Chem. Pharm. Bull. 37(9) 2410—2412 (1989) Vol. 37, No. 9

Inhibitory Effect of a Lichen Polysaccharide Sulfate, GE-3-S, on the Replication of Human Immunodeficiency Virus (HIV) *in Vitro*

Kazuhiro HIRABAYASHI,[a] Susumu IWATA,[a] Masahiko ITO,[b] Shirou SHIGETA,[b] Takao NARUI,[c] Takeo MORI[a] and Shoji SHIBATA*,[d]

Research Laboratory, Minophagen Pharmaceutical Co.,[a] Komatsubara, Zama, Kanagawa 228, Japan, Department of Microbiology, Fukushima Medical College,[b] Fukushima 960, Japan, Meiji College of Pharmacy,[c] Nozawa 1–35–23, Setagaya-ku, Tokyo 154, Japan and Shibata Laboratory of Natural Medicinal Materials,[d] c/o Minophagen Pharmaceutical Co., Yotsuya 3–2–7, Shinjuku-ku, Tokyo 160, Japan. *Received February 13, 1989*

A sulfate (GE-3-S) prepared by chlorosulfonic acid treatment of GE-3, a partially acetylated β(1→6) glucan of the lichen *Umbilicaria esculenta*, inhibited the cytopathic effect of human immunodeficiency virus (HIV) and suppressed the HIV-antigen expression in Molt-4 (clone 8) cells. GE-3-S also suppressed the giant cell formation of HIV-infected Molt-4 cells, and inhibited HIV-induced plaque formation by 50% at the dose of 19.5 μg/ml and completely at 250 μg/ml in MT4 cells. GE-3-S had no direct effect on the reverse transcriptase of HIV.

Keywords polysaccharide sulfate; anti-human immunodeficiency virus (HIV) activity; GE-3-S; cytopathic effect

Human immunodeficiency virus (HIV) is now considered to be the etiological agent of acquired immune deficiency syndrome (AIDS).[1,2] The HIV infection of T4 cells involves the recognition and binding process between the HIV gp 120 envelope glycoprotein and the cellular receptor CD4.[3–5]

HIV-infected lymphocyte cultures show the formation of syncytia, multinucleated giant cells, which is caused by the fusion of T4 cells.[6,7] According to the foregoing researches, cell surface expression of infected T4 cells induces fusion with uninfected T4 cells.[8] The syncytia formation is followed by cytolysis and cell death, and this process has generally been recognized as the principal mechanism of T4 depletion *in vivo*.[9]

Several anti-HIV compounds are known, *e.g.* ribavirin,[10] phosphonoformic acid,[11] recombinant interferon-α,[12] 3′-azido-2′,3′-dideoxythymidine (AZT)[13] and 2′,3′-dideoxynucleosides.[14] Glycyrrhizin, a saponin of licorice root, has been found by Ito *et al.*[15] to inhibit HIV replication *in vitro*, while it has been demonstrated in clinical trials that the CD4/CD8 ratio recovers in HIV carriers upon the administration of glycyrrhizin to approach that of the normal population.[16]

Recently, Ito *et al.*[17] reported that dextran sulfate and heparin effectively inhibited HIV infection and replication at concentrations which did not show any cytotoxicity to the host T4 cells. In connection with this, we have tested the anti-HIV activity of lichen polysaccharides and their sulfates *in vitro*, since various homo- and hetero-glycans are readily available from lichens, GE-3,[18] partially acetylated pustulan,[19] which is widely distributed in the lichens of *Umbilicaria* (=*Gyrophora*, *Lasallia*) spp., is a β(1→6)glucan showing a host-mediated antitumor activity against Sarcoma 180 and Erlich's carcinoma in mice.[18,20]

In this paper we report the inhibitory activity of sulfated GE-3 (GE-3-S) against HIV replication *in vitro*.

Experimental

Preparation of GE-3-S GE-3 was sulfated with chlorosulfonic acid in dimethylformamide (DMF) at room temperature under continuous stirring for 4 h in an N_2 atmosphere. The reaction mixture was poured into water, and the pH was adjusted to 10 with 1 N NaOH. After dialysis the crude sulfate (sodium salt) was purified by column chromatography and lyophilization. GE-3-S is readily soluble in water: $[\alpha]_D$ −25.0° (c=2.0, H_2O); molecular weight 200000, as determined by high performance liquid chromatography (HPLC) using pulullan as a standard. S: 13.6%; IR ν (cm^{-1}): 1240 (S=O), 910 (β-linkage), 820 (e); the SO_3Na group is located on the 2- and/or 3-position of the D-glucosyl unit.

Cells and Virus The HTLV-I-transformed cell line, MT4, and the human leukemic T-cell line, Molt-4 (clone 8),[21] were used. The cells were cultured and maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 IU of penicillin G and 100 μg/ml streptomycin. The HIV was obtained from the culture supernatant of the Molt-4/HTLV-III cell line.[22]

Assay for HIV-Induced Cytopathic Effect Molt-4 (clone 8) cells were infected with HIV at a multiplicity of infection (moi) of 0.002, and incubated for 1 h at 37 °C. After virus adsorption, the infected cells were washed and resuspended in culture medium. The number of Molt-4 (clone 8) cells was adjusted to 1×10^5 cells/ml, and they were placed in wells of flat bottomed 96-well plastic microtiter trays containing various concentrations of the test compounds (GE-3-S and other polysaccharides). After incubation for 4 d at 37 °C in a CO_2-incubator, half of the culture medium was exchanged with the fresh medium. After incubation for another 2 d at 37 °C, the number of viable cells was counted microscopically in a hematocytometer by the trypan blue exclusion method.[15]

Assay for HIV-Specific Antigen Expression Virus-specific antigen expression in the HIV-infected Molt-4 (clone 8) cells was determined by immunofluorescence (IF) using a polyclonal antibody from a sero-positive anti-HIV human serum and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human immunoglobulin G (IgG) (Dakopatts A/S, Copenhagen, Denmark). More than 500 cells were counted under a fluorescence microscope, and the percentage of fluorescent cells was calculated on day 3 after infection.

Reverse Transcriptase (RT) Assay RT activity was assayed by the method of Harada *et al.*[22] Supernatants of the Molt-4/HTLV-III were concentrated 100 times by sucrose gradient ultracentrifugation. The viral pellets were disrupted by addition of 5 mM Tris–HCl (pH 8.1) containing 0.5 M KCl, 0.1 mM dithiothreitol (DTT) and 0.1% Triton X-100. The assay for RT activity was performed at 37 °C for 1 h with 10 μl of disrupted HIV in a final volume of 50 μl of 50 mM Tris–HCl (pH 8.4) containing 2 mM DTT, 100 mM KCl, 10 mM $MgCl_2$, 0.01% Triton X-100, 1 μCi [^{3}H-methyl]thymidine triphosphate (57 μCi/mmol: Amersham, Buckinghamshire, UK) and 50 μg/ml poly (rA): poly (dT) (P-L Biochemicals Inc., Milwaukee, Wis., U.S.A.). The reaction was stopped with 5% trichloroacetic acid (TCA), and precipitates were collected on glass fiber filters and counted in a liquid scintillation counter. The assays were carried out in triplicate.

Plaque Assay of HIV To evaluate the inhibitory effect of GE-3-S on HIV infection in MT-4 cells, a plaque assay using an agarose overlay with various concentrations of GE-3-S was performed as previously described.[23] To fix MT4 cells onto culture vessels, 35 mm polystyrene tissue culture dishes were coated with poly-L-lysine (PLL (MW 120000): Sigma Chemical Co., St Louis, Mo., U.S.A.). A suspension of 2.25×10^6 MT4 cells in 1.5 ml was put on to each PLL-coated dish and incubated for 1 h at room temperature. The dishes were gently washed with phosphate buffered saline (PBS) to remove nonadherent cells. An appropriate dilution (100 μl) of the virus stock was slowly added and incubated with the cells for 1 h at room temperature. After the adsorption period, 1 ml of agarose

overlay consisting of RPMI 1640 medium with 10% FCS, antibiotics and 0.6% agarose (Sea Plaque Agarose, Marine Colloid Corp., Rockland, Me., U.S.A.) was poured into each dish. The dishes were incubated at 37 °C for 3 d, and 1 ml of agarose overlay containing neutral red was added. The dishes were incubated for another 3 d and the number of visible plaques was counted. All experiments were carried out in triplicate.

Results and Discussion

Anti-HIV activity of GE-3-S was determined by counting the viable cells using trypan blue exclusion as described by Yamamoto *et al.*[15] When Molt-4 (clone 8) cells were infected with HIV, the viability of the cells was gradually reduced by the viral cytopathic effect (CPE). On day 3 after infection, the number of viable cells was only 3×10^5 cells/ml as compared with 10×10^5 cells/ml in the uninfected controls, whereas in the presence of 31 μg/ml of GE-3-S the host cells were completely protected against viral CPE. Unifected Molt-4 (clone 8) cells were unaffected by GE-3-S at this concentration (Fig. 1).

When Molt-4 (clone 8) cells were infected with HIV and examined by immunofluorescence (IF), 52.4% of the cells showed positive for viral antigen on day 3 after infection. A dose-dependent inhibition of IF was observed in the HIV-infected Molt-4 (clone 8) cells which had been incubated in the presence of various concentrations of GE-3-S. At a concentration of 31 μg/ml of GE-3-S, viral antigen expression was almost completely suppressed (Table I). At the same concentration, the Molt-4 (clone 8) cells were protected against HIV cytopathogenicity (Fig. 1).

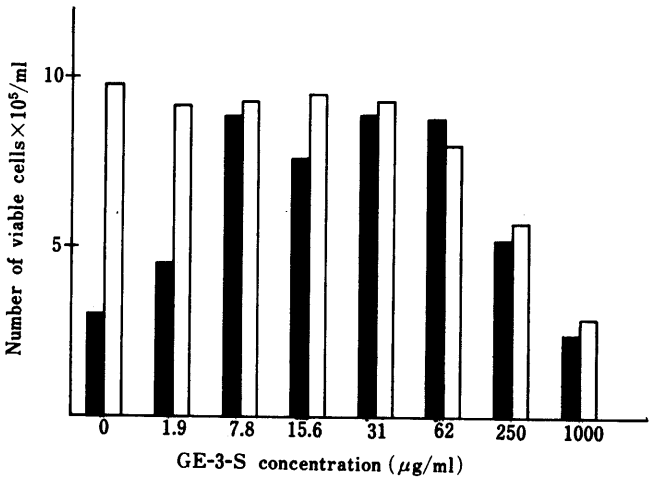

Fig. 1. Inhibition of the Cytopathogenicity of HIV for Molt-4 (Clone 8) Cells by GE-3-S

The HIV-infected cells are indicated by solid columns (■) and control uninfected cells by open columns (□).

TABLE I. Inhibitory Effects of GE-3-S on the Expression of HIV-Specific Antigens in Molt-4 (Clone 8) Cells

GE-3-S concentration (μg/ml)	Percentage of indirect immunofluorescence (IF)-positive cells
0	52.4
1.9	27.2
7.8	16.2
15.6	13.2
31.0	7.9
62.0	7.9
250.0	5.1
1000.0	2.7

The anti-HIV activity of GE-3-S was also demonstrated by the inhibition of syncytia formation of HIV-infected Molt-4 cells (Fig. 2).

GE-3-S brought about a *ca.* 45% reduction of the plaque formation in HIV-infected MT4 cells at 15.6 μg/ml and a complete inhibition at 250 μg/ml. The inhibitory effect was dosedependent, with ID_{50} 19.5 μg/ml (Fig. 3).

GE-3-S had no effect on the cell-free HIV reverse transcriptase (RT) activity even at 250 μg/ml (Table II),

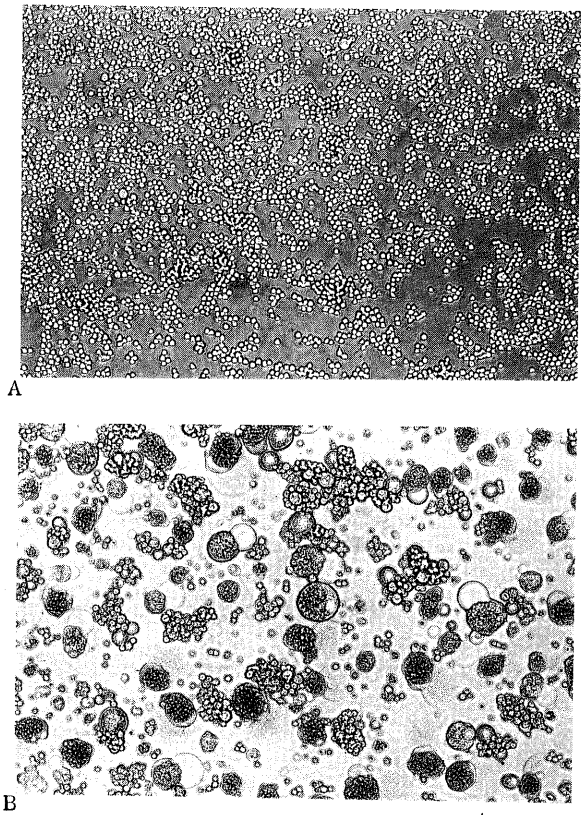

Fig. 2. Giant Cell Formation of Molt-4 (Clone 8) Cells Following Infection with HIV and Incubation for 7 d at 37 °C in a CO_2-Incubator in the Absence (B) and Presence (A) of GE-3-S (62.5 μg/ml) (× 100)

No giant cells are observed in panel A, whereas several can be seen in panel B.

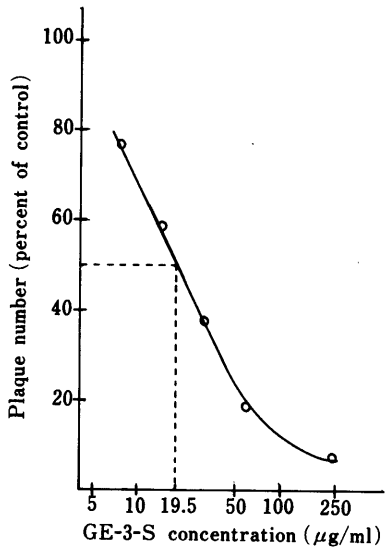

Fig. 3. Inhibitory Effect of GE-3-S on HIV-Induced Plaque Formation in MT4 Cells

Table II. Effect of GE-3-S on the Cell-Free Reverse Transcriptase (RT) Activity of HIV

GE-3-S concentration (μg/ml)	RT activity ($\times 10^{-3}$ cpm)
0	102
7.8	127
15.6	108
31.0	100
62.0	88
250.0	84

Table III. Anti-HIV Activities of Polysaccharides

	Compound	Source	Anti-HIV activity
GE-3	Partially acetylated pustulan ($\beta(1\rightarrow6)$-glucan)	*Umbilicaria esculenta*[19]	−
UGE-3	Urea-treated GE-3[20]		−
GE-3-S	GE-3 sulfate		+
PC-3	$\alpha(1\rightarrow3)(1\rightarrow4)$glucan (1 : 1)	*Parmelia caperata*[24]	−
PC-3-S	PC-3 sulfate		−
Pachyman	$\beta(1\rightarrow3)$glucan with some branched chain	*Poria cocos*[25]	−
U-Pachyman	Urea-treated pachyman		−
Pachyman-S	Pachyman sulfate		−
Lichenan	$\beta(1\rightarrow3)(1\rightarrow4)$glucan	*Cetraria islandicum*[26]	−
Lichenan-S	Lichenan sulfate		−

which almost completely prevented HIV replication in the Molt-4 (clone 8) cells.

The anti-HIV activities of other lichen and fungal polysaccharides and their sulfates were examined in comparison with that of GE-3-S. Of those tested, GE-3-S was the only compound that exhibited anti-HIV activity (Table III).

The presence of the sulfate group in the molecule of GE-3-S is essential for the anti-HIV activity, since GE-3, the parent compound, is not effective, while a structural specificity is also present in GE-3-S, since some other sulfated polysaccharides, PC-3-S, pachyman-S and lichenan-S, are not effective. It is noteworthy that dextran sulfate is effective against HIV proliferation whereas dextran is ineffective.

The mechanism of anti-HIV activity of GE-3-S has not yet been elucidated, but it seems probable that GE-3-S, having no anti-RT activity, could be effective against HIV infection by a similar way to heparin and dextran sulfate[15] preventing adsorption of HIV particles on the surface of T4 lymphocytes by interference at the site of either viral envelope glycoprotein pg 120 or lymphocyte receptor CD4.

These polysaccharide sulfates show weak toxicity to animals. GE-3-S showed no acute toxicity in ddY mice at 400—1000 mg/kg (i.v. or i.p.).

Acknowledgement The authors wish to thank Mr. A. Sato and Mr. S. Mori, Fukushima Medical College, for their cooperation. Thanks are also due to the members of Central Analytical Laboratory, Meiji College of Pharmacy, for spectral analyses.

References

1) F. Barre-Sinoussi, J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rosenbaum and L. Montagnier, *Science*, **220**, 868 (1983).
2) M. Popovic, M. G. Sarngadharan, E. Read and R. C. Gallo, *Science*, **224**, 497 (1984).
3) A. G. Dalglish, P. C. L. Beverley, PP. R. Clapham, D. H. Crawford, M. F. Greaves and R. A. Weiss, *Nature* (London), **312**, 763 (1984).
4) Q. J. Sattentau, A. G. Dalgleish, R. A. Weiss and P. C. L. Beverley, *Science*, **234**, 1120 (1986).
5) J. S. McDougal, M. S. Kennedy, J. M. Sligh, S. P. Cort and A. Mawle, *Science*, **231**, 382 (1986).
6) D. Zagury, J. Bernard, R. Leonard, R. Cheynier, M. Feldman and P. S. Sarin, *Science*, **231**, 850 (1986).
7) T. Falks, D. M. Powell, M. M. Lightfoote, S. Benn, M. A. Martin and A. Fauci, *Science*, **231**, 600 (1986).
8) J. D. Lifson, M. B. Feinberg, G. R. Reyes, L. Rabin, B. Banapour, S. Chakrabarti B. Moss, F. Wong-Staal, K. S. Steimer and E. C. Engleman, *Nature* (London), **323**, 725 (1986).
9) J. Sodroski, W. C. Goh, C. Rosen, K. Campbell and W. A. Haseltine, *Nature*, (London), **322**, 470 (1986).
10) J. B. McCormic, J. P. Getchell, S. W. Mitchell and D. R. Hicks, *Lancet*, ii, 1367 (1984).
11) E. G. Sandstrom, J. C. Kaplan, R. E. Byington and M. S. Hirsch, *Lancet*, i, 1480 (1985).
12) D. D. Ho, K. L. Hartshorn, R. T. Rota, C. A. Amdrews, J. C. Kaplan, R. T. Schooley and M. S. Hirsch, *Lancet*, i, 602 (1985).
13) H. Mitsuya, K. J. Weihold, P. A. Furman, D. W. St. Clair and S. Broder, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 7096 (1985).
14) H. Mitsuya and S. Broder, *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 1911 (1986).
15) M. Ito, H. Nakashima, M. Baba, R. Pouwels, E. DeClercq, S. Shigeta and N. Yamamoto, *Antiviral Res.*, **7**, 127 (1987).
16) Y. Goto, K. Yamada and M. Minamitani, *Igaku No Ayumi*, **140**, 619 (1987).
17) M. Ito, M. Baba, A. Sato, R. Pouwels, E. DeClercq and S. Shigeta, *Antiviral Res.*, **7**, 361 (1987).
18) Y. Nishikawa, T. Takeda, S. Shibata and F. Fukuoka, *Chem. Pharm. Bull.*, **17** 1910 (1969).
19) B. Lindberg and J. MacPherson, *Acta Chem. Scand.*, **8**, 985 (1954); C. Helleqvist, B. Lindberg and K. Samuelsson, *ibid.*, **22**, 2736 (1968).
20) M. Watanabe, K. Iwai, S. Shibata, K. Takahashi, T. Narui and T. Tashiro, *Chem. Pharm. Bull.*, **34**, 2532 (1986).
21) R. Kikukawa, Y. Koyanagi, S. Harada, N. Kobayashi, M. Hatanaka and N. Yamamoto, *J. Virology*, **57**, 1159 (1986).
22) S. Harada, Y. Koyanagi and N. Yamamoto, *Virology*, **146**, 272 (1985).
23) S. Harada, S. Koyanagi and N. Yamamoto, *Science*, **229**, 563 (1985).
24) T. Takeda, Y. Nishikawa and S. Shibata, *Chem. Pharm. Bull.*, **18**, 1074 (1970).
25) G. Chihara, J. Hamuro, Y. Maeda, Y. Arai and F. Fukuoka, *Nature* (London), **225**, 943 (1970).
26) W. L. Cunningham and D. J. Manners, *Biochem. J.*, **90**, 596 (1964).