Short Communication

Anti-viral Activity of Water-solubilized Lignin Derivatives *in Vitro*

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Two lignin derivatives were examined to see whether or not they have anti-viral activity *in vitro*. One of them, EP3, was a high molecular weight substance chromatographically fractionated from an extract of the culture medium of the Japanese edible mushroom, *Lentinus edodes*, mycelia (LEM). Another, lignosulfonate (LS), was obtained from the waste liquor of the acid sulfite pulping process of wood. To investigate the anti-viral activity of the drugs, VERO-317 cells, derived from African green monkey kidney, were used. Both drugs completely inhibited the occurrence of the cytopathic effects (CPE) of herpes simplex viruses (type 1 and type 2), and western equine encephalitis (WEE) virus, and partially inhibited those of poliovirus, measles virus, and mumps virus, when the drugs were added to the culture medium after viral infections.

Quite recently, Suzuki *et al.*¹⁾ reported that EP3 inhibited the CPE of human immunodeficiency virus (HIV) *in vitro* and found that the active principle of EP3 was a water-solubilized lignin. Further, they demonstrated that lignosulfonate (LS) which activates murine macrophages and causes proliferation of bone marrow cells,²⁾ also has the anti-HIV activity.³⁾ This study has been designed to investigate whether or not the lignin derivatives, EP3 and LS, show anti-viral activity for various viruses *in vitro*.

The preparation methods of lignin derivatives were described in our previous papers.^{1,2)}

For this study, VERO-317⁴⁾ (a stable subline of VERO) was used. VERO-317 cells were continuously cultured in glutamine-deprived, glutamate- and biotin-supplemented Eagle's minimum essential medium without serum, proteins, and lipids (EM #317). The cells were grown on 24-well plastic culture plates (Corning, N.Y., U.S.A.) in a chemically defined medium (EM #317) for subsequent experiments. The viral infection was done after the cells reached confluency.

Herpes simplex viruses, UR-3 (type 1) and MS (type 2) were kindly supplied by Dr. K. Miyamura of the National Institute of Health, Tokyo, Japan. Herpes simplex virus, UW (type 2) was generously supplied by Dr. B. Simizu of Chiba University, Chiba, Japan. Measles virus (Sugiyama strain), poliovirus (type 1), WEE virus, and vaccinia virus were kindly given by Dr. T. Yamanaka of Chiba Prefecture Serum Institute, Chiba, Japan. Herpes simplex virus (Yasumura strain, type 1) and mumps virus were isolated in this laboratory of Dokkyo University School of Medicine.

These viruses were grown in VERO-317 cells in 25-ml glass bottles in the EM #317. After the cells caused CPE completely, the cells were frozen and thawed three times in culture media. Then, the cell lysates were centrifuged at 5,000 rpm for 30 min, and the supernatants were titrated by appearance of cytopathic effects in the same cells. These stocks of the viruses were stored at -70°C until use.

Before virus inoculation, the cells were washed with phosphate-buffered saline, pH 7.4, without Mg^{2+} and Ca^{2+} (PBS^{-}). A viral solution (0.2 ml) containing 100 TCID was added to each well and kept 90 min for adsorption at room temperature ($\sim 25^{\circ}\text{C}$). Then the solution was removed from the well and fresh culture medium with and without each of the lignin derivatives was added.

When the VERO cells infected with herpes simplex virus, type 1 (Yasumura strain) were

Fig. 1. Inhibitory Effects of LS and EP3 on Cytopathic Effects Induced with Various Viruses.

VERO cells were incubated with various viruses for 1.5 hr at room temperature, and then the viral solution was removed and 1 ml of fresh culture medium with and without LS or EP3 was added. After the cells were cultured for 7 days at 37°C, they were fixed with methanol for 10 min at room temperature and then stained with 10% Giemsa solution (Merck).

cultured in fresh medium with and without lignin derivative, EP3, at various concentrations for 4 days at 37°C, EP3 completely inhibited the occurrence of the cytopathic effects at 100 µg/ml and strongly inhibited them at 50 µg/ml. However, no significant effect was observed at 5–10 µg/ml. This drug did not affect the VERO cell growth at these concentrations. Similar results were obtained with liginosulfonate (LS). Therefore, the effects of these drugs were examined at 100 µg/ml.

The VERO cells were infected with various viruses and cultured in the drug-including media. Each drug completely inhibited CPE by

Table I. INHIBITORY EFFECTS OF EP3 AND LS ON CYTOPATHIC EFFECTS BY VIRUSES

Virus	EP3	LS
Vaccinia	(+)	(+)
Herpes simplex		
(Yasumura, type 1)	+++	+++
(UR-3, type 1)	+++	+++
(UW, type 2)	+++	+++
(MS, type 2)	+++	+++
WEE	+++	+++
Polio	+	++
Mumps	+	+
Measles (Sugiyama)	+	+

+++ , complete inhibition; ++ , a few cytopathogenic effects were observed; + , a small effect was observed; (+) a small drug effect was observed until 4 days of culture.

herpes viruses, and their inhibitory effects were independent not only of viral types but also viral strains (Fig. 1, Table I). Although it is likely that the drugs did not show their effect on vaccinia virus at 7 days (Fig. 1), the small inhibitory effects were observed until 4 days of culture. The drugs also completely inhibited CPE by WEE virus. LS almost completely inhibited the poliovirus-induced CPE and EP3 showed clear inhibition. Similarly, the inhibitory effect of LS was larger than that of EP3 in mumps virus. No difference in the inhibitory effect between the drugs was observed with measles virus.

In general, antibiotics are not effective on viral diseases. Recently, Suzuki *et al.* showed that lignin derivatives, EP3¹⁾ and LS,³⁾ had anti-viral activity for HIV *in vitro*. Furthermore, the oral administration of LEM, which contains EP3, improved the hepatic functions of hepatitis-B patients *in vivo* without serious side effects.⁵⁾ Judging from these results, the lignin derivatives may show anti-viral activity for other viral diseases *in vivo*.

It was reported that alkaloids⁶⁾ and anti-cancer agents⁷⁾ also inhibited the growth of herpes virus (type 1) *in vitro*. LS inhibited plaque formation by herpes simplex virus *in vitro*.⁸⁾ In this study, the lignin derivatives EP3 and LS completely inhibited the appearance of CPE by herpes viruses (type 1 and type 2). These drugs showed inhibitory effect on the oc-

currence of CPE by various viruses, when the drugs were in the cell culture medium. Therefore, lignin derivatives may be promising antiviral compounds, even though we do not understand the inhibitory mechanisms of these drugs on various viruses *in vivo* as well as *in vitro*.

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