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SHORT COMMUNICATIONS

Inhibition of HIV-1 infectivity with curdlan sulfate *in vitro*

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Antiviral activity of sulfated polysaccharide compounds was first reported by Gerber *et al.* in 1958 [1], who found that embryonic eggs were protected against influenza B and mumps virus by agar polysaccharides extracted from *Gelidium cartilagenium* and carrageenan from *Chondrus crispus*. The following two decades, various sulfated polysaccharides, such as dextran sulfate [2, 3], heparin [4], sulfated glycosaminoglycan [5] or sulfated polysaccharide from species of Dumontiacea [6], were found to inhibit viral replication of herpes simplex, coxsackie A9, yellow fever, and polio viruses.

The emergence of AIDS in 1981 [7] caused by HIV infection [8, 9] afforded an opportunity to evaluate sulfated polysaccharide compounds in antiviral activity against HIV. Carrageenan [10], heparin [11], dextran sulfate [12], fucoidan [12], pentosan polysulfate [13], polysulphated polyxylan [14] and mannan sulfate [15] showed anti-HIV activity *in vitro*. For example, the chemical bond of these polysaccharides consists of sulfated linear D-galactans with alternate 1→3-α and 1→4-β linkages in carrageenan, sulfated linear D-glucans with 1→6-α linkages in dextran sulfate and regular sequences of a tri-sulfated 1→4-β linked disaccharide repeating unit in heparin. Besides these sulfated polysaccharides, potent anti-HIV activity has been observed in sulfated 1→3-β-D-glucan with branched chain of 1→6-β-D-glucan such as lentinan sulfate [16]. Therefore, based upon the findings of the characteristic properties of sulfated polysaccharides against HIV infectivity, curdlan sulfate (CRDS) with 1→3-β-D-glucan as a main chain has been synthesized in order to evaluate its inhibitory activity against HIV and therapeutical availability in preliminaries.

Materials and methods

Compounds. CRDS is a sulfated curdlan. Curdlan is a straight 1→3-β-D-glucan. The sulfation procedure was described elsewhere [17]. The chemical structure of CRDS is shown in Fig. 1a. Its molecular weight (M.W.) defined by gel permeation chromatography has been in the range of 2×10^4 to 25×10^4 daltons according to sulfation conditions. Sulfur content (S.C.) of CRDS defined by ion chromatography has been in the range from 10.5 to 16.2%, that is, the number of sulfate groups per glucose unit has varied between 0.8 and 1.7.

Anti-HIV assay. Anti-HIV activity of CRDS was analysed by the cellular viability using the trypan blue dye exclusion method and by HIV-1 antigen expression using the indirect immunofluorescence assay [18].

Cells. MT-4 cells, a T4 lymphotropic cell line carrying human T-cell lymphotropic virus type 1 [19].

Virus. HIV-1 was obtained from the culture supernatant of a MOLT-4 cell line persistently infected with HTLV-IIIB [9].

Results and discussions

CRDS (M.W. = $(7.9 \pm 0.6) \times 10^4$ daltons, S.C. = $15.2 \pm 0.3\%$) has completely inhibited HIV-antigen expression on MT-4 cells, and made MT-4 cells grow normally, at a concentratioon of 3.3 μg/mL or more (Fig. 1b). Moreover, syncytium formation has been blocked (data not shown). Cytotoxic activity of CRDS against uninfected MT-4 cells has not been observed at a concentration of up to 5000 μg/mL (Fig. 1b).

Anti-HIV activity of CRDS is closely related to M.W. and S.C. of CRDS. It has been found that CRDS should have a M.W. greater than 5.0×10^4 daltons and contain greater than 13.5% sulfur in order to possess inhibitory action on HIV-1 infection at a CRDS concentration of 3.3 μg/mL (Fig. 2).

In order to evaluate the inhibitory effect of a compound against viral infection, it is important to see whether the

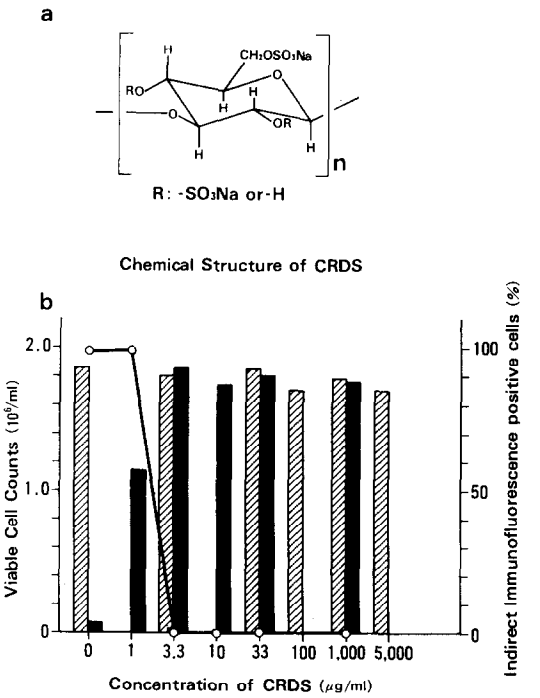

Fig. 1. (a) The chemical structure of CRDS. (b) The inhibitory effect of CRDS against HIV-1 at various concentrations of the compound, and the direct toxicity of CRDS to MT-4 cells. (■) Viable cell counts of MT-4 in co-cultivation of MT-4, MT-4/HIV-1 and CRDS on day 6. (▨) Viable cell counts of MT-4 in co-cultivation of MT-4 and CRDS on day 6. (○) Percentage of HIV-1 antigen positive cells assessed with the indirect immunofluorescence assay on day 6 after co-cultivation of MT-4, MT-4/HIV-1 and CRDS. MT-4 cells were suspended at a concentration of 12×10^5 cells/mL, and CRDS solutions were prepared as 2, 6.6, 66, 200, 2000 and 10,000 μg/mL. MT-4 cell suspension (0.25 mL) and CRDS solution (0.5 mL) were mixed in microtiter wells, and 0.25 mL of HIV-1 producing MT-4 cell suspension with cell density of 2.4×10^3 mL was added to those microtiter wells. The cell number ratio of MT-4/HIV-1 vs MT-4 was 0.002. Cultivation was carried out at 37°, 5% (v/v) CO_2 for 6 days in order to examine the anti-HIV-1 activity.

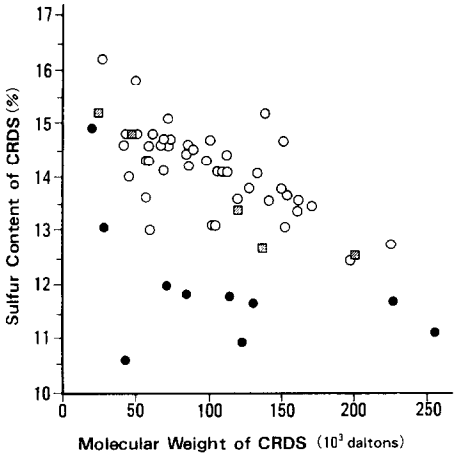

Fig. 2. The relation of anti-HIV-1 activity of CRDS to sulfur content and molecular weight of the compound. CRDSs with various M.W. and S.C. were synthesized and anti-HIV-1 activity of those compounds were assayed employing the assay system indicated in Fig. 1b in order to find the optimal M.W. and S.C. of CRDS for inhibitory activity against HIV-1 infection. Anti-HIV-1 activity of each CRDS was found at CRDS concentration of 3 μg/mL (○); 10 μg/mL (▨) or >10 μg/mL (●).

infectivity of the virus will appear again in a compound-depleted culture following co-cultivation of the compound with infected cells and target cells. Nevertheless, in studying HIV no compound has been positively evaluated by employing such an assay system. Therefore, we assayed HIV-1 infectivity in the CRDS-depleted cell culture following co-cultivation of HIV-1 infected MT-4 (MT-4/HIV-1), MT-4 and CRDS at cultivation times of 24, 48, 72 or 168 hr under the cell number ratio of MT-4/HIV-1 vs MT-4 with 0.002 or 0.5. The antigen expression of HIV-1 on the cell surface in CRDS depleted cell culture completely disappeared 12 days after co-cultivation of MT-4/HIV-1, MT-4 and CRDS for 168 hr, at a CRDS concentration of 5 μg/mL in (MT-4/HIV-1)/(MT-4) of 0.5 (Fig. 3), and normal cell proliferation was observed (data not shown). The same result was obtained with (MT-4/HIV-1)/(MT-4) of 0.002 (data not shown). Thus, MT-4 cells were never infected after incubation with CRDS.

On the other hand, toxicities of CRDS were preliminarily examined employing animal models. LD_{50} of CRDS in intravenous injection was found as around 2 000 mg/kg employing mice or rats, and neither death nor hemorrage observed in consecutive administration of CRDS for two weeks at doses of 50 mg/kg/day in Sprague–Dawley rats (Table 1). Since sulfated polysaccharides were characterized as anti-coagulating agents [20], anti-coagulant activity of CRDS has been determined to be 14 to 16 units/mg (Table 1), which is approximately one tenth of that of heparin, 130 to 150 units/mg. A CRDS concentration of 20 μg/mL in blood increased APTT of CRDS in guinea-pigs or rats by two times as much as in control blood. No antigenicity has been observed in CRDS using a guinea-pig assay system (Table 1).

The half-life of CRDS in plasma has been found to be different depending on its molecular weight, that is, it was 60 min in M.W. of 7×10^4 daltons and 180 min in 17×10^4 daltons by employing a rat model (Table 2). Therefore, in order to maintain an effective CRDS concentration of 5 μg/mL in blood to inhibit HIV-1 infection, the CRDS dosage for continuous infusion is calculated as 6.5–19.5 mg/hr in humans depending on M.W. of CRDS. Total dosage required per day is calculated as approximately 160–

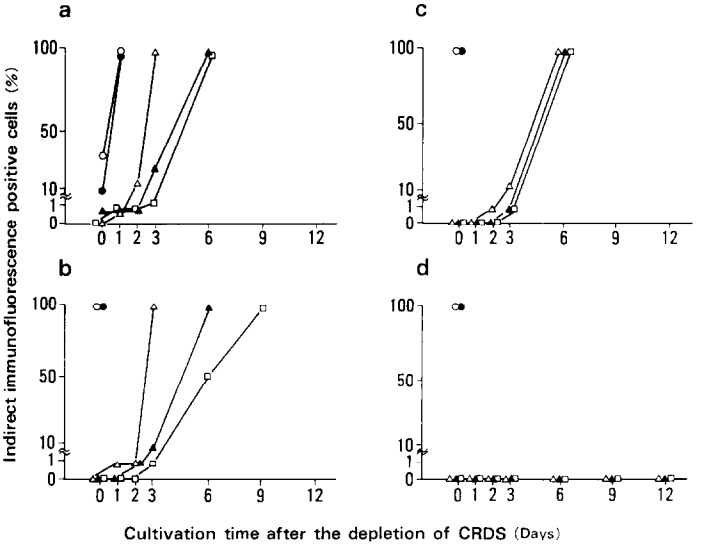

Fig. 3. Inhibitory effect of CRDS (M.W. $=(7.9 \pm 0.6) \times 10^4$ daltons, S.C. $= 15.2 \pm 0.3\%$) against HIV-1 in CRDS depleted cell culture following co-cultivation of MT-4. MT-4/HIV-1 and different concentrations of CRDS [(○) 0 μg/mL, (●) 0.5 μg/mL, (△) 5 μg/mL, (▲) 50 μg/mL and (□) 500 μg/mL] for different co-cultivation periods [(a) 24 hr, (b) 48 hr, (c) 72 hr or (d)168 hr]. MT-4 cells were suspended at a concentration of 12×10^5 cells/mL and CRDS solution were prepared at concentration of 1.0, 10, 100, 1000 μg/mL. MT-4 cell suspension (0.25 mL) and CRDS solution (0.5 mL) were mixed in microtiter wells. HIV-1 producing MT-4 cell suspension (0.25 mL) with cell density of 6×10^5 cells/mL was added to these microtiter wells ((MT-4/HIV-1)/MT-4 = 0.5). After incubation of 24 hr, 48 hr, 72 hr or 168 hr at 37° and 0.5% (v/v) CO atmosphere, each cultured cell suspension was centrifuged to separate cells from supernatant, and separated cells were suspended in the CRDS-free culture medium. These cell suspensions were centrifuged again and the separated cells were suspended in the CRDS-free culture medium for further cultivation. On day 0, 1, 2, 3, 6, 9, 12 after cultivation without CRDS, HIV-1 antigen expression was examined.

Table 1. Toxicity, anti-coagulent activity and antigenicity of CRDS

a. LD_{50} in intravenous injection of CRDS	ICR mice	
	Male (N = 10)	2400 mg/kg
	Female (N = 10)	1900 mg/kg
	Sprague–Dawley rats	
	Male (N = 10)	1920 mg/kg
	Female (N = 10)	2240 mg/kg
b. Clinical sign in consecutive 2-week intravenous injection of CRDS	Neither incidence of death nor hemorrage at doses of 0.5–50 mg/kg/day in Sprague–Dawley rats (N = 10)	
c. Anti-coagulant activity	14–16 units/mg CRDS	
d. Antigenicity	Negative in guinea-pigs	

M.W. and S.C. of CRDS were $(7.9 \pm 0.6) \times 10^4$ daltons and $15.2 \pm 0.3\%$, respectively.

The anti-coagulant activity of CRDS is expressed as heparin equivalent units, taken at normal rat plasma APTT doubling dosages.

No symptoms of active systemic anaphylaxis were seen when guinea-pigs, sensitized by 3 weekly injections of a solution of CRDS and Freund's complete adjuvant, were challenged 2 weeks after the last sensitization.

Table 2. The half-life of CRDS in rat *in vivo*

CRDS	M.W. (dalton)	Sulfur content (% W/W)	$T_{1/2}$ (min)
Lot A	7 × 104	14.6	60
Lot B	17 × 104	14.5	180

CRDS was intravenously injected to Wistar male rats at doses of 50 mg/kg. The blood samples (0.25 mL) were taken from carotid vein under ether anesthesia at 2, 10, 30 min, 2, 6 and 24 hr after injection. Two hundred millilitres of blood sample was mixed with 20 mL of sodium citrate (38 mg/mL) and plasma was separated by centrifugation at 3000 rpm for 10 min. One hundred millilitres of plasma was mixed with 15 mL of trichloroacetic acid (1 g/mL) and supernatant was separated by centrifugation at 3000 rpm for 10 min. The supernatant (100 mL) was charged to the column (TSK-G2500TW-XL: TOYO SODA Co, Ltd) for gel permeation chromatography and eluted with 0.05 M PBS (pH 6) at an elution rate of 1 mL/min. Sulfur atom in the eluate was measured by the inductivery coupled plasma method (SPS Model 1100; SEIKO Co., Ltd). CRDS concentration in plasma was calculated using the following equation:

$$\text{CRDS (mg/mL)} = (A/B) \times 100$$

where A is the sulfur content of CRDS injected plasma (mg/mL) and B is the sulfur content of CRDS (% w/w).

470 mg/day/man; that is, 3.2–9.4 mg/kg/day. These suggested dosages for intravenous injection of CRDS to humans should be carefully examined by further toxicological analysis.

With respect to the mechanisms of action of CRDS, it is most likely that CRDS blocks HIV virion adsorption to target cell surfaces as already reported for the mechanism of other kinds of sulfated polysaccharides [21, 22]. Virus secreted from infected cells and viral antigens expressed on cell surfaces will be blocked by CRDS. Therefore, the viral envelope will not have any contact mechanism to infect the cell surface, resulting in complete inhibition for both viral replication and syncytium formation. Concerning whether sulfated polysaccharides will bind to viral or cellular surfaces, it was indicated that sulfated polysaccharides would bind to viral surfaces, resulting in inhibition of viral binding to the cell surface [23–27]. It is important to make clear which molecule in the envelope glyprotein of HIV-1 that CRDS will bind to.

The possibility that HIV-1 exists as a latent state has been examined by mitogen stimulation assay with phytohemagglutinin or concanavalin A, or by addition of fresh MT-4 cells to the culture, and neither cytopathic effect nor antigen expression was observed in the culture (data not shown). The latent period of HIV-1 might differ in each infected cell; therefore, it will be necessary to investigate the effectiveness of CRDS on HIV-1 by employing other cell lines such as H9, U937 or CEM. In addition, a combined usage of CRDS with cell-stimulating agents such as interleukines or cytokines will make the latent period shorten by provoking viral replication to be faster resulting in amplification of the effectiveness of CRDS.

In summary, Curdlan sulfate (CRDS), a synthetic sulfated polysaccharide with straight 1→3-β-D-glucopyranoside linkage as a mainchain, has been firstly found to display a prominent inhibitory activity against human immunodeficiency virus type 1 (HIV-1) *in vitro* at a concentration of 3.3 μg/mL (approx. 0.04 μM) or more, without any direct toxicity to cells up to a CRDS concentration of 5000 μg/mL.

Furthermore, activity of CRDS against HIV-1 was assessed in CRDS-depleted cell culture following incubation with MT-4/HIV-1, MT-4 cells and CRDS for different periods *in vitro*. The HIV-1 cellular infectivity

completely disappeared in CRDS-depleted cell culture after the incubation for 168 hr at CRDS concentration of 5 μg/mL or more. Thus, the cells were never infected after incubation with CRDS.

The experimental results of CRDS on the LD_{50} by intravenous administration, clinical signs in consecutive intravenous injection for 2 weeks, the anti-coagulant activity, the antigenicity and the half-life have suggested that it would be worthwhile to investigate CRDS in AIDS clinical research with further toxicological examinations.

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Liver esterases and soman toxicity in the rat following partial hepatectomy

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It is well established that soman (1,2,2′-trimethylpropyl methylphosphonofluoridate) is detoxificated through hydrolysis catalysed by the unspecific enzyme somanase (EC 3.1.8.1, organophosphorus compound hydrolase [1]) and binding to serine esterases other than acetylcholinesterase (EC 3.1.1.7) such as carboxylesterase (EC 3.1.1.1; carboxylic ester hydrolase) and cholinesterase (EC 3.1.1.8). These enzymes are widely distributed in the body. In rat liver, which possesses a high capacity of soman detoxification [2] as indicated by great activity, sensitivity and affinity of carboxylesterases and somanases towards the agent [3], the carboxylesterase and cholinesterase activities were inhibited to a lesser extent than in other organs after soman poisoning [4, 5].

The importance of liver in soman detoxification had been previously investigated both by soman perfusion through rat liver [6] and by alteration of liver carboxylesterase and cholinesterase activities after pretreatment with various agents such as phenobarbital [4, 7], triorthocresyl phosphate (TOCP) [4] or 2-(*o*-cresyl)-4H-1:3:2-benzodioxaphosphorin-2-oxide (CBDP) [7–9], tetraisopropyl pyrophosphoramide (iso-OMPA) [7, 10] and *N,N′*-diisopropylphosphordiamidofluoridate (mipafox) [10]. So far, no data has been available concerning the influence of partial hepatectomy on acute toxicity of organophosphorus compounds. The purpose of this study was to compare the abilities of intact and partially removed rat livers to detoxify soman as well as to examine the effect of the widely used method of patial hepatectomy on the acute soman toxicity in rats.

Materials and methods

Animals. All experiments were carried out on male Wistar rats weighing 170–190 g which had food and water *ad lib.* Rats under slight ether anesthesia underwent partial hepatectomy according to the procedure of Waynforth [11]. The technique (originally described by Higgins and Anderson [12]), based on removal of the main lobes (median and left lateral) which comprise approximately two-thirds of the liver, is tolerated well by the animals and simple to perform. However, the ablation of 85% of the liver was not tolerated as well.

Materials. Soman was obtained from the Chemistry Section at the Military Technical Institute. It was 98.8% pure as determined by nuclear magnetic resonance spectroscopy. Other chemicals were obtained from various commercial sources: tributyrin and physostigmine sulfate (Serva, Heidelberg, F.R.G.); methyl butyrate (Fluka, Buchs, Switzerland); ethyl butyrate and atropine sulfate (Sigma Chemical Co., Poole, U.K.); triorthotolyl phosphate (Eastman Kodak, Rochester, NY) and oxime HI-6 (pyridinium, 1-(((4-carbamoylpyridinio)-methoxy) methyl-2-(hydroxyiminomethyl) dichloride) (Institute SBS Sarajevo, Yugoslavia).

Toxicology. Acute toxicity of soman based on 24 hr mortalities was calculated by the method of Weil [13]. TOCP (250 mg/kg, s.c.) was administered 24 hr before performing partial hepatectomy.

Enzyme determinations. Rats were pretreated with physostigmine sulfate (0.2 mg/kg, s.c.) + atropine sulfate (20 mg/kg, i.m.) + HI-6 (50 mg/kg, i.m.) 20 min prior to the s.c. injection of soman. This treatment schedule was adopted to ensure that there were survivors at the 30 min sampling period. Such pretreatment did not affect normal carboxylesterase and cholinesterase activity in rat liver.

Liver homogenate (1:10) in saline was prepared in Potter–Elvehjem homogenizer and centrifuged at 3000 rpm for 10 min.

Carboxylesterase activities were assayed by the pH-stat technique using tributyrin (final concentration 6.5 mmol/L), ethyl (9 mmol/L) and methyl (5 mmol/l) butyrates as substrates. Butyric acid, liberated from enzymatic degradation of the substrates, is titrated with 0.01 N NaOH solution. To a reaction vessel 10 mL of substrate solution was added and then titrated to pH 7.60 at 25°. The amounts of liver homogenate supernatant added were: 0.2 mL for tributyrin and 0.05 mL for ethyl and methyl butyrate hydrolysis. Enzyme activities were calculated from the rate of addition of NaOH during the first 5 min after homogenate was added (and expressed as nmoles of substrate hydrolysed per min per mg of protein). All solutions were CO_2-free. Cholinesterase activity in liver was assayed towards acetylthiocholine iodide as substrate (and expressed as nmoles of substrate hydrolysed per min per mg protein) according to the method of Ellman *et al.* [14]. Protein content was determined by the method of Lowry *et al.* [15].

Statistics. Significance of difference was examined by Student's *t*-test; $P < 0.05$ was considered statistically significant.

Results and discussion

The results presented in Table 1 show that partial hepatectomy markedly increased acute toxicity of soman in rats. Treatment with 250 mg/kg of TOCP, which caused complete inhibition of plasma and skin carboxylesterase and 85% of liver carboxylesterase activities [3], strongly potentiated the toxicity of soman. After TOCP treatment most soman binding sites are already occupied and cholinesterases are then widely exposed to the agent. Under these circumstances detoxification of this compound is greatly altered since the reduction of soman LD_{50} to 15% of its initial value (Table 1) cannot further be attributed to carboxylesterases but to enzymatic hydrolysis. Therefore,