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Anti-HIV (human immunodeficiency virus) activity of sulfated paramylon

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Summary

Sulfated derivatives of paramylon, a water-insoluble (1-3)-β-D-glucan from *Euglena gracilis*, significantly inhibited the cytopathic effect of human immunodeficiency virus (HIV-1, HIV-2) and the expression of HIV antigen in cultured MT-4, MOLT-4 cells and human peripheral blood mononuclear cells. Native paramylon, *N,N*-dimethylaminoethyl paramylon, *N,N*-diethylaminoethyl paramylon, 2-hydroxy-3-trimethylammoniopropyl paramylon chloride, and carboxymethyl paramylon had little or no anti-HIV activity. The anti-HIV activity of the sulfated paramylon derivatives depended on the number of sulfate groups, and the molecular weight. Paramylon sulfate significantly inhibited HIV-1 binding to MT-4 cells. The anti-coagulant activity of the sulfated paramylon derivatives also depended on the number of sulfate groups, but was generally lower than that of dextran sulfate. The results point to the potential of paramylon sulfate in the treatment of HIV infection.

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Abbreviations: HIV, human immunodeficiency virus; PBMC, human peripheral blood mononuclear cells; DMAE, *N,N*-dimethylaminoethyl; DEAE, *N,N*-diethylaminoethyl; HAP, 2-hydroxy-3-trimethylammoniopropyl; CM, carboxymethyl; SR, substitution ratio; CC_{50}, 50% cytotoxic concentration; EC_{50}, 50% effective concentration; SI, selectivity index.

Sulfated paramylon; HIV; Virus adsorption

Introduction

Paramylon is a water-insoluble (1-3)-β-D-glucan (molecular weight: 122 kD) isolated from *Euglena gracilis* (Clark and Stone, 1960). The yield of paramylon from *Euglena gracilis* amounts to approximately 60–70% of the dried cells. However, industrial utilization of paramylon has been limited to the production of some cosmetic materials. We recently reported that paramylon modified with positively charged groups, such as *N*,*N*-dimethylaminoethyl (DMAE), *N*,*N*-diethylaminoethyl (DEAE) and 2-hydroxy-3-trimethylammoniopropyl (HAP), induced potent antimicrobial activity in mice, whereas native paramylon and its derivatives with a negatively charged group did not (Sakagami et al., 1989, 1991). We report here that among these paramylon derivatives, only paramylon sulfate has anti-HIV activity, further confirming the importance of sulfate groups in the anti-HIV activity of polyanions (De Clercq, 1986; Ito et al., 1987; Ueno and Kuno, 1987; Nakashima et al., 1987b; Baba et al., 1988a; Mizumoto et al., 1988). We also examined paramylon sulfate samples of different sulfate content, and investigated the mode of action of the paramylon sulfate and its anti-coagulant activity.

Materials and Methods

Materials

Paramylon was prepared from *Euglena gracilis* E-6 (NIES 48), and modified with DMAE, DEAE, HAP or a sulfate group, as described previously (Sakagami et al., 1989). The molecular weight of paramylon, determined by Manner's method (Manner et al., 1971) and the GPC method, was 122 kD. The substitution ratio (SR) of the introduced groups was determined by the Kjeldahl method (Kjeldahl, 1983), the neutralization titration of free OH, or the sulfur content (with a Micro Colorometric Titrating System, DOHRMANN).

Glucose (MW 180) was obtained from Merck, Darmstadt. Laminaribiose (MW 342), laminaritriose (MW 504), laminaritetraose (MW 667), laminaripentaose (MW 829), laminarihexaose (MW 991) and laminariheptaose (MW 1153), prepared by hydrolysis of pachyman from *Poria cocos* (Whelan, 1962), were obtained from Seikagaku Kogyo (Tokyo). Laminarin (mean MW 5430) was obtained from Sigma (St. Louis, MO). Partially hydrolyzed products of paramylon, Eu-Oligo (I) (mean MW 606) and Eu-Oligo (II) (mean MW 1440), were prepared as described previously (Sakagami et al., 1992). These samples were sulfated as previously described (Sakagami et al., 1989), except that they were desalted by ACILYZER with MW 100 and 300 membrane (Asahi Chem. Ind., Tokyo).

Curdlan (TAK) (87 kD), a water-insoluble (1-3)-β-D-glucan, isolated from *Alcaligenes faecalis* var. *myxogenes*, IFO 13140 (Harada et al., 1968), was modified with a DMAE group, as described previously (Sakagami et al., 1989).

The following reagents were obtained from the indicated companies: Dextran sulfate (8 kD) (Kowa, Tokyo); Curdlan sulfate (300 kD) (Ajinomoto, Tokyo); Pentosan polysulfate (3.1 kD) (Sigma); RPMI 1640 medium (Gibco, Grand Island, NY); fetal calf serum (FCS) (Whittaker Bioproduct, MD); Histopaque-1077 (Sigma); phytohemagglutinin (Sigma); recombinant human interleukin 2 (IL-2) (Shionogi, Osaka); (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) (Wako Pure Chemical, Osaka); fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG (Cappel Organon Teknika, West Chester, PA); p24 antigen assay kit (Abbott Lab. Chicago); poly (rA) : oligo(dT) 12–18 (rAdT) (Pharmacia Fine Chemicals, Uppsala); [methyl-^{3}H]thymidine 5′-triphosphate (30 Ci/ml) (Amersham International plc); [^{14}C]glucose (250–360 mCi/mmol) (NEN Research Product, Du Pont).

Cell lines

Human T lymphotropic virus type I (HTLV-1)-positive T cell line, MT-4, and lymphoblastoid T-cell line, MOLT-4 (clone no. 8) (Kikukawa et al., 1986) were subcultured twice a week at a concentration of 3×10^5/ml in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). Human peripheral blood mononuclear cells (PBMC) from healthy donors were obtained by centrifugation through Histopaque-1077, stimulated with phytohemagglutinin for 3 days, and then cultured in RPMI 1640 medium containing 20% FCS, antibiotics and 5 ng/ml of IL-2.

Virus

A strain of HIV-$1_{HTLV\text{-}IIIB}$ was prepared from the culture supernatant of MOLT-4/HTLV-IIIB cells which was persistently infected with HTLV-IIIB. A strain of HIV-1_{A012B} (clinical isolate) was obtained from an HIV-1 infected patient (Larder et al., 1989). A strain of HIV-2_{ROD} was prepared from the culture supernatant of HIV_{ROD}-infected MT-4 cells. HIV stocks were titrated in MT-4 cells. The virus titers were determined as 50% tissue culture infectious doses or plaque forming units (Harada et al., 1985).

For the RT experiments, HIV-1-infected TALL-1 cells were used as a source of HIV-1 (Mizumoto et al., 1988).

Assay for cell fusion

A cell-mixing syncytium-induction assay was used to evaluate the virus-induced cell fusion (Lifson et al., 1986). The HIV-infected cells were observed under a light microscope at × 200 magnification to determine the number of multinuclear giant cells (syncytia) formed. The extent of syncytium formation was evaluated, based on the following criteria. Wells with no multinucleated

giant cells were scored negative (−). Wells with rare, but unequivocal multinucleated giant cells with more than four nuclei within a common cell membrane, were scored as 1. Wells with more frequent giant cells, but in which most of the × 200 field did not contain giant cells was scored as 2. Wells that contained giant cells in most but not all of the × 200 field, were scored as 3, and wells that contained syncytia in all of the × 200 field were scored as 4.

Inhibition of HIV cytopathicity

Test samples of solution were added to CD-4 positive MT-4 cells (2.5×10^4/well) infected with HTLV-IIIB (7×10^5 PFU/ml) or HIV-2_{ROD} (1×10^6 PFU/ml) at a multiplicity of infection (MOI) of 0.01, in 96-well microtiter plates (Falcon 3071, Becton Dickinson, NJ). To test cytotoxicity, a sample of the solution was added to MT-4 cells without infection. The wells were incubated in a CO_2 incubator at 37°C for 5 days, and the number of surviving cells was determined by the MTT method (Pauwels et al., 1988). The effect of each sample was evaluated by the 50% effective virus-inhibitory concentration (EC_{50}), 50% cytotoxic concentration (CC_{50}), and selectivity index ($SI = CC_{50}/EC_{50}$).

Inhibition of expression of HIV-specific antigen

A test sample solution (500 μl at various concentrations) was added to 500 μl of MT-4 cell suspension (3×10^5/ml) infected with HTLV-IIIB or HIV-2_{ROD} at a MOI of 0.01 in each well, and incubated at 37°C for 5 days. The reaction mixture was then placed in a test tube and centrifuged. The precipitated cells were subjected to laser flow cytofluorographic analysis of HIV-specific antigen (anti-HIV human polyclonal antibody for HTLV-IIIB and mouse monoclonal antibody for HIV-2_{ROD}), as previously described (Nakashima et al., 1987a; Pauwels et al., 1987). The percentage of antigen-positive fluorescent cells (F-cells) was calculated as follows:

$$\frac{(\%\text{ of F-cells in test well}) - (\%\text{ of F-cells in mock MT-4})}{(\%\text{ of F-cells in HIV-infected MT-4}) - (\%\text{ of F-cells in mock MT-4})} \times 100$$

The assay procedure for measuring the anti-HIV-1 activity of the compounds in PBMC was based on the quantitative detection of HIV-1 p24 antigen in the culture supernatant, as described previously (Nakashima et al., 1992a). Phytohemagglutinin-stimulated PBMC (10^6 cells/ml) were exposed to HTLV-IIIB at a MOI of 0.1. After virus adsorption for 1 h, the cells were extensively washed to remove unadsorbed virus particles, suspended in IL-2 (5 ng/ml) containing RPMI 1640 medium, and then cultured at 37°C in the presence of various concentrations of the test compounds. On days 4 and 8 after infection, the cells were subcultured with fresh cultured medium with the appropriate concentration of test compound at a ratio of 1:3. The amount of p24 antigen in the culture supernatant was determined with a sandwich enzyme-linked immunoadsorbent assay kit.

Reverse transcriptase (RT) assay

Extracellular HIV content was measured by RT activity, according to the method of Daniel (Daniel et al., 1985) with slight modification (Mizumoto et al., 1988). MOLT-4 cells were mixed with culture supernatant of HIV-1-infected TALL-1 cells at a MOI of 0.01 in 2 ml of RPMI 1640 medium and incubated for 2 h at room temperature. The cells were washed three times with culture medium, and further incubated for 6 days in 24 well culture plates (Nunc, InterMed) in the presence of test samples. The virus in the culture supernatant was pelleted, and resuspended on ice in 10 μl of dissociation buffer (10 mM Tris-HCl (pH 7.8), 1% NP-40, 1 mM EDTA). To this was added 90 μl of a buffer (40 mM Tris-HCl (pH 7.8), 100 mM $MgCl_2$, 45 mM KCl, 100 μg poly(rA): oligo(dT) 12–18 (rAdT)/ml, 4 mM dithiothreitol) and 2.5 μl [methyl-^{3}H]thymidine 5′-triphosphate (1 μCi/tube). After incubation at 37°C for 60 min, acid-insoluble radioactivity was determined by a liquid scintillation counter.

Inhibition of HIV-binding to MT-4 cells

As previously reported (Schols et al., 1989), MT-4 cells were exposed to HIV-1 stock (MOLT-4/HTLV-IIIB, 7×10^7 PFU/ml) in the presence of various concentrations of the test samples. After incubation at 37°C for 1 h, the cells were washed twice to remove unbound virus. The cells were then processed for indirect immunofluorescence using a human polyclonal anti-HIV-positive serum as the first antibody and an FITC-conjugated rabbit anti-human IgG as the second antibody. After immunofluorescence staining, the cells were washed twice with phosphate-buffered saline (PBS), resuspended in 0.37% paraformaldehyde in PBS, and analyzed by laser flow cytometry (Schols et al., 1989). The binding inhibitory activity ratio (BI) was calculated as follows:

$$BI = \left(1 - \frac{\%\ MF\ (VS) - \%\ MF\ (CS)}{\%\ MF\ (V) - \%\ MF\ (C)}\right) \times 100$$

where MF = mean fluorescence; VS = HIV-infected cells treated with test sample; CS = control cells (not exposed to HIV) treated with test sample; V = HIV-infected cells without test samples; C = control cells (not exposed to HIV and not treated with test sample).

Assay for anti-coagulation activity

After blood was collected from normal human volunteers, it was distributed into 10-ml tubes. Samples dissolved in PBS were then added to achieve a concentration of 10 or 100 μg/ml. 10 min later, the blood was spun down at 2500 rpm for 15 min. The plasma was collected and placed into 10-ml tubes. It was subjected to prothrombin time (PT) assay by the Quick one step method, and to partial thromboplasmin time (PTT) assay by the inactivation method.

Absorption of paramylon sulfate in mice

[^{14}C]Paramylon sulfate was prepared from the *Euglena gracilis* (3×10^9/150 ml), which had been cultured for 24 h with 2.5 mCi [^{14}C]glucose in vitamin-enriched glucose/ammonium medium, and then sulfated so as to obtain SR = 14%. The final product showed a specific activity of 4.9×10^6 dpm/mg.

Female ICR mice (Sankyo Labo. Service Co.) (25 g) were treated orally or intravenously with 0.5 mg (1.24×10^6 dpm) of [^{14}C]paramylon sulfate.

At the indicated times thereafter, 0.65 ml of heparinized blood was collected from the heart of anesthetized mice, mixed with 1.4 ml of 0.9% NaCl, and centrifuged at 2000 rpm for 5 min. The pelleted cells were washed once with 2 ml of 0.9% NaCl. The radioactivity of the combined supernatants was determined in a liquid scintillation counter.

Results

Anti-HIV activity of paramylon derivatives

Paramylon sulfate [substitution ratio (SR): 12.6%] significantly inhibited the cytopathicity of HIV-$1_{HTLV\text{-}IIIB}$ in MT-4 cells (Fig. 1). This effect appeared at 0.14 μg/ml and reached a maximum level at 3.7 μg/ml. Paramylon sulfate (SR: 12.6%) also suppressed the cytopathic effect induced by HIV-2_{ROD} infection (Fig. 1). Cationized paramylons such as DMAE-paramylon (SR: 3.7–6.3%), DEAE-paramylon (SR: 10%) and HAP-paramylon (SR: 4.3%) were inactive (Table 1). Light microscope observation revealed that paramylon sulfate inhibited syncytium formation at 10–100 μg/ml, reducing the score to 1, but the

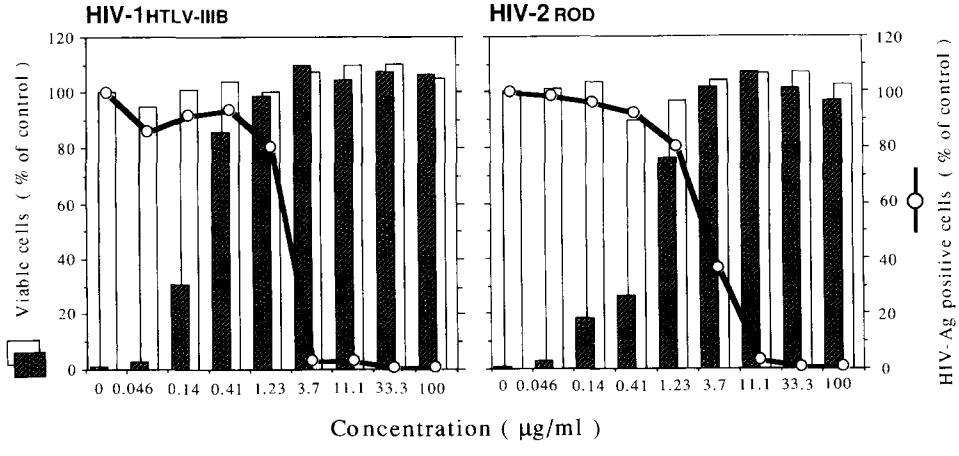

Fig. 1. Inhibition of HIV-induced cytopathicity by sulfated paramylon (SR: 12.6%). Viability of HIV-infected treated MT-4 cells (black bars) and mock-infected treated MT-4 cells (open bars) was expressed as percent of mock-infected and untreated control cells. The number of HIV-1 antigen-positive cells (open circles), determined by indirect immunofluorescence and laser flow cytometry, was expressed as percent of virus-infected and untreated control cells.

TABLE 1

Inhibition of the cytopathicity of HIV-$1_{HTLV\text{-}IIIB}$ by sulfated paramylons

Substance	SR (%)	MW (kD)	CC_{50}		EC_{50}		SI
			(μg/ml)	(μM)	(μg/ml)	(μM)	
DMAE-paramylon	3.7	128	>1000	>7.8	>1000	>7.8	<1
	5.0	130	>1000	>7.7	>1000	>7.7	<1
	6.3	133	>1000	>7.5	>1000	>7.5	<1
DEAE-paramylon	10.0	145	392	2.7	>1000	>6.9	<1
HAP-paramylon	4.3	137	996	7.3	>1000	>7.3	<1
DMAE-curdlan	5.0	87	>1000	>11.5	>1000	>11.5	<1
Paramylon sulfate	0.07	122	>1000	>8.2	>1000	>8.2	<1
	0.08	123	>1000	>8.1	>1000	>8.1	<1
	4.0	132	>1000	>7.6	50.1	0.38	>20
	4.1	132	>1000	>7.6	3.7	0.028	>274
	7.2	139	>1000	>7.2	4.0	0.029	>252
	8.7	143	>1000	>7.0	3.7	0.026	>273
	9.2	144	>1000	>6.9	3.5	0.024	>284
	12.6	151	>1000	>6.6	2.5	0.017	>407
	14.0	156	>1000	>6.4	1.8	0.012	>563
	15.6	159	>1000	>6.3	2.8	0.018	>356
Eu-oligo (I) sulfate			226		>300		<1
Eu-oligo (II) sulfate			241		>300		<1
Glucose sulfate			232		>300		<1
Laminaribiose sulfate			180		>300		<1
Laminaritriose sulfate			187		>300		<1
Laminaritetraose sulfate			198		>300		<1
Laminaripentaose sulfate			238		>300		<1
Laminarihexaose sulfate			254		>300		<1
Laminariheptaose sulfate			247		>300		<1
Laminarin sulfate			240		>300		<1
Dextran sulfate		8	>1000	>125	0.9	0.11	>1130
Curdlan sulfate		300	>1000	>3.3	0.6	0.002	>1748
Pentosan polysulfate		3	>1000	>333	1.2	0.39	>819

Lower molecular weight substances, such as Eu-oligo (I, II), glucose and laminarioligosaccharides and laminarin were sulfated under the same conditions used to produce paramylon sulfate (SR: 14.0%).

other (inactive) derivatives did not (score: 3–4) (data not shown). Most paramylon derivatives, except for DEAE-paramylon (CC_{50}: 392 μg/ml), showed little or no cytotoxicity (CC_{50} >1000 μg/ml) (Table 1).

Paramylon sulfate (SR: 12.6%) and dextran sulfate reduced the extracellular concentration of HIV-1, as monitored by RT activity. Their 50% effective concentration (EC_{50}) was 5 and <1 μg/ml, respectively (Fig. 2). DMAE-paramylon, DEAE-paramylon and CM-paramylon (SR: 1.4%) were inactive at a concentration below 100 μg/ml (Fig. 2).

The inhibitory activity of paramylon sulfate was dependent on the sulfate content. Anti-HIV activity of paramylon sulfate was significantly increased

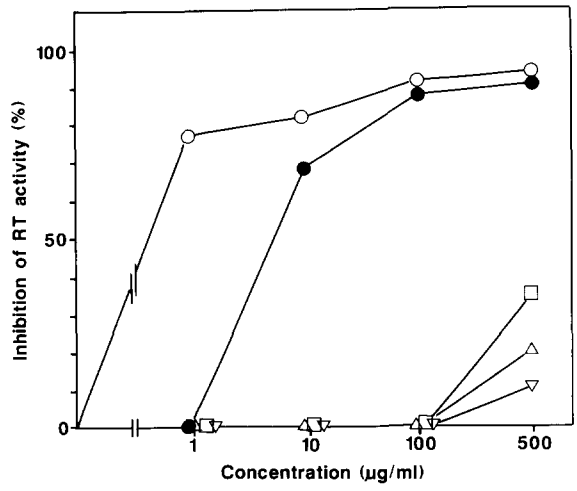

Fig. 2. Effect of paramylon derivatives on extracellular RT activity. RT activity was determined in the supernatant of HIV-$1_{HTLV\text{-}IIIB}$-infected Molt-4 cells, cultured in the presence of the indicated amounts of intact paramylon (▽), paramyxlon sulfate (SR: 14%) (●), DMAE-paramylon (SR: 5%) (△), CM-paramylon (SR: 1.4%) (□) or dextran sulfate (SR: 8%) (○). Control RT activity was 12,386 cpm.

when SR of the sulfate groups exceeded 4.0% (Table 1). The EC_{50} values of the paramylon sulfates were 2.5 μg/ml (0.017 μM) for SR12.6%; 1.8 μg/ml (0.012 μM) for SR14.0%; and 2.8 μg/ml (0.018 μM) for SR15.6%. The selectivity index (SI) of these compounds was > 407, > 563 and > 356, respectively. However, anti-HIV activity of paramylon sulfate was somewhat lower than that of dextran sulfate (SI > 1130), Curdlan sulfate (SI > 1748) or pentosan polysulfate (SI > 819) (Table 1).

Sulfated derivatives of partially hydrolyzed paramylon, Eu-Oligo (I) (mean MW 606) and Eu-Oligo (II) (mean MW 1440), had no apparent anti-HIV activity (SI < 1), but had higher cytotoxicity (CC_{50}: 226–241 μg/ml). The sulfated derivatives of glucose (MW 180), laminarioligosaccharides (MW 342–1153) and laminarin (mean MW 5430) were also inactive (SI < 1) (Table 1).

Effect on HIV antigen expression

Fig. 1 shows that paramylon sulfate (SR: 12.6%) caused a concentration-dependent reduction of the expression of HIV-specific antigens in MT-4 cells. The 50% inhibitory concentrations for HIV-1 and HIV-2 were 2.2 and 2.9 μg/ml, respectively.

Paramylon sulfate samples (SR: 12.6%, 14.0%) also caused a concentration-dependent inhibition of HIV-$1_{HTLV\text{-}IIIB}$ p24 antigen expression in PBMC (EC_{50}: <0.32 μg/ml) (Fig. 3A) and of antigen expression of a clinical isolate (HIV-1_{A012B}) in MT-4 cells (EC_{50}: 0.1–1 μg/ml) (Fig. 3B).

HIV-binding assay

As shown in Fig. 4, paramylon sulfate (SR: 12.6%) caused a concentration-

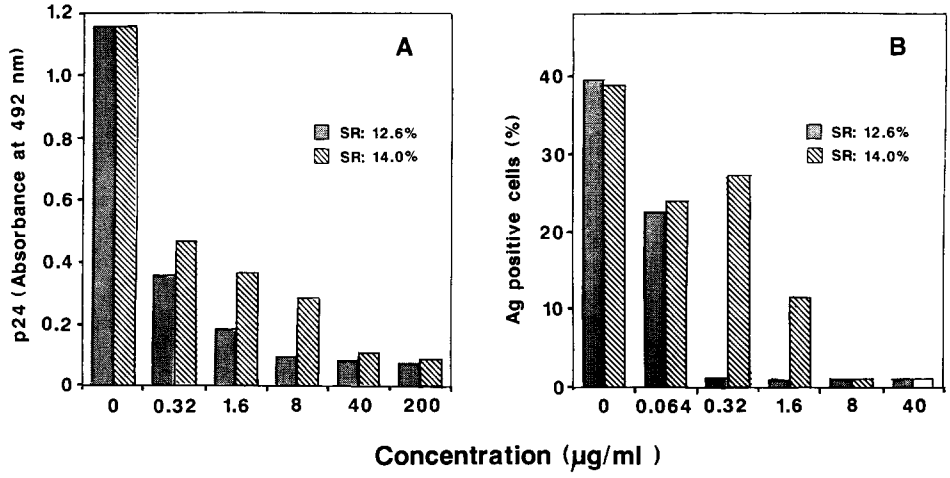

Fig. 3. (A) Inhibition of the expression of HIV-$1_{HTLV\text{-}IIIB}$ p24 antigen in PBMC by paramylon sulfate (SR: 12.6%) and paramylon sulfate (14.0%). (B) Inhibition of antigen (Ag) expression of the clinical isolate HIV-1_{A012B} in MT-4 cells by paramylon sulfate (SR: 12.6%) and paramylon sulfate (14.0%). Black columns, paramylon sulfate (SR: 12.6%); hatched columns, paramylon sulfate (SR: 14.0%).

dependent inhibition of HIV-1 binding to MT-4 cells. The 50% inhibitory concentration was 0.82 μg/ml.

Anti-coagulant activity

The PT and PTT were measured to assay the anti-coagulant activity of ten paramylon sulfates (Table 2). The PT value of the untreated blood was 8–12 s. Treatment of the blood with any of the 8 paramylon sulfates (SR: 0.07, 0.08,

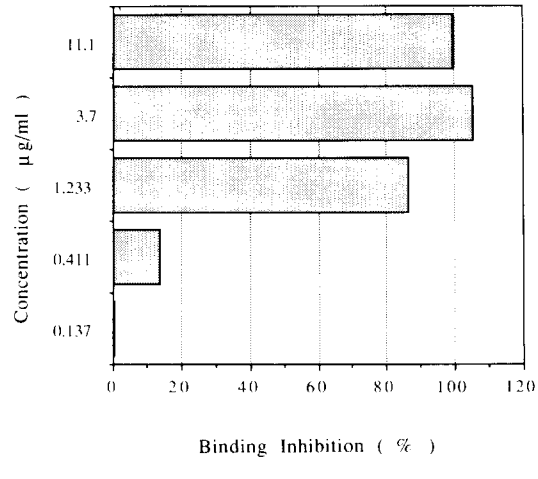

Fig. 4. Inhibition of HIV-1 binding by paramylon sulfate. MT-4 cells were incubated with HIV-1 in the absence (control) or in the presence of the indicated concentrations of paramylon sulfate (SR: 12.6%).

TABLE 2

Anti-coagulant activity of paramylon sulfates

Treatment	Conc. (μg/ml)	Time (sec)	
		PT	PTT
Untreated (control)	–	8–12	38–60
Paramylon sulfate (SR = 0.07%)	10	12.0	50
	100	11.4	49
Paramylon sulfate (SR = 0.08%)	10	12.3	50
	100	11.6	43
Paramylon sulfate (SR = 4.0%)	10	12.2	49
	100	11.7	49
Paramylon sulfate (SR = 4.1%)	10	12.2	51
	100	11.7	47
Paramylon sulfate (SR = 7.2%)	10	13.6	60
	100	19.4	300
Paramylon sulfate (SR = 8.7%)	10	12.9	51
	100	13.1	159
Paramylon sulfate (SR = 9.2%)	10	12.9	48
	100	12.9	98
Paramylon sulfate (SR = 12.6%)	10	13.4	65
	100	18.2	300
Paramylon sulfate (SR = 14.0%)	10	12.4	48
	100	11.7	43
Paramylon sulfate (SR = 15.6%)	10	12.2	48
	100	11.6	41
Dextran sulfate (MW 8000; SR = 8%)	10	16.2	>300
	100	13.8	>300

4.0, 4.1, 8.7, 9.2, 14.0 or 15.6%) (10–100 μg/ml) did not significantly change the control PT value (11.6–13.1 s), but treatment with 2 of the paramylon sulfates (SR: 7.2 or 12.6%) or dextran sulfate increased the PT values to 19.4, 18.2 and 16.2 s, respectively.

The PTT value of the untreated blood was 38–60 s. Treatment of the blood with 6 of the paramylon sulfates (SR: 0.07, 0.08, 4.0, 4.1, 14.0 or 15.6%) did not significantly change the PTT value (41–51 s). Although treatment with 4 of the paramylon sulfates (SR: 7.2, 8.7, 9.2 or 12.6%) increased the PTT value (48–300 s), their anti-coagulant activity was significantly lower than that of dextran sulfate (PTT value >300 s) (Table 2). More specifically, paramylon sulfate samples with SR of 14.0 and 15.6% had a markedly reduced anti-coagulant activity as compared to that of dextran sulfate.

Discussion

The present report revealed that sulfated paramylons, but not unsulfated paramylon nor other paramylon derivatives (DMAE, DEAE, HAP or CM), have potent activity against HIV-$1_{HTLV\text{-}IIIB}$, HIV-1_{A012B} and HIV-2_{ROD} in both MT-4 cells and PBMC. This points to the importance of the sulfate groups in the anti-HIV activity of these polymers. The anti-HIV activity of paramylon sulfates was found to depend on both molecular weight and sulfate content (Table 1), which confirms previous findings (Daniel et al., 1985; Itoh et al., 1990; Baba et al., 1990b; Witvrouw et al., 1991).

We found that paramylon sulfate completely inhibited HIV-binding to cells, in a dose range effective for anti-HIV activity (Figs. 1 and 4). Thus, the anti-HIV activity of paramylon sulfate could be attributed, at least in part, to inhibition of HIV binding. This is consistent with previous reports on dextran sulfate (Baba et al., 1988b; Mitsuya et al., 1988; Schols et al., 1989; Nakashima et al., 1989), but quite different from the incomplete inhibition of HIV binding by polyphenolic compounds such as tannins (Nakashima et al., 1992b) and lignins (Manabe et al., 1992; Nakashima et al., 1992c).

Sulfated polysaccharides are generally endowed with antithrombin activity (Baba et al., 1990a). Surprisingly, all 10 paramylon sulfates (SR: 0.07–15.6%) showed much lower anti-coagulant activity than dextran sulfate in terms of PTT (Table 2). These data were verified and found to be reproducible.

Clinical reports have revealed the poor absorption (bioavailability, tissue distribution) of sulfated polysaccharide (Abrams et al., 1989; Lorentsen et al., 1989). In fact, we found that only 0.1% of the radioactivity of orally administered [^{14}C]paramylon sulfate appeared in the plasma fraction (corresponding to 1.0 ml of blood) between 0.5 and 18 h after administration (Table 3). It is also apparent that the intravenously administered [^{14}C]paramylon sulfate rapidly disappeared from the plasma (Table 3). Roughly, about 8.8 μg of paramylon sulfate per ml blood was present in the

TABLE 3

Absorption of [^{14}C]paramylon sulfate in mice

Time after administration (h)	Radioactivity (dpm) in plasma (1 ml blood)	
	Oral administration	i.v. administration
0.5	1268 ± 298 (0.10%)	21656 ± 3154 (1.75%)
1	659 ± 74 (0.05%)	1466 ± 373 (0.12%)
3	1645 ± 71 (0.13%)	441 ± 59 (0.04%)
8	1321 ± 37 (0.11%)	351 ± 3 (0.03%)
18	660 ± 173 (0.05%)	279 ± 42 (0.02%)

Female ICR mice were treated with 0.5 mg (1.24×10^6 dpm) of [^{14}C]paramylon sulfate by the indicated routes. At the indicated times thereafter, the radioactivity in the plasma fraction (corresponding to 0.65 ml of blood) was determined as described in Materials and Methods, and expressed as the value per 1 ml of blood. The number in parentheses is the ratio (%) of recovered radioactivity vs. initially administered radioactivity. Each value is mean ±S.D. from 2–3 mice.

plasma 30 min after i.v. administration. Future studies on the effectiveness of sulfated polysaccharides in the treatment of HIV infection should be conducted with a parenteral rather than oral formulation, as has been suggested earlier (Lorentsen et al., 1989).

It has been reported that sulfated polysaccharides and azidothymidine (AZT) synergistically inhibit HIV replication (Ueno and Kuno, 1987; Schols et al., 1991). Combination of paramylon sulfate and AZT might thus lead to a decrease of the EC_{50} value of the individual compounds.

The antiviral activity of paramylon sulfate is restricted to HIV. We previously reported that paramylon sulfate (10–100 μg/ml) did not inhibit plaque formation of influenza virus (Harada et al., 1991) or herpes simplex virus (Fukuchi et al., 1989). The present results confirm the anti-HIV activity of sulfated polysaccharides prepared from other sources (Mizumoto et al., 1988; Itoh et al., 1990). Since these polysaccharides have been reported to show mitogenic activity (Sugawara et al., 1982, 1984), some immunopotentiating activity might be expected in vivo. We found that introduction of a sulfate group into paramylon significantly improved the water-solubility of paramylon, which was originally highly insoluble in water. Furthermore, this polysaccharide can be obtained in large quantity from the cultured *Euglena gracilis* (Sakagami et al., 1989). Paramylon sulfate may seem to be a good candidate drug, to be pursued further for its potential in the treatment of HIV infections.

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