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INHIBITION OF INFECTION WITH HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 BY SULFATED GANGLIOSIDES

Atsushi Handa, Hiroo Hoshino*, Katsuyuki Nakajima**, Masakazu Adachi**, Kiyoshi Ikeda***, Kazuo Achiwa***, Takeshi Itoh*** and Yasuo Suzuki***

Department of Hygiene, Gunma University School of Medicine, Maebashi, Gunma 371, Japan

**Japan Immunoresearch Laboratory Co, Ltd., Takasaki, Gunma 370, Japan

***School of Pharmaceutical Sciences, University of Shizuoka , Shizuoka 422, Japan

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SUMMARY: Four kinds of gangliosides, namely GM1a, GD1a, GD1b and GT1b and their sulfated derivatives were examined for antiviral activities against human immunodeficiency virus type 1 and abilities to modulate CD4 antigen on the cell surface. The infection of human T cells with the virus was markedly inhibited by treatment with the sulfated gangliosides at a concentration of 10 μg/ml, while the non-sulfated gangliosides had only weak antiviral activities. The sulfated gangliosides completely inhibited syncytium formation induced by HIV-1 at 30 μg/ml. The CD4 antigen on the surface of T cells became hardly detectable after treatment with them. They did not damage cells, nor prolong the activated partial thromboplastin time at concentrations of up to 100 μg/ml, suggesting that they may have little side effect *in vivo*.

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immune deficiency syndrome (AIDS) (1-3). CD4 glycoprotein is a major receptor for HIV-1 and thus HIV-1 preferentially infects CD4-positive T lymphocytes and monocytes (4-6). Virus replication will result in destruction of T cells (1-3). Gangliosides are a member of glycolipids, which contain glucose,

* To whom all correspondence should be addressed.

Abbreviations: HIV-1, human immunodeficiency virus type 1; FCS, fetal calf serum; DMSO, dimethyl sulfoxide; FITC, fluorescent isothiocyanate; IFA, indirect immunofluorescence assay; IC50, 50% inhibitory concentration.

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galactose, fucose, hexosamines, ceramide and sialic acids. Gangliosides are present in many tissue, especially rich in the brain. It was reported recently that GM1 or GM1a, one of gangliosides, binds to the CD4 antigen on the cell surface of T cells, down-regulate its expression and inhibit HIV-1 infection when examined under serum-free culture conditions (7-9). We purified four different gangliosides GM1a, GD1a, GD1b and GT1b from bovine brains and their derivatives were prepared by sulfation. We examined these compounds for anti-HIV-1 activities and their effects on expression of CD4 molecules on the cell surface.

MATERIALS AND METHODS

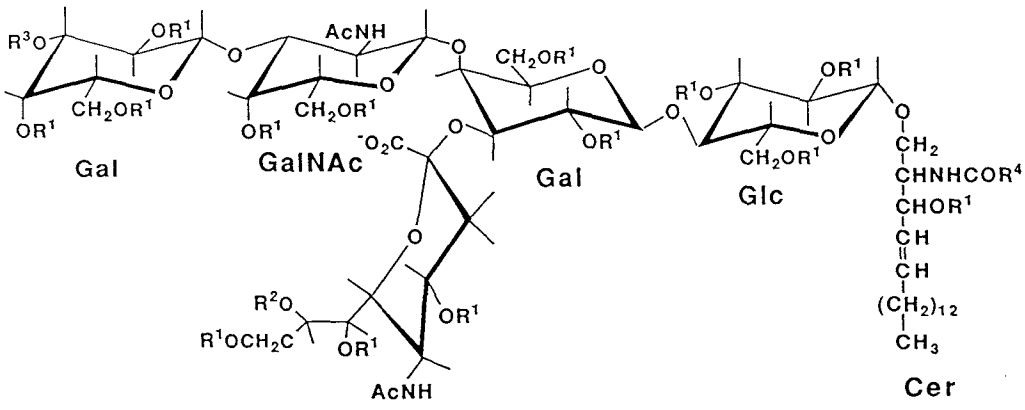
Cells and virus: MT-4 cells are a human T-cell line harboring human T-cell leukemia virus type 1 and very susceptible to HIV-1 (10). MOLT-4 clone 8 cells were used for syncytium formation assays (11). These cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 50 μg/ml kanamycin. Stocks of HIV-1 was prepared from culture supernatant of MOLT-4/HTLV-IIIB cells and frozen at -70°C. MOLT-4/HTLV-IIIB cells were MOLT-4 cells persistently infected with HTLV-IIIB strain of HIV-1(12).

Compounds: Gangliosides GM1a, GD1a, GD1b and GT1b were purified from bovine brains (13) and sulfated derivatives were prepared by successive treatment with sulfurtrioxide trimethylamine complex (2 eq.) in dimethylformamide at 50° C for 20 hours and with trifluoroacetic acid (1.5 eq.) in dichloromethane followed by gel filtration through Sephadex LH-20 (chloroform-methanol-water/10:10:1, by vol.) (14). The structures of these gangliosides were shown in Fig. 1. The structures of the per-*O*-sulfated gangliosides were supported by the positive ion fast atom bombardment (FAB) mass spectrometry (JMS-SX102, JEOL, Tokyo). High molecular weight, high sulfite-containing dextran sulfate (average molecular weight, 500,000; sulfite content, 17%) and heparin were purchased from Pharmasia (Uppsala) and WAKO PURE CHEMICAL INDUSTRIES, LTD. (Osaka), respectively. These compounds were dissolved in dimethyl sulfoxide (DMSO) at 10 mg/ml and store at -20°C until use.

Assay for anti-HIV-1 activity: MT-4 cells were seeded in 24-well plates in an amount of 1.0×10^5/ml. Then gangliosides or sulfated derivatives were added into wells at concentrations of 0.3-100 μg/ml. After incubation for two hours, the cells were infected with HIV-1 at the multiplicity of infection (m.o.i) of 0.05. Four days later HIV-1 antigen-positive cells were detected by indirect immunofluorescence assay (IFA). As the first and second antibodies, serum of an AIDS patient and fluorescent isothiocyanate(FITC)-conjugated rabbit anti-human IgG were used. HIV-1 production was measured by reverse transcriptase assay as described elsewhere (15).

Syncytium formation assay, which has been used to examine effects of compounds on early steps of HIV-1 infection (16), was performed as follows: 1.6×10^5 MOLT-4 clone 8 cells and 4×10^4 MOLT-4/HTLV-IIIB cells were cocultured in the presence of gangliosides for 24 hours. Numbers of syncytia formed were counted using a microscope after fixation with 5% formalin.

Detection of CD4 antigen on the cell surface: The presence of CD4 antigen on the surface of MT-4 cells was examined by a flow cytometer (CytoACE 150, JASCO Co. Ltd., Tokyo) using Leu3a anti-CD4 monoclonal antibody. For this, MT-4 cells were seeded at 1×10^6/ml, treated with gangliosides (1-100 μg/ml) for one hour and then reacted with FITC-conjugated Leu3a monoclonal antibody.



	R^1	R^2	R^3	R^4: Alkyl
GM_{1a}:	H	H	H	
GD_{1a}:	H	H	NeuAc	
GD_{1b}:	H	NeuAc	H	
GT_{1b}:	H	NeuAc	NeuAc	
GM_{1a}-SO_3H:	SO_3H	SO_3H	SO_3H	
GD_{1a}-SO_3H:	SO_3H	SO_3H	NeuAc-SO_3H	
GD_{1b}-SO_3H:	SO_3H	NeuAc-SO_3H	SO_3H	
GT_{1b}-SO_3H:	SO_3H	NeuAc-SO_3H	NeuAc-SO_3H	

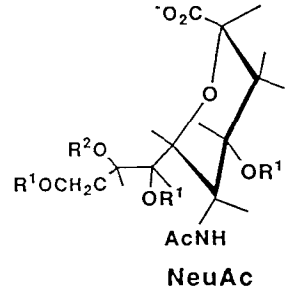


Fig. 1. Structures of gangliosides and sulfated derivatives: Cer, ceramide; Glc, glucose; Gal, galactose; GalNAc, N-acetylgalactosamine; NeuAc, N-acetylneuraminic acid.

After incubation for 30 min at 4°C, the cell were fixed with 1% paraformaldehyde and subjected to flow cytometry.

Activated partial thromboplastin time (APTT): APTT of plasma from a normal subject was examined in the presence of sulfated gangliosides by using an automatated machine. For this, platerin plus activator was obtained from Organon Teknika Corporation (Durham). Human plasma (100 µl) was mixed with platerin plus activator (100 µl) and test compounds and then APTT was measured.

RESULTS

Anti-HIV-1 activities of non-sulfated gangliosides

When MT-4 cells were infected with HIV-1 and cultured for four days in the absence of gangliosides, over 95% MT-4 cells were positive for viral antigens. Although one of gangliosides GM1 or GM1a was reported to have anti-HIV-1 activity under serum-free culture conditions (7-9), effects of GM1a and other gangliosides were examined in RPMI 1640 medium containing 10% FCS (Fig. 2A). They had weak anti-HIV-1 activities. Even at a concentration of 100 µg/ml, 20-60% of MT-4 cells became immunofluorescent after HIV-1 infection. Fifty %

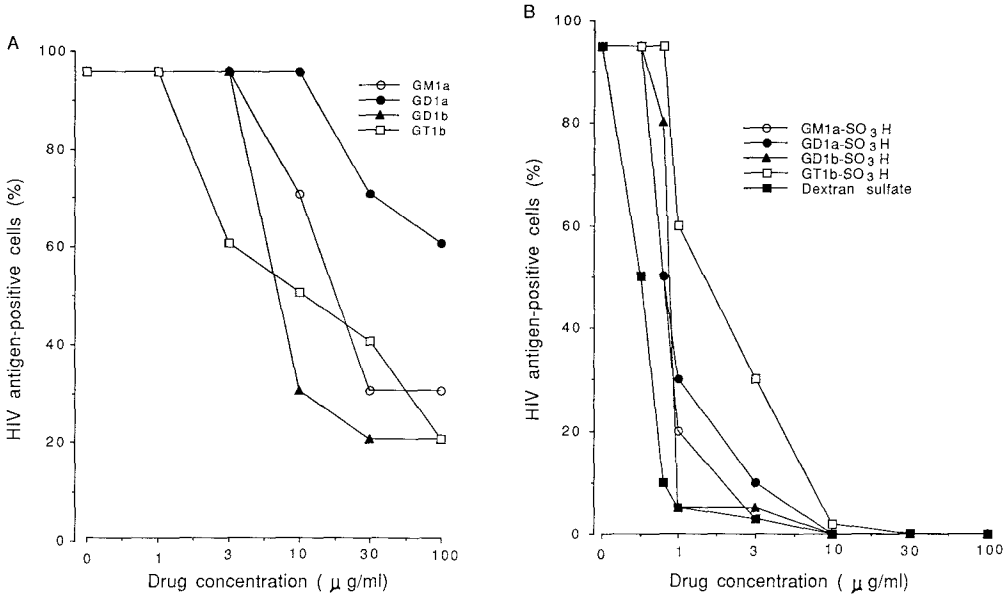


Fig. 2. Effects of gangliosides and sulfated derivatives on infection of MT-4 cells with HIV-1. MT-4 cells were infected with HIV-1 in the presence of non-sulfated (A) or sulfated (B) gangliosides. Expression of HIV-1 antigen was detected by IFA after cultivation for four days.

inhibitory concentrations (IC_{50}'s) for GM1a, GD1a, GD1b and GT1b were estimated from dose-response curves and shown in Table I.

Anti-HIV-1 activities of sulfated gangliosides

Because sulfated derivatives of polysaccharides have been reported to have much stronger anti-HIV-1 activities than non-sulfated polysaccharide (17-

Table I. Effects of gangliosides and sulfated derivatives on HIV-1 infection and CD4 expression on the surface of MT-4 cells

Compound	HIV-1 infection (IC_{50})		CD4 expression (%)	
	Non-sulfated	Sulfated	Non-sulfated	Sulfated
GM1a	30*	0.8	66**	18
GD1a	>100	0.8	69	16
GD1b	10	0.9	65	18
GT1b	10	2.0	65	10
Dextran	ND***	0.5	ND	ND

* Concentrations (μg/ml) of compounds at which 50% of MT-4 cells expressed HIV-1 antigens.
** CD4-positive cells (%) detected by flow cytometry after treatment with gangliosides at 100 μg/ml for one hour.
*** ND, not done.

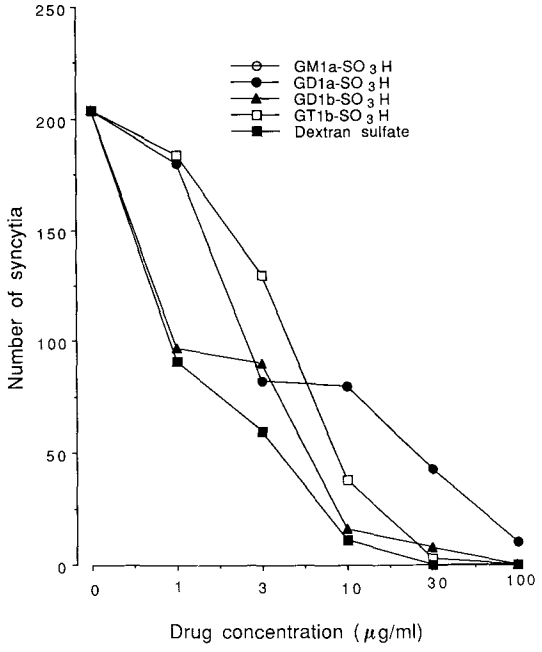


Fig. 3. Inhibition of syncytium formation induced by HIV-1 by sulfated gangliosides. MOLT-4 cells were cocultivated with HIV-1-producing MOLT-4/HTLV-IIIB cells for 24 hours in the presence of sulfated gangliosides. Syncytia in wells were counted using an inverted microscope.

22), the gangliosides were sulfated and their anti-HIV-1 activities were examined (Fig. 2B). The sulfated gangliosides had potent anti-HIV-1 activities: Infection with HIV-1 was completely inhibited at 10 μg/ml. IC_{50}'s for sulfated GM1a, GD1a, GD1b and GT1b and dextran sulfate were estimated (Table I). Thus anti-HIV-1 activities of gangliosides were enhanced five to hundred times by sulfation. HIV-1 production by MT-4 cells after infection as detected by reverse transcriptase assays was also markedly inhibited in the presence of sulfated gangliosides (data not shown).

Next whether the sulfated gangliosides inhibited syncytium formation was examined in the culture medium containing 10% FCS (Fig. 3). Syncytium formation was inhibited almost completely at a concentration of 30 μg/ml. These results suggested that the sulfated gangliosides would act on at least an early step of HIV-1 infection, namely, adsorption or penetration.

Expression of CD4 antigen on the cell surface

It was examined whether non-sulfated and sulfated gangliosides would modulate expression of CD4 antigen on the surface of MT-4 cells (Fig. 4). Most of MT-4 cells (97%) were judged to be CD4 antigen-positive by flow cytometry. By treatment with sulfated GM1a at 100 μg/ml for one hour, CD4 antigen became hardly detectable, although non-sulfated GM1a had weak effect (Fig. 4A). Slight down-regulation of CD4 antigen was detectable by treatment with non-

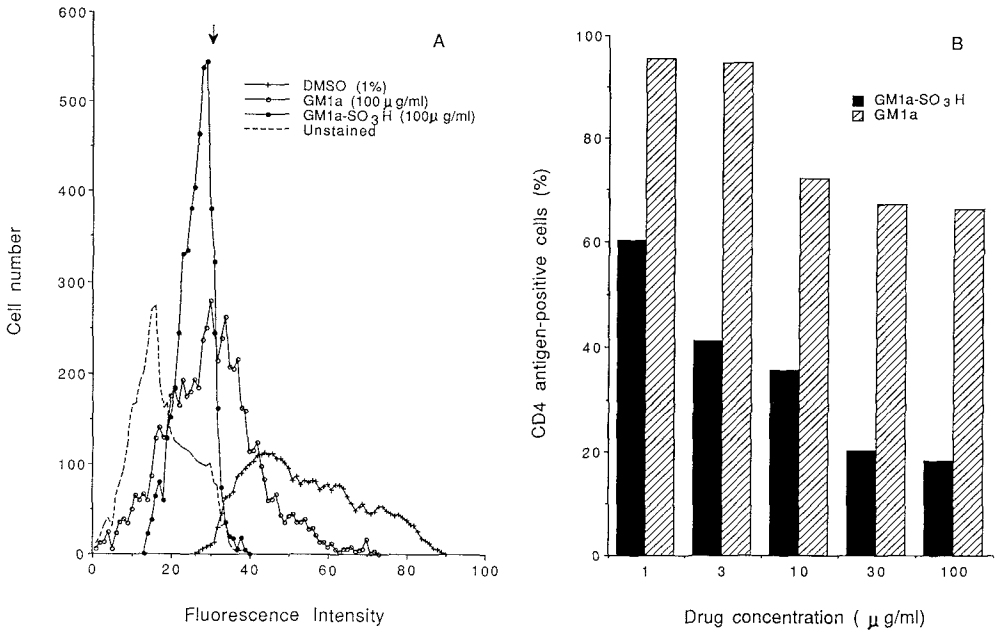


Fig. 4. Down-regulation of CD4 antigen by sulfated GM1a. (A) MT-4 cells were treated with non-sulfated (100 μg/ml) or sulfated (100 μg/ml) GM1a or 1% DMSO for one hour at 37°C. Then the cells were reacted with FITC-conjugated Leu3a anti-CD4 monoclonal antibody and examined by a flow cytometer. Flow cytometry of unstained MT-4 cells is shown by the dotted line. The arrow indicates a cut-off value for fluorescence intesity. Ninety seven % of MT-4 cells treated with 1 % DMSO (solvent control) were judged to be positive for CD-4 antigen. (B) Expression of CD4 antigen on the surface of MT-4 cells treated with indicated concentrations of non-sulfated (hatched symbol) or sulfated (closed symbol) GM1a.

sulfated and sulfated GM1a at concentrations of 10-100 and 1 μg/ml, respectively (Fig. 4B). Other non-sulfated and sulfated gangliosides, namely GD1a, GD1b and GT1b, gave similar results. Representative data on CD4 expression after treatment with the gangliosides are shown in Table I.

Lack of side effect of sulfated gangliosides

MT-4 cells were cultivated in the presence of sulfated gangliosides for four days and then viable cell numbers were counted. These compound did not inhibit growth of MT-4 cells at all (Table II). Next effects of sulfated gangliosides on APTT of human plasma were examined. The sulfated gangliosides did not prolong APTT (Table II). Dextran sulfate and heparin markedly prolonged APTT. These findings suggested that the sulfated gangliosides would have little side effect *in vivo*.

DISCUSSION

GM1 or GM1a has been reported to down-regulate CD4 antigen and inhibit HIV-1 infection in serum-free culture (7-9). This activity is blocked by

Table II. Effects of sulfated gangliosides on cell growth and APTT

Compound	Concentration (μg/ml)	Cell number($x10^{-5}$)	APTT (sec)
Sulfated			
GM1a	100	6.4*	50**
GD1a	100	6.4	57
GD1b	100	6.5	52
GT1b	100	6.5	51
Dextran sulfate	100	6.5	168
Heparin	100	ND***	>276
DMSO	1%	6.6	53

* Number of viable MT-4 cells after cultivation for four days in the presence of compounds. DMSO is a solvent control.
** APTT (sec) of normal human plasma in the presence of compounds.
***ND, not done.

addition of FCS or bovine and human serum albumin, which suggests the formation of ganglioside-albumin complex. We purified GM1a, GD1a, GD1b and GT1b from bovine brains and sulfated them. The sulfated gangliosides had much more potent anti-HIV-1 activities than non-sulfated gangliosides and they modulated CD4 antigen on the surface of human T cells much more efficiently than the non-sulfated gangliosides (Table I and Figs. 2 and 4). Infection of T cells with HIV-1 is mediated through CD4 antigen, which is an HIV-1 receptor. Thus potent anti-HIV-1 activities of the sulfated gangliosides can be explained by their effects on modulation of CD4 antigen. These compounds markedly down-regulated CD4 antigen on the cell surface even in the presence of 10% FCS and thus they could inhibit binding of HIV-1 to its receptors on MT-4 cells.

Whereas most non-sulfated polysaccharides seem to have little anti-HIV-1 activities (17,18), non-sulfated gangliosides still had weak anti-HIV-1 activities (Table I). Anti-HIV-1 activities of both types of compounds are markedly potentiated by sulfation. As for sulfated polysaccharides, their molecular weights and sulfite contents seem to be important for their antiviral activities (12,16,23,24). Low molecular weight (2,000) dextran sulfate was reported to have little anti-HIV-1 activity (23). IC_{50} of dextran sulfate estimated in this experiment was much smaller than those of dextran sulfate reported in some literatures (23, 24). This might be due to the use of high molecular weight, high sulfite-containing dextran sulfate. Molecular weights of the sulfated gangliosides used in this experiment were about 2,000-3,500. Nevertheless IC_{50}'s of the sulfated gangliosides and dextran sulfate were similar (Table I). Dextran sulfate was reported to be ingested badly and be unstable in the plasma

of rats: Its half life was 20-480 min (25). It remains to be investigated whether sulfated gangliosides have similar disadvantages.

Many sulfated polysaccharides having anti-HIV-1 activities are known to prolong APTT (16, 20-22). On the other hand, the sulfated gangliosides did not prolong it (Table II), indicating that they would not have anti-coagulant activity *in vivo*. They did not affect growth of T cells *in vitro* (Table II). The sulfated gangliosides had potent anti-HIV-1 activities even when T cells were cultured in the presence of FCS. These properties of the sulfated gangliosides may be therapeutically advantageous if we should consider the possibility of their clinical use. GM1 was really administered to humans to treat neurological disorders (Alzheimer's disease), its half life being 60-75 hours in blood (26). Gangliosides have been reported to influence biological activities, such as cellular interactions or induction of differentiation of leukemic cells (27, 28). It also remains to be investigated whether sulfation of gangliosides will affect these activities.

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