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INHIBITION OF THE INFECTIVITY AND CYTOPATHIC EFFECT OF HUMAN IMMUNODEFICIENCY VIRUS BY WATER-SOLUBLE LIGNIN IN AN EXTRACT OF THE CULTURE MEDIUM OF Lentinus edodes MYCELIA (LEM)

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Inhibition of the infectivity and cytopathic effect of human immunodeficiency virus type 1 (HIV-1) by the immunoactive fractions obtained from LEM, which is an extract of the culture medium of Lentinus edodes mycelia, is reported. A purified fraction, EPS4, obtained from LEM by ethanol precipitation followed by hydrophobic chromatography and gel filtration chromatography completely inhibited the HIV-1 induced cytopathic effect in vitro at concentrations of $\geq$10 μg/ml. Chemical and spectral analysis revealed that EPS4 is composed of water-soluble lignins containing minor amounts of protein (3.2%) and sugars (12.2%). Taken together with the previously reported observation that EPS4 promotes the activation of macrophages and the proliferation of bone marrow cells, the fraction appears to possess both an immunostimulating activity and an anti-HIV effect in vitro.

Acquired immune deficiency syndrome (AIDS) is a pandemic immunosuppressive disease which results in life-threatening opportunistic infections and malignancies[1-3]. The pursuit of effective chemotherapeutic agents for AIDS has yielded various candidate drugs including 2',3'-dideoxynucleoside analogs[4], recombinant CD4 proteins[5] and dextran sulfate[6-8], all of which show a potent inhibitory activity against human immunodeficiency virus (HIV) in vitro. Indeed, one of the 2',3'-dideoxynucleoside analogs, AZT[9] has been proved to be effective in clinical use[10,11].

LEM is an extract of a mycelial culture of the Japanese edible mushroom Lentinus edodes, which was grown in solid medium composed of sugar-cane bagasse and rice bran. We have previously reported that some fractions obtained from LEM exert immunostimulating activities: augmentative effects on both the glucose consumption of murine macrophages and thymidine uptake of bone marrow cells[12,13]. Recently,

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LEM and its ethanol precipitated fraction have been reported to have an inhibitory effect on the cytopathogenicity of HIV in vitro[14]. In this communication, we describe an anti-HIV effect of our purified immunoactive fractions derived from LEM.

MATERIALS AND METHODS

LEM[15]: LEM is a product of SUN LEM Co., Ltd. (former Noda Shokukin Co., Ltd.). It is a brownish spray-dried powder of the hot-water extract of a mycelial culture of the Japanese edible mushroom, Lentinus edodes (shiitake). LEM contains water soluble metabolites together with components originating from both the autolyzed mycelia and enzymatically degraded constituents of the culture medium.

Fractionation of LEM (Fig. 1): (1)Ethanol precipitation. All procedures were done at 0-4 °C. LEM (100 g) was dissolved in 1000 mL of distilled water, and the pH was adjusted to 7.2 by aqueous NaOH. Cold ethanol (600 ml) was then added, and the precipitates formed were removed by centrifugation at 10,000 x g for 20 min. Further addition of ethanol to the supernatant (400 ml, 50 %v/v ethanol in total) gave precipitates designated as neoPPT1[12], which were collected by centrifugation and lyophilized. (2) Hydrophobic chromatography. NeoPPT1 was further fractionated by hydrophobic chromatography using Phenyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden). Three buffer systems with increasing orders of hydrophobicity were employed for the elution: buffer-1 (10 mM phosphate-Na containing 1M $(NH_4)_2SO_4$, pH 6.8), buffer-2 (10 mM phosphate-Na, pH 6.8) and buffer-3 (buffer-2 containing 75 %v/v ethyleneglycol). NeoPPT1 was dissolved in buffer-1 and applied to the column (80 mm i.d. x 400 mm) equilibrated with the same buffer. Successive elution with buffer-1, 2 and 3 followed by dialysis against water and lyophilization of each eluate gave fractions EP1, EP2 and EP3, respectively. (3) Gel filtration chromatography. EP3 was dissolved in 50 mM phosphate-Na buffer, pH 7.2, and further fractionated by gel filtration chromatography using a column (26 mm i.d. x 600 mm) of Sephacryl S-300 (Pharmacia). The eluates were collected into 4 fractions (EPS1 to EPS4) by the order of elution. Approximate molecular weights were: EPS1, 1,100-1,500 Kd; EPS2, 400-1,100 Kd; EPS3, 100-400 Kd; and EPS4, 10-100 Kd.

IR spectra : IR spectra of EPS4 and wheat straw lignin were recorded as KBr tablets on a Shimadzu IR-435 infrared spectrophotometer.

Cell and virus: The HTLV-I carrying the $CD4^+$ human T cell clone ATH8[9,16], was used in this study. This clone is highly sensitive to the cytopathic effect of HIV. ATH8 cells were cultured in RPMI-1640 medium supplemented with 15%v/v FCS, 15%v/v TCGF (Advanced Biotechnologies Inc.), 4 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin. HIV-1 was obtained from the culture supernatant of HIV-1/HTLV-III_B producing H9 cells (Kindly provided by Drs. M.Popovic and R.C.Gallo).

Assay for HIV-induced cytopathic effect: The antiviral activity of the LEM fractions against the HIV replication was determined by its capacity to inhibit the virus-induced cytopathogenicity, as previously described[9,16]. Briefly, the following procedure was employed: ATH8 cells were pretreated with 2 µg/ml polybrene for 30 min at 37 °C. The cells were then exposed to 2000 HIV-1 virions per cell for 45 min at 37 °C and resuspended in the culture medium in the presence or absence of the test samples and incubated for 7 days at 37 °C. The number of viable cells was counted by the trypan blue exclusion method.

Reverse transcriptase assay: The inhibitory effect of the LEM fractions on the activity of purified HIV-1 reverse transcriptase (a kind gift from Dr. Sarngadharan) was examined using $p(rA)\cdot(dT)_{12-18}$ as a template as previously described[17].

RESULTS

Figure 1 represents a schematic diagram for the fractionation of LEM. Figure 2 shows the inhibitory effect of the LEM fractions, neoPPT1, EP1, EP2 and EP3, on the HIV-induced cytopathogenicity to the target ATH8 cells. When the target ATH8 cells are exposed to HIV-1 in the form of cell-free virions, most ATH8 cells are destroyed without forming detectable syncytia and distinct morphological changes are not usually detected on the microscope. NeoPPT1, which was obtained as a 37.5% ethanol-soluble/50% ethanol-insoluble fraction, appeared to be effective at the concentrations of $\geq$50 μg/ml, although the cytotoxicity to ATH8 cells was predominant appeared at $\geq$20 μg/ml. Three fractions, EP1, EP2 and EP3, fractionated from neoPPT1 by the hydrophobic chromatography exhibited a more clear-cut anti-HIV activity. Among them, EP3, the most hydrophobic fraction, showed the highest activity. EP3 virtually completely inhibited the cytopathic effect of HIV-1 at 20 μg/ml, and was only moderately cytotoxic to the target ATH8 cells at the highest concentration tested (200μg/ml).

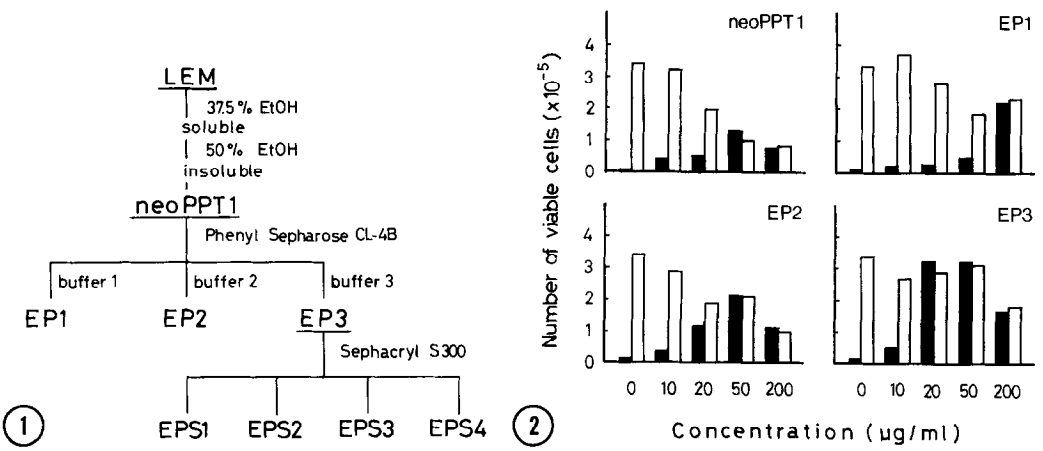

Fig.1 Schematic diagram of fractionation from LEM. The scheme was slightly modified from that of our previous work in which immunoactive substances effective for chronic hepatitis B have been aimed to purify from LEM[9].

Fig.2 Inhibitory effect of neoPPT1, EP1, EP2, and EP3 on HIV-1-induced cytopathic effect to ATH8 cells. ATH8 cells ($2x10^5$) were exposed to $HTLV\text{-}III_B$ (2000 virions per cell) in the presence or absence of various concentrations of the samples. Control cells (open columns) were similarly treated without exposure to virus. The total number of viable cells was counted after 7 days. ATH8 cells are extremely sensitive to the cytopathic effect of HIV-1 in vitro and the number of total viable cells that would have been otherwise destroyed by the cytopathic effect of HIV-1 represents the number of the cells that were protected by the drug against the infectivity and cytopathic effect of the virus[4].

EP3 was further fractionated by a gel filtration chromatography on a Sephacryl S-300 column. The elution showed one broad peak which ranged from the void volume (1,500Kd) to 10Kd, and the eluate was expediently collected into 4 fractions, EPS1, EPS2, EPS3 and EPS4, according to the order of elution. As shown in Fig. 3, EPS4, having the smallest molecular size(10-100 Kd), exhibited the most potent anti-HIV activity among the fractions. EPS4 could completely suppressed the infectivity and cytopathic effect at the concentrations of $\geq$10 μg/ml and did not affect the growth of ATH8 cells at concentrations of up to 200 μg/ml.

The chemical characteristics of EPS4 obtained are: (1) the elemental analysis; C, 44.6%; H,4.68%; N,1.74%. (2) the protein content; 3.2% (amino acid analysis), (3) the neutral sugar content; 12.2% (Somogyi-Nelson's method), and (4) the uronic acid content, 3.7% (carbazole-H_2SO_4 method). These data indicate that neither protein nor sugars are the major constituents (above 80%) of this fraction at all.

The IR spectrum of EPS4 showed strong bands due to carboxylate (1600 cm^{-1}) and aromatic rings (1600, 1500 and 1420 cm^{-1}). In addition to these, many absorption bands specific to lignin structure were observed and they coincided with those of Bjorkman lignin, a water-insoluble low molecular weight lignin purified from wheat straw. Furthermore, the solid state ^{13}C-NMR (CP-MAS NMR) spectrum of EPS4 also gave large signals of carboxyl, aromatic and methoxyl carbons together with sugar carbons (data not shown). From these data the greater part of EPS4 was assigned to lignin having large number of carboxyl groups.

The IR spectra of EP3 and its further chromatographed fractions were almost identical to those of lignins. This observation, together with our finding that EP3 gave only a broad peak, and the anti-HIV

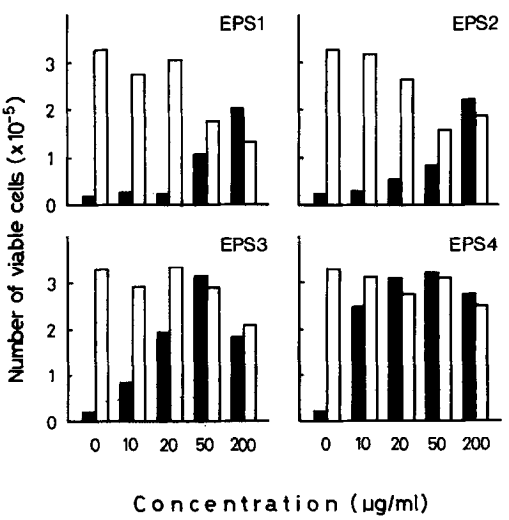

Fig.3 Inhibition of the infectivity and cytopathic effect of HIV-1 against ATH8 cells by EPS1, EPS2, EPS3, and EPS4. Procedures and the usage of symbols are described in the legend to Fig.2.

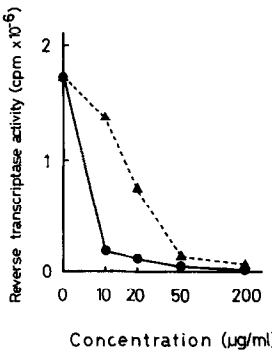

Fig.4 Inhibitory effect of neoPPT1(▲···▲) and EP3(●—●) on reverse transcriptase activity from HIV-1.

activity was observed in a relatively wide range with regard to molecular sizes, indicates that EP3 is an assembly of structurally similar lignins with diversely distributed molecular weights.

The LEM fractions obtained in this study were assayed for HIV-1 reverse transcriptase activity using $p(rA)\cdot(dT)_{12-18}$ as a template. EP3 and neoPPT1 had an apparent inhibitory effect as shown in Fig. 4. Ninety percent of RT activity was suppressed by 10 μg/ml of EP3 and 50 μg/ml of neoPPT1, respectively.

DISCUSSION

In this study, we demonstrated that the fractions derived from LEM had an apparent inhibitory effect on the HIV replication in vitro and the specific anti-viral activity increased along with further fractionation. EPS4, which is the lowest molecular weight fraction of EP3, showed a remarkable inhibitory effect on the HIV-induced cytopathogenicity to ATH8 cells at a concentration of 10 μg/ml. Neither the cellular viability nor the growth of uninfected ATH8 cells was affected at the concentrations of up to 200 μg/ml EPS4. In addition, EP3 inhibited the the activity of purified HIV-1 reverse transcriptase in vitro.

LEM has been reported to have multiple biological activities[12,13,18-22]. They could be summarized in two types of action; one is a direct anti-viral activity and the other is a modulation of immune functions. LEM has been reported to directly inhibit the infection of tobacco mosaic virus (TMV) by blocking the initial stage of the replicative cycle[18]. Modulations of host-mediated immunological activities by LEM and its fractions have also been reported: (1) suppressions of the proliferation of rat ascites hepatoma AH414 and its hepatocarcinogenesis[19], (2) a protective effect against the liver cell injury induced by antibody-dependent cell-mediated cytotoxicity (ADCC)[20], (3) an enhancement of IL-1 production[20], (4) an activation of murine macrophage functions[12] and a stimulation of proliferation of

murine bone marrow cells in vitro[13]. In an open clinical trial on LEM performed in 16 medical centers in Japan, orally administrated LEM has been shown to promote seroconversions in patient of chronic hepatitis B without any apparent side effects[21-23].

In the current study, we demonstrated that the main component of the EP3 fraction was a water-soluble lignin whose molecular weight ranged from 10 to 1,500 Kd, and EPS4, the lower molecular portion of EP3, was superior in the anti-HIV activity than other molecular weight fractions. Lignin is a complex dehydrogenative polymerization product of cinnamyl alcohol derivatives. Considering that some basidiomycetes are generally known to have a high ability to degrade wood lignin and that the sugar-cane bagasse used in this study contains 20% of lignin, it is plausible that the present strain of Lentinus edodes decomposes oxidatively a bagasse lignin to produce carboxylated water-soluble lignin derivatives.

In our previous work, EP3 and EPS4 have been shown to strongly augment the glucose consumption by murine macrophages and to induce the proliferation of murine bone marrow cells in vitro[13]. It is worth noting that the same immunoactive fractions appears to represent the direct anti-viral activity. These water soluble lignins are thought to have a polyanionic nature brought about by large number of carboxyl groups.

Polyanionic macromolecules including polyacrylic acid, dextran sulfate, have been shown to inhibit the penetration of certain viruses into the cell[24]. Certain anionic substances such as dextran sulfate[7,9], heparin[6] and suramin[25] have recently been reported to be effective in inhibiting the HIV replication in vitro. Thus, polyanionic nature of the water soluble lignins in LEM might contribute to the inhibition of the HIV-induced cytopathic effect.

It should be stressed, however, that the activity of an agent against HIV in vitro does not ensure that the agent will be clinically applicable in the setting of HIV-infection. Bioavailability, metabolic features, toxicities, and other factors may negate the usefulness of a given agent. LEM has been administered orally as a natural nutrient for over fifteen years in Japan and this history suggests that LEM might not bring about at least major side effects in human beings. However, it remains to be asked whether LEM can be absorbed orally to achieve the effective concentrations in plasma and whether LEM can exert its antiviral effect against HIV in the face of 100% plasma. Also we do not know how quickly LEM is degraded to lose its antiviral effect when introduced into the blood stream. Nevertheless, our findings described here and previous observations that EPS4 appears to have multiple biological activities including apparent immunopotentiating capability together with an activity against HIV in vitro warrant for further investigation.

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