions which are dependent on induction of T4 cells (1-2). Since AIDS is a disease in which the HIV destroys the T4 cells slowly but continuously, it is apparent that any attempt to treat AIDS must include anti-HIV therapy. Furthermore, attempts must be made to reconstitute immunologic disfunction caused by the virus.

Recently, it has been reported that a sea algal extract (SAE) from Schizymenia pacifica inhibits viral adsorption to the cells and reverse transcriptase (RT) of HIV without interfering with cell growth in vitro (3-4). The active substance in the SAE was found to be a member of the carrageenan family, a sulfated polysaccharide. It has also been shown that a number of sulfated compounds such as Evans blue and suramin were anti-HIV substances in vitro (5).

The following represents a continuation of this team's studies on the chemical modification of compounds for inhibitors against HIV. In this study, the immunomodulator lentinan and other nonsulfated polysaccharides were sulfated and their anti-HIV effects were studied in vitro.

MATERIALS AND METHODS

Compounds. Curdlan galactose sulfate(CGS), curdlan arabinose sulfate(CAS), and lentinan sulfate(LS) are sulfated compounds of D-galactosyl curdlan(CG), oligo-D-arabinosyl curdlan(CA) and lentinan, respectively. CG and CA were synthesized by methods described previously (6). D-galactosyl curdlan was synthesized by the reaction of acetyl galactose orthoester(3,4,6-tri-O-acetyl-(1,2-O-ethylorthoacetyl-α-D-galactopyranose)) with curdlan acetate, followed by the deesterification of acetylgroups with dilute sodium hydroxide solution. The ratio, D-galactose unit (side chain)/D-glucose unit (main chain), was 0.20. Oligo-D-arabinosyl curdlan was synthesized by the reaction of benzoyl D-arabinose orthoester(3-O-benzoyl-(1,2,5-O-orthobenzoyl)-β-D-arabinofuranose))

with curdlan acetate, followed by deesterification with 1M sodium methoxide-methanol solution. The ratio, D-arabinose unit (side chain)/D-glucose unit (main chain), was 1.05. The sulfation of free polysaccharide was carried out as described elsewhere (7). The sulfur contents of CGS, CAS and LS were 9.55%, 12.29%, and 13.93%, respectively. The number-average molecular weight of lentinan sulfate was about 2 X 10^4 dalton which was obtained by gel permeation chromatography using dextran as reference, and about 3 X 10^4 dalton using pullulan as reference.

Anti-HIV assay. Anti-HIV activity of compounds was analyzed by measuring the decrease in the number of viable cells and indirect immunofluorescence (IF) method using MT-4 cells (8).

Inhibition of syncytia formation. In the study for multinucleated giant cell formation due to cell to cell infection, a human T cell line, MOLT-4 and its HIV producing cell, MOLT-4/$HIV_{HTLV-IIIB}$ were used(9). MOLT-4 and MOLT-4/$HIV_{HTLV-IIIB}$ cells were mixed (in a ratio of 1:1) adjusting to a final cell density of $5x10^5$ cells/ml, as described (10).

Reverse transcriptase (RT) inhibition assay. The assay for RT inhibitory activity of compounds was performed at 37°C for 30 min with 1U of purified avian myeloblastosis virus (AMV) reverse transcriptase mixed with various concentrations of compounds by methods described previously(11).

Anticoagulant activity. The anticoagulant activity of lentinan sulfate was determined using bovine serum as described previously (12). Dextran sulfate (Meito Sangyo NC-1032, Tokyo) was used as a control.

RESULTS

When the inhibitory effect of LS, CGS and CAS was examined on viral antigen expression in HIV-infected MT-4 cells, these compounds strongly inhibited viral antigen synthesis at a concentration of 10 μg/ml. (Table 1). This concentration was the same as that required for complete protection against HIV-cytopathogenicity. (Fig. 1)

Table 1. Expression of viral antigen in HIV-infected MT-4 cells.

Concentration (μg/ml)	IF-positive cells (%)					
	LS	CAS	CGS	L	CA	CG
500	0	0.2	0.2	>90	>90	>90
100	0.2	0.2	0.2			
10	0	0	0.4			
1	>90	>90	>90			
0	>90					

Measured by indirect immunofluorescence method on the 6th day after infection.

The protective effect of these three sulfated compounds on HIV-induced cytopathic effect (CPE) was assessed on the 3rd and 6th days after infection. All three compounds completely protected MT-4 cells against destruction by HIV infection at concentrations above 10 μg/ml. Whereas their parental unsulfated compounds, lentinan CG and CA showed almost no inhibitory effect on HIV even at the concentration of 500 μg/ml. At the concentration of 500 μg/ml, these three compounds and their sulfated counterparts did not inhibit the growth of the MT-4 cells. (Fig. 1)

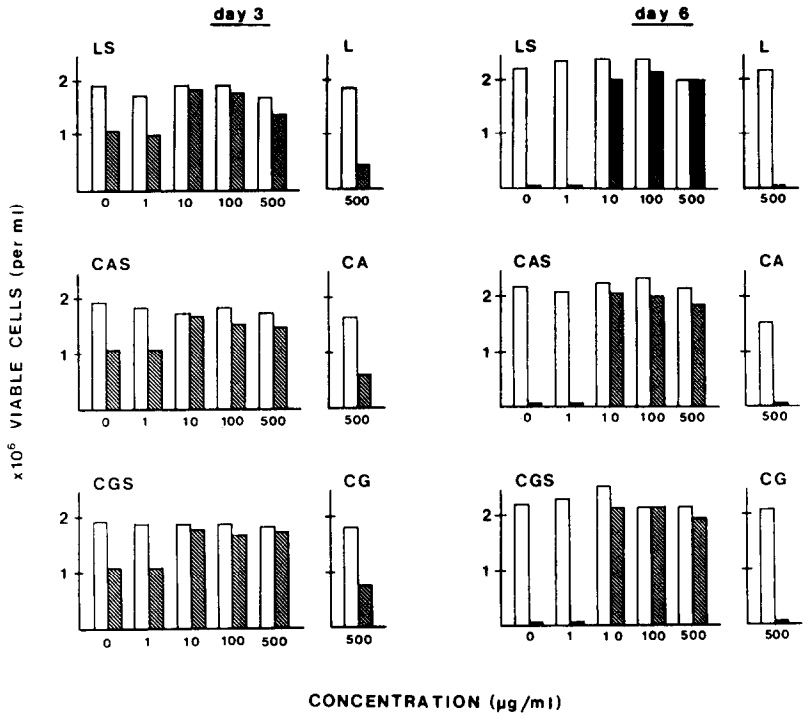

Fig.1 Effect of LS, CAS, CGS, L(Lentinan), CA, CG on cell growth and HIV-induced cytopathic effect. MT-4 cells(open bars) and HIV-infected MT-4 cells(slash bars) were adjusted at 3 X 10^5 cells/ml and cultured in the presence of various concentrations of the drugs. On the 3rd day after infection, half of the medium was changed. The effects of the drugs were monitored by counting viable cells(trypan blue dye exclusion method) 3 and 6 days after infection.

The effect of the sulfated polysaccharides on syncytia formation between MOLT-4 and MOLT-4/$HIV_{HTLV-IIIB}$ cells was then examined. After 20 hr of cocultivation, the multinucleated giant cell formations were evident in the control cultures without drugs. On the other hand, all sulfated compounds at concentrations of 10-100 μg/ml clearly blocked syncytia induction. (Table 2)

Table 2. Effect of various polysaccharides on syncytia formation

Concentration (μg/ml)	Fusion index* (% of inhibition)		
	LS	CAS	CGS
100	0.02	0.13	0.085
10	0.25	0.27	0.50
1	5.7	2.1	1.4
0.1	4.7	4.7	4.9
0	3.4		

MOLT-4 and MOLT-4/$HIV_{HTLV-IIIB}$ cells were mixed 1:1 in proportion to cell number and were adjusted to a final concentration of 5 X 10^5 cells/ml. After 20 hr of cocultivation, the number of cells was counted by trypan blue dye exclusion method. The calculation of the fusion index was done as follows:

$$\text{Fusion index} = \frac{\text{Cell number in control well}}{\text{Cell number in test well}} - 1$$

*Control wells were cultured only with MOLT-4 cells.

Table 3. Inhibition of various polysaccharides against the RT activity of AMV.

Concentration (μg/ml)	% Inhibition					
	LS	CAS	CGS	L	CA	CG
100	98	98	99	6	7	9
10	76	90	79			
1	31	51	61			
0.1	6	36	42			

Various concentrations of the compounds were mixed with 1 unit of AMV-RT enzyme and inhibitory activity was assessed.
Percentage of inhibition was calculated as the

$$\text{ratio} = \left(1 - \frac{\text{counts in the presence of inhibitor}}{\text{counts in the absence of inhibitor}}\right) \times 100$$

The inhibitory effect of these compounds was then examined on RT activity of AMV. All three sulfated compounds LS, CGS and CAS, showed >98% reduction of RT activity at the concentration of 100 μg/ml, and 50% inhibitory concentrations of LS, CGS and CAS were 2.5 μg/ml, 1 μg/ml and 0.3 μg/ml, respectively. However, all of the unsulfated compounds had no inhibitory effect on RT activity at all. (Table 3)
Finally, the anticoagulant activity of lentinan sulfate was studied and was determined as 23 unit/mg. This value is almost equivalent to that (20.6 unit/mg) of a commercial dextran sulfate.

DISCUSSION

Any attempt at AIDS therapy must include not only the suppression of viral replication thereby halting the destruction of T4 cells, but also a strategy to enhance the immune system. Thus, it is practical to design certain drugs utilizing a chemical modification of the substances which originally possess such activities, i.e. which modify the immune system or induce immunologic reconstitution in AIDS. The present study was carried out with this point in mind. Under the experimental conditions mentioned previously 10-500 μg/ml of LS, CAS and CGS almost completely inhibited HIV-induced CPE and viral antigen synthesis. Synthetic sulfated polysaccharides apparently contained RT inhibiting activity in a cell-free assay system. Since the original substances, lentinan and curdlans did not contain the ability to inhibit RT, it was apparent that the addition of sulfate residues endowed the compound with such ability (13). It should, however, be noted that no data are available at present to show that they can be taken up into the cells. Moreover, it is still possible that even if they are taken up, they may be subject to cleavage by lysosomal enzymes before they could act as RT inhibitors. Therefore, the anti-HIV activity of sulfated polysaccharides can not be attributed to RT inhibition without this information. On the other hand, these sulfated compounds efficiently blocked cell to cell infection of HIV in MOLT-4 cells. This strongly suggests that these compounds inhibit adsorption of HIV to CD4 receptors, an initial step of HIV infection.

The back bone of all three compounds (lentinan, CA and CG) was β-1,3-glucan with 1,6 branches, their branches being glucose, arabinose and galactose, respectively. The effective doses of these three compounds were all 10 μg/ml against cell destruction by HIV infection. Moreover, these compounds also inhibited giant cell formation.

Previously it was reported that dextran sulfate (β-1,6-glucan structure) at concentrations of 10-100 μg/ml inhibited HIV-induced CPE, giant cell formation and RT activity (14).

With respect to immunomodulatory activity of lentinan sulfate, curdlan arabinose sulfate or curdlan galactose sulfate, these compounds completely abolished antitumor activity in transplanted allogenic or syngenic tumor models, such as S 180-ICR or P 815-DBA/2, in vivo, whereas the non-sulfated polysaccharides lentinan, arabinosyl curdlan or galactosyl curdlan showed strong antitumor activity. The antitumor activity of lentinan depends on its molecular weight, that is, its activity was observed in a range of molecular weights from 1×10^4 to 1×10^6 dalton. Sulfation of lentinan decrease molecular weight to $2 - 3 \times 10^4$ dalton. Therefore,the abolishment of antitumor activity in sulfated polysaccharides will be caused by sulfation, but not by decrease in molecular weight. Further experiments are necessary to clarify the difference in immunomodulatory activity between non-sulfated and sulfated polysaccharides.

Finally, it should be noted that sulfation of the substances apparently added the ability not only to inhibit cell-free infection of HIV in MT-4 cells but also ability to block cell fusion reactions of MOLT-4 cells. At present it is not clear why sulfation of the compounds results in the generation of such activities.

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