The Inhibitory Effect of the Crude Extract from a Seaweed of *Dygenea simplex* C. AGARDH on the *in Vitro* Cytopathic Activity of HIV-1 and It's Antigen Production

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The crude water extract (NS-1) from a seaweed (*Digenea simplex* C. AGARDH Rhodomelaceae) exhibited anti-human immunodeficiency virus (HIV)-1 activity *in vitro*. The inhibitory effect of the extract on the cytopathic activity of HIV-1 and it's antigen production was examined using a microplate method, immunofluorescent assay, and an HIV antigen detection kit (Abbott). NS-1 inhibited both the cytopathic effect of HIV-1 to MT-4 cells and the giant cell formation of Molt-4 cells infected with HIV-1.

Key words anti-human immunodeficiency virus drug; digenea simplex; cytopathic effect; antigen production

A hot water extract from the seaweed of *Digenea simplex* C. AGARDH Rhodomelaceae has been used for ascariasis as a natural drug. Research for anti-human immunodeficiency virus (HIV) drugs has been done enthusiastically and some partially effective drugs such as 3'-azidothymidine (AZT) or dideoxyinosine (DDI) have been used clinically, accompanied by side effects and the appearance of HIV strains resistant to the drugs. On the other hand, research for an anti-HIV drug with low side effects from among old natural drugs has been done, and some natural drugs with anti-HIV activity have been reported.[1,2] We also found anti-HIV activity in the crude water extract (NS-1) from dried seaweed of *Digenea simplex*. In the present study, we are going to show an inhibitory effect of the crude extract on both the cytopathic effect (CPE) of the virus to human lymphocytes and on HIV-1 antigen production.

Experimental

Chemicals Five grams of small cut pieces of *Digenea simplex* C. AGARDH Rhodomelaceae were boiled under reflux two times with 1000 ml of distilled water for 2 h. Both extracts were combined and concentrated to 200 ml *in vacuo*, and centrifuged at $2000 \times g$ for 20 min. The supernatant liquid was dialyzed with a dialysis membrane tube (Spectro/Por 6 mwco: 50000) at 4 °C for 6 d against 1000 ml of distilled water. The non-dialysable fraction was freezedried, and the remaining material was applied to our experiments as crude water extracts (NS-1).

Measurement of Average Molecular Weight of NS-1 Size exclusion chromatography[3] was utilized in order to determine the molecular weight of NS-1 using HPLC. Polyethylene oxides (MW 26000—885000) were used as standards. HPLC conditions were as follows. HPLC system: Shimazu LC-6AD pump with Shimazu RID-6A detector and Shimazu C-6A integrator. Column: TSKgelGMPW$_{XL}$ (7.8 mm i.d. × 30 cm, Tosoh) × 2, mobile phase: 50 mM NaCl, flow rate: 1.0 ml/min, column temperature: 40 °C, detector: refractive index (RI) 8×10^{-6} RIU, sample concentration: 1 mg/ml 50 mM NaCl, loading volume: 100 μl.

Identification and Quantitation of Sugar Constituents 2 ml of 2 N sulfuric acid was added to NS-1 (5 mg) and heated at 100 °C for 6 h. After cooling, the NS-1 was neutralized with barium carbonate, centrifuged, and the supernatant was dried. The residue was dissolved in 0.1 ml of 1 M ammonia, then 1 ml of $NaBH_4$ solution (0.2 g/5 ml dimethyl sulfoxide (DMSO)) was added and the solution was kept at 40 °C for 90 min. Next, 0.1 ml of 8 M acetic acid, 0.2 ml of 1-methyl imidazol and 2 ml of acetic anhydride were added, and after stirring the reaction mixture for 10 min, 5 ml of distilled water was added. After cooling, the reaction mixture was extracted with dichloromethane,[4] and the dichloromethane fraction was analyzed using a gas chromatograph/mass spectrometer (GC-MS) following the conditions below. Identification of sugar was carried out by comparison of the retention time in the TLC chromatogram and the mass chromatogram of the standard of each sugar with those of NS-1.

GC-MS conditions were as follows. GC-MS system: a JEOL Auto Mass 150 mass spectrometer equipped with a Hewlett-Packard 5890 Series II gas chromatograph. Column: DB-1 (J&W Scientific) 0.25 mm × 30 m, column temperature: 70 °C (1 min)–20 °C/min–150 °C (0 min)–5 °C/min–250 °C (20 min), injection port temperature: 250 °C. Carrier gas flow rate: He 1.0 ml/min, ionization voltage: 70 eV, ion source temperature: 250 °C. Scan range: *m/z* 50—*m/z* 600. Electron impact (EI) mode.

Quantitation of Sulfate Radical After dissolving the NS-1 (20 mg) in 2 ml of distilled water, 1 ml of 5 M NaOH was added and heated at 100 °C for 60 min. After cooling, the reaction solution was neutralized with 2.7 M HCl and adjusted to 20 ml with distilled water. The concentration of the sulfate radical in the NS-1 was measured using ion chromatography under the following conditions.

Ion Chromatography Conditions: Equipment, IC-200 ion chromatography (Yokokawa). Column: PAX 1 (precolumn) and SAX 1, eluting solution: 4 mM Na_2CO_3 and 4 mM $NaHCO_3$, eliminating solution: 15 mM H_2SO_4, flow rate: 2.0 ml/min.

Cells Human leukemic T-cell line, Molt-4,[5] HIV-1 continuously infected with Molt-4[5] (Molt-4/HTLV-III), and an HTLV-1 carrying cell line, MT-4,[6] were used in this study. The cells were cultured and maintained in RPMI-1640 medium supplemented with 10% fetal calf serum.

Assays for Virus Titer The virus titer was assayed using the microplate method.[7,8] Briefly, at the first step an MT-4 cell pellet (6×10^6) was incuvated with 50 μl of virus solutions serially diluted from 10^{-1} to 10^{-7} for 1 h at 37 °C, the 6×10^4 infected cells were distributed into each well and cultured for 4 d at 37 °C in a CO_2 incubator. On the 5th day, 50 μl of the recollected culture and 100 μl of fresh medium per well was cultured for 2 d as the second step. The 50% tissue culture infectious dose ($TCID_{50}$; μg/ml) of the original virus solution was estimated with the CPE in culture on the 7th day.

Virus and Virus Infection The culture medium of Molt-4/HTLV-III was centrifuged by $1800 \times g$ and filtrated by millipore filter. The supernatant was used as the virus solution. MT-4 cells were infected with HIV at the titer of 100 $TCID_{50}$. MT-4 cell pellet (6×10^6) was incubated with 50 μl of titer-adjusted HIV solution with (B) and without (A) drug (NS-1), and then cultured in a medium containing serialy diluted drug. The culture was maintained as follows: HIV infected MT-4 cells were cultured for 3 d. On the 4th day, two-thirds of the culture was recollected and fresh medium containing the drug was added to the remaining one-third of the culture, then the cells were cultured for another 3 d. On the 7th day, the same process was repeated. Recollected cells

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and supernatants on the 4th, 7th, and 10th days were applied for the assays (Chart 1).

Assays for HIV-Induced CPE An HIV-induced CPE was detected by the giant cell formation assay and by the decreasing viability of the MT-4 cells infected with HIV. The viable cells were detected by the Trypan blue exclusion method.

Staining of HIV-Specific Antigens Viral antigen expression in HIV infected MT-4 cells was detected by indirect immunofluoresence assay (IFA) using a commercial IFA kit (VIRGO HIV IFA Kokusai) according to the technical pamphlet. Briefly, acetone-fixed cells were incubated with diluted sero-positive anti-HIV human serum for 30 min at 37 °C. Cells were then washed with phosphate-buffered saline (PBS) twice for 15 min and incubated with the fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG for 30 min at 37 °C. After washing with PBS as described above, fluorescent-positive cells were detected with a fluorescent microscope.

Quantitation of HIV-Specific Antigen Production After spinning the cells at $1500 \times g$ for 10 min the supernatant of the culture medium was tested for HIV antigen by enzyme-linked immunosorbent assay (ELISA) (HIV Ag EIA Abbott, Abbott).

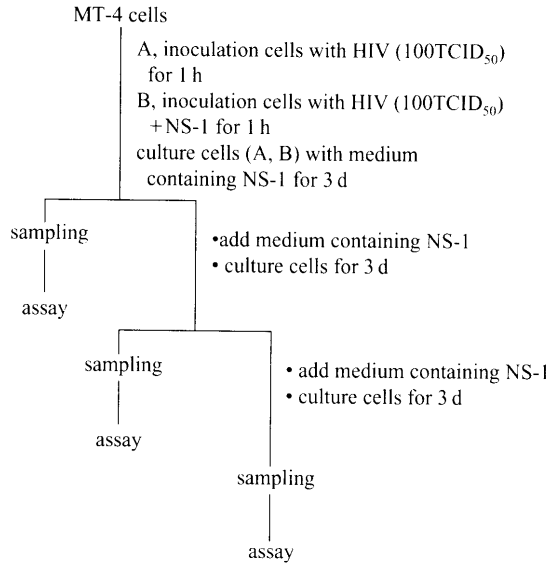

Chart 1. *In Vitro* Assay of Anti-HIV Drug

Assay for Virus Titer of Cultures Incubated with Drugs After spinning the cells at $1500 \times g$ for 10 min, the supernatant of the culture medium was tested for virus titer using the microplate method as described above.

Inhibition Test of HIV-Induced Giant Cell Formation The inhibitory effect of drugs in HIV-induced giant cell formation was tested by co-cultivation of Molt-4 cells and continuously HIV-infected Molt-4 cells.[9] Briefly, HIV-infected Molt-4 cells and the uninfected Molt-4 cells at a concentration of 2.5×10^5 in 250 μl, respectively, were added to 500 μl of medium containing tested drugs as test samples, HIV-infected Molt-4 cells and uninfected Molt-4 cells at the same concentration were added to 500 μl of a medium without drugs as a positive fusion control, and 500 μl of uninfected Molt-4 cells were added to 500 μl of medium as a negative control. These cells were cultured for 20 h at 37 °C in a humidified, 5% CO_2 incubator. The number of viable cells in those cultures was counted and the fusion index was calculated. The formula of the fusion index follows:

$$\text{fushion index} = \frac{\text{number of viable cells in negative control well}}{\text{number of viable cells in mixed test well}} - 1$$

Results

Inhibitory Effect of NS-1 on HIV-Induced CPE in MT-4 Cells NS-1 showed an inhibitory effect on HIV-induced CPE in MT-4 cells (Fig. 1). On the 4th day there was the tendency for the viability of HIV-infected MT-4 cells cultured at a high concentration to be higher than those at a low concentration, but the viability was not significantly low even in the culture without the drug. On the 7th day, the viability showed a remarkable decline in the culture at a low concentration. At concentrations of 62.5 and 125 μg/ml, NS-1 showed higher viability in experiment B (ex. B) than in experiment A (ex. A), showing that the drug had a higher inhibitory effect in ex. B (Fig. 1).

HIV Antigen Production On the 4th day, the amount of HIV antigen produced was very small at every drug concentration (Fig. 2). On the 7th day there was a small

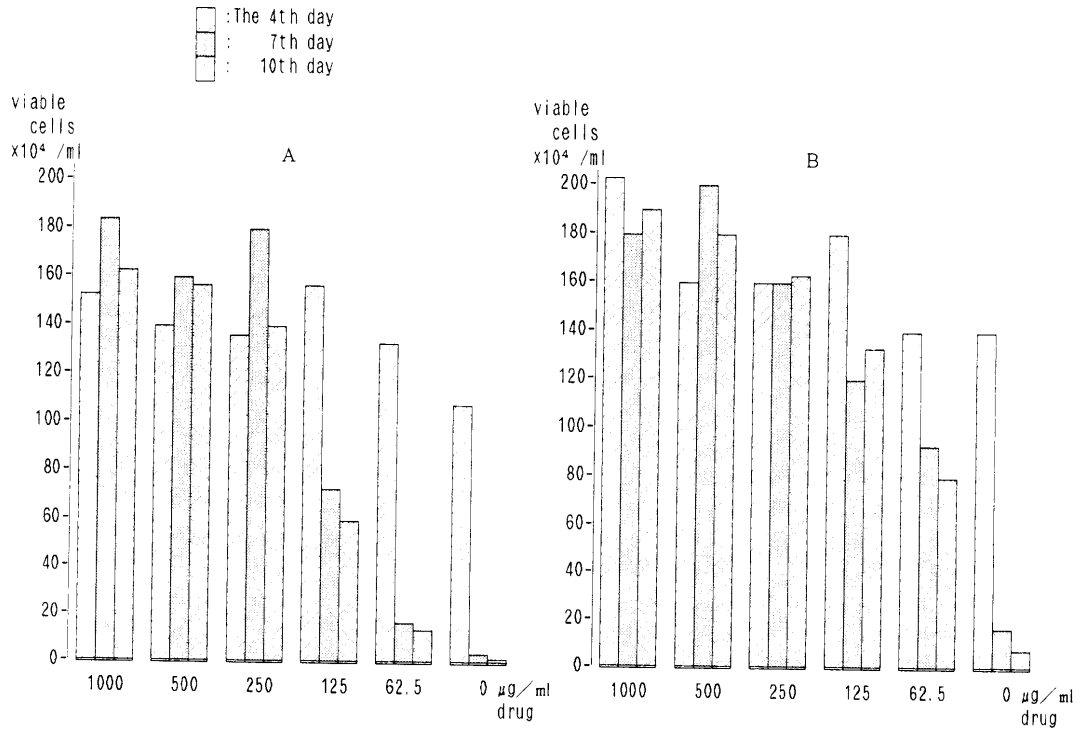

Fig. 1. Inhibitory Effect of NS-1 on HIV-Induced CPE in MT-4 Cells

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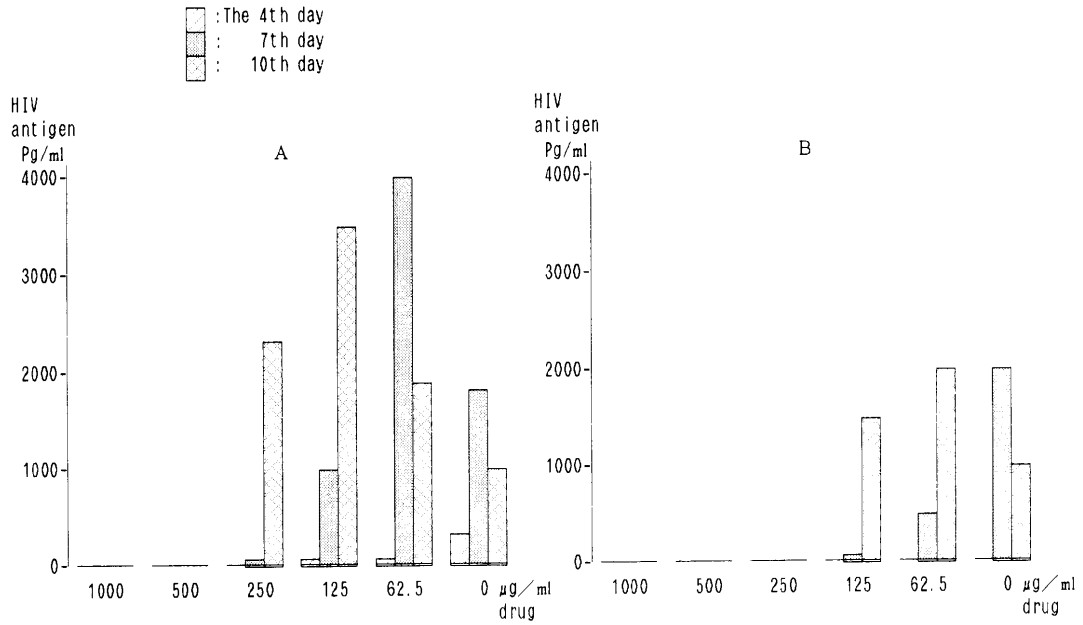

Fig. 2. Inhibitory Effect of NS-1 on HIV Antigen Production in MT-4 Cells

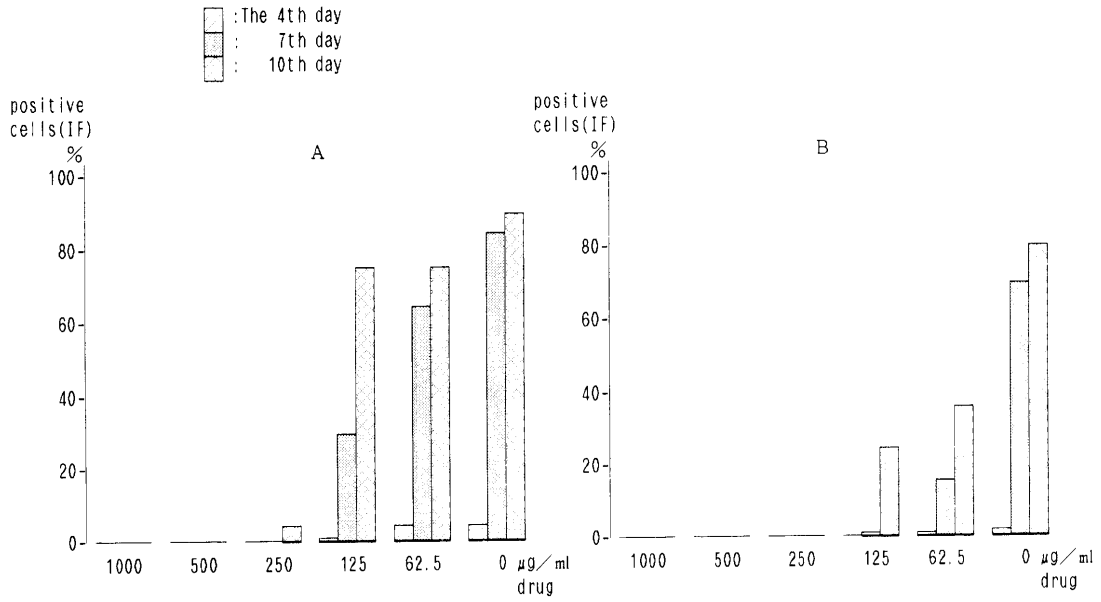

Fig. 3. Inhibitory Effect of NS-1 on HIV Antigen Expression in MT-4 Cells

amount of antigen production at concentrations higher than 250 μg/ml. Considerable antigen production was observed in ex. A at the concentration lower than 125 μg/ml and in ex. B at the concentration lower than 62.5 μg/ml. The peak of antigen production was observed at the concentration of 62.5 μg/ml in ex. A and at the concentration of 0 μg/ml in ex. B (Fig. 2). On the 10th day, the antigen production was detected at a concentration less than 250 μg/ml in ex. A and at a concentration less than 125 μg/ml in ex. B. Peak antigen production was observed at the concentration of 125 μg/ml in ex. A and at the concentration of 62.5 μg/ml in ex. B (Fig. 2).

HIV Antigen Expression On the 4th day, the ratio of HIV antigen expressed cells to total culture cells was zero or very low (Fig. 3). On the 7th day the ratio of HIV antigen positive cells to total cells increased at the concentration less than 125 μg/ml in ex. A and at concentrations less than 62.5 μg/ml in ex. B with a decrease in drug concentration. On the 10th day the HIV antigen positive rate increased at a concentration less than 125 μg/ml in both ex. A and ex. B. In cultures of less than 125 μg/ml the rate was higher than 70% in ex. A, but less than 40% in ex. B except 0 μg/ml (Fig. 3).

Assays for Virus Titer The infection titer (log$TCID_{50}$) of harvested culture supernatant was zero at a concentration of more than 500 μg/ml in ex. A and more than 250 μg/ml in ex. B. The infection titer at the concentration more than 62.5 μg/ml in ex. B was zero on the 4th day. Therefore, considerably high titers were observed in both ex. A (less than 250 μg/ml) and ex. B (less than 125 μg/ml) (Fig. 4).

Inhibition Test of HIV-1 Induced Giant Cell Formation The value of the fusion index was small when NS-1 was added to the culture. There is not an ovbious

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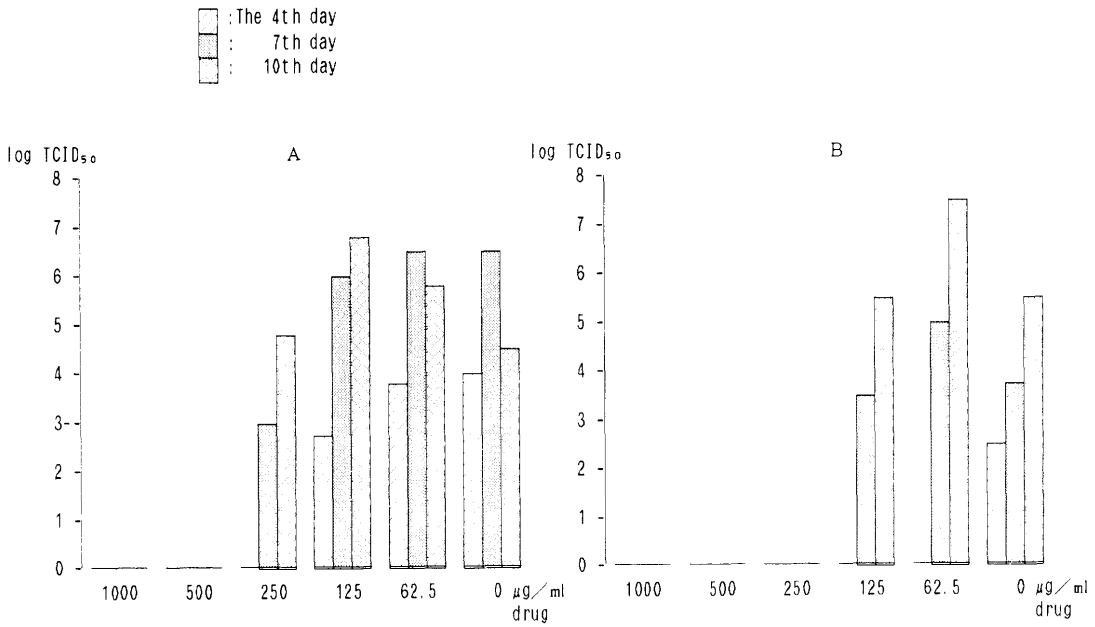

Fig. 4. Inhibitory Effect of NS-1 on Virus Titer

Table 1. Inhibitory Effect of NS-1 on the Giant Cell Formation of Molt-4 Cells

Drug concentration (μg/ml)	Fusion index
1000.0	1.6
500.0	2.2
250.0	2.0
125.0	1.8
62.5	2.0
0.0[a]	3.5

a) Fusion control.

Table 2. Molecular Weight, Sugar Constituents and Sulfate Contents of NS-1

Average molecular weight[a]	1.40×10^5
Sugar constituents	Galactose,[c] uronic acid,[c] glucose, xylose, mannose, rhamnose
Sulfate contents[b] (as SO_4, %)	5.2

a) Calculated from a major peak of the size exclusion chromatography. b) Measured with ion chromatography. c) Major sugars.

correlation between the index number and the administered dose of NS-1 (Table 1).

Physicochemical Properties of NS-1 Average molecular weight was approximately 140 kilodaltons (kDa). In regard to sugar constituents, NS-1 consisted mainly of galactose and uronic acid. The sulfate content of NS-1 was 5.2% as SO_4. These physicochemical properties of NS-1 are shown in Table 2.

Discussion

The anti HIV-1 activity of NS-1 was confirmed in our study. The higher activity in ex. B than in ex. A suggests that NS-1 would have an inhibitory activity against viral adsorption to the taget cell. That corresponds with the results of the giant cell formation inhibition test. The number of living cells decreased gradually as the dose of NS-1 decreased and the duration of culture was prolonged, but the peaks of HIV antigen production and the virus titer were seen at a concentration with 125 μg/ml (ex. A) and with 62.5 μg/ml (ex. B) on the 10th day. The reason why the antigen production and the virus titer did not show a peak in the culture with less than 62.5 μg/ml (ex. A) and 0 μg/ml (ex. B) appears to be that a shortage of NS-1 might have caused cell death and the lack of host cells for virus replication. In the inhibition test of HIV-induced giant cell formation there was not a correlation between the inhibitory effect and the dose of NS-1. This result suggested that the mechanisms of anti-HIV activity of NS-1 in MT-4 cells and Molt-4 cells were different.

Physicochemical analysis showed that NS-1 contained sulfate. Various sulfate polysaccharides have been reported to have anti-HIV activity, especially an inhibitory effect of dextran sulfate has been reported.[10] In regard to the anti-HIV components of NS-1, we proposed that the anti-HIV activity consisted in a component other than dextran sulfate due to the following. The sugar constituent and sulfate content of dextran sulfate are glucose and more than 50% as SO_4, respectively. On the other hand, the sugar constituents of NS-1 were mainly galactose (51.6%) and uronic acid (17.8%), with small amounts of glucose (4%), xyrose (3%), mannose and rhamnose (1.4%), and the sulfate content of NS-1 was 5.2% as SO_4 (Table 2). Therefore, we guessed that the anti-HIV component of NS-1 consisted in a sulfate fraction other than dextran sulfate. We found little anti-HIV activity in the outer fraction of membrane dialysis (MWCO: 50000). And we were unable to detect any anti-HIV-1 activity in kainic acid (data not shown). We are going to further investigate the anti-HIV activity in fractionated samples from NS-1 in order to elucidate the compoennts containing the activity.

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References

1) Ito M., Nakashima H., Baba M., Pauwel R., Clercq E. D., Shigeta S., Yamamoto N., *Antivir. Res.*, **7**, 127 (1987).
2) Ngan F., Chang R. S., Tabba H. B., Smith M., *Antivir. Res.*, **10**, 107 (1988).
3) Mori S., "Saizuhaijo Chromatography," Kyoritu Shuppan, Tokyo, 1991, p. 49.
4) Blankeney A. B., Harris P. J., Henry R. J., Stone B. A., *Carbohydrate Research*, **113**, 291 (1983).
5) Harada S., Koyanagi Y., Yamamoto N., *Virology*, **146**, 272 (1985).
6) Harada S., Koyanagi Y., Yamamoto N., *Science*, **229**, 563 (1985).
7) Mise K., Miki T., Ohonuki N., *et al.*, *Annu. Rep. Natl. Inst. Hygi. Sci.*, **108**, 128 (1990).
8) Ohotake T., Ueha N., Kunita S., Kurimura K., *J. Jpn. Assoc. Infect. Dis.*, **63**, 676 (1987).
9) Yosida O., Nakajima H., Yamamoto N., The 36th Annual Meeting of the Society of Japanese Virologists, 1988, p. 119 (abstract 177).
10) Ito M., Baba M., Sato A., Pauwel R., De Clercq E., Shigeta S., *Antivir. Res.*, **7**, 361 (1987).

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