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Antiviral Activity of Virus-Like Particles From *Lentinus edodes* (Shiitake)

Brief Report

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With 1 Figure

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Summary

Interferon (IF) induction and antiviral activity by purified spherical (S) or filamentous (F) virus-like particles and S-derived RNA was studied. A single administration of S particles prior to virus challenge reduced significantly the mortality of mice infected with western equine encephalitis (WEE) virus.

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Some of the viruses or virus-like particles from species of *Penicillium* and *Aspergillus* have been found to inhibit animal virus multiplication *in vitro* or *in vivo*. It was subsequently shown that the active principle was double-stranded (ds) RNA contained in the fungal virus particles and that ds-RNAs are highly active in inducing interferon (IF) and host-resistance to viral infections (1, 6, 7, 10). In a previous report (11), we have demonstrated that several kinds of virus-like particles are detected in both mycelia and fruiting bodies of *Lentinus edodes*. Two kinds of spherical (S, 36 and 45 nm in diameter) and filamentous (F, 17×200 to 1200 nm) particles have been partially purified by density gradient centrifugation in CsCl and characterized in part. In recent years, Ishida *et al.* (5, 15) have reported that the ds-RNA fraction extracted from the spores and mycelia of *L. edodes* is highly active as an IF inducer. However, it has not been resolved whether a fungal virus RNA in the mushroom extracts is responsible for IF induction. We report here the IF induction and antiviral activity by purified virus-like particles from *L. edodes* and RNA derived from the particles.

Fresh extract from fruiting bodies of *L. edodes* in 0.033 M phosphate buffer (PB, pH 6.8) was used as a starting material for the purification of virus-like particles (VLP) which was performed by the modified method reported by Hol-

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LINGS (3) and DIELEMAN-VAN-ZAAYEN (2). The crude extract was treated with a mixture of 15 per cent butoxy- and 30 per cent ethoxyethanol in 2.5 M PB (pH 6.8). Further purification of S and F particles was carried out by density gradient centrifugation at $105{,}000 \times g$ for 18 to 20 hours in 35 per cent CsCl (11). The nucleic acid preparation from purified S particles was obtained by the cold phenol method. The protein and RNA content of S and F particles or of the nucleic acid fraction were determined by the method of LOWRY *et al.* (9) and orcinol reaction, respectively.

Preparations isolated from the lower band in the CsCl column showed polyhedral or spherical particles 45 nm in diameter, buoyant density of 1.41, and a maximal UV absorption at 260 nm. Another preparation from the upper band contained filamentous particles of 17×200 to 1200 nm in diameter. Filamentous particles had a buoyant density of 1.33 and maximal UV absorption at 280 nm (Fig. 1).

The cell cultures used in these experiments included continuous cell lines of rabbit kidney (RK-13), baby hamster kidney (BHK-21) and mouse fibroblasts (L). These cells were grown in Eagle's minimum essential medium (MEM) containing 0.03 per cent L-glutamine, supplemented with 5 per cent inactivated calf serum. Stock suspensions of vesicular stomatitis virus (VSV, New Jersey strain) were prepared in primary chick embryo cells. Western equine encephalitis (WEE) virus, Rockefeller Institute standard strain, was used as a 10 per cent infected mouse brain homogenate in Hanks' balanced salt solution which was centrifuged at $12{,}000 \times g$ for 15 minutes (14). RK-13 and L cell monolayers containing about

Table 1. *Inhibitory effects of S or F particles and of the RNA extracted from S particles on the virus growth and cytopathic effect by VSV in RK-13 cells*[a]

Inducer (μg/ml)	MOI (PFU/cell)	CPE at 48 hours	VSV yields (PFU/ml)	
			24 hours	48 hours
S 7.0	0.01	−	6.0×10^1	9.4×10^1
RNA 1.0		−	2.5×10^5	2.0×10^2
F 9.0		+ to ++	2.6×10^6	7.0×10^4
Untreated		+++	2.7×10^6	1.4×10^6
S 7.0	1.00	+	NT[b]	2.8×10^3
RNA 1.0		−	2.2×10^5	4.4×10^4
F 9.0		+++	2.0×10^6	NT[b]
Untreated		+++	5.2×10^6	3.6×10^6

[a] Rabbit kidney cell (RK-13) cultures were exposed to S (7.0 μg/ml), RNA (1.0 μg/ml) extracted from the purified S particles, or F (9.0 μg/ml), and incubated at 37° C for 18 to 24 hours. The medium was then removed and cell monolayers were washed 3 or more times with phosphate buffer saline. The cells were infected with the input multiplicity (MOI) of 0.01 and 1.00 PFU/cell of VSV. Antiviral effects on VSV infection were assayed by determining the virus yields at 24 and 48 hours post-infection (p.i.). The virus yields were assayed in BHK-21 cells. The extent of inhibition of the cytopathic effect (CPE) was also evaluated at 48 hours p.i., as follows: +++, complete destruction of monolayer cells; + to ++, extensive CPE; −, no CPE

[b] Not tested

1×10^6 cells were exposed to S-F particles or the RNA at the indicated concentration. The cell cultures were incubated at 37° C for 18 to 24 hours, and culture fluid samples were saved for IF assay. For selective removal of the ds-RNA

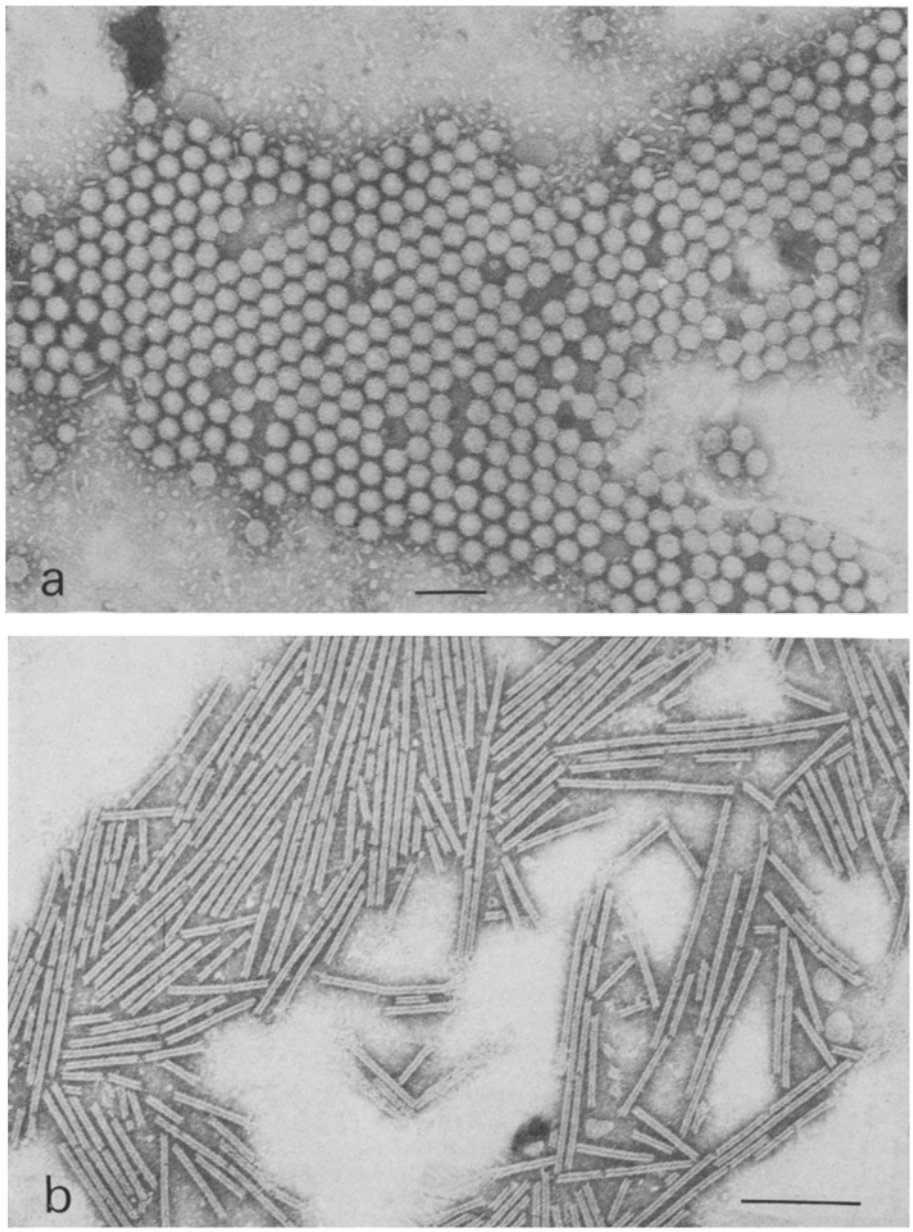

Fig. 1. Electron micrograph of VLP preparation from *L. edodes*. *a* purified spherical (S) particles (Bar = 100 nm; ×100,000); *b* purified filamentous (F) particles (Bar = 200 nm; ×80,000)

inducer from IF samples, pancreatic ribonuclease (10 μg/ml) was added to each sample and the mixtures were incubated overnight at 20° C before assaying for antiviral activity. IF was usually assayed by the 50 per cent plaque reduction method using vesicular stomatitis virus (VSV) in RK-13 or L cells (12). IF titers were also determined by inhibition of cytopathic effect (CPE). Other antiviral activity *in vitro* against VSV was assayed by determining the virus yields at 24 and 48 hours post-infection (p.i.) in the RK cells treated directly with each of the materials (13).

Table 1 shows the antiviral activities in RK-13 cells treated with S or F particles and the RNA extracted from S particles. Highest resistance to VSV infection was shown by treatment with S particles. Inhibition of the viral growth was lower in the cells exposed to F than to S or the RNA. Table 2 summarizes the results of IF induction in RK-13 cells using S or F particles and the RNA. The IF titers induced by S or RNA ranged from 80 to 640 units/ml by the plaque reduction method while slightly lower titers were obtained by the CPE inhibition method. The RNA derived from S particles was highly active in inducing IF production even after the addition of an amount as small as 1.0 μg/ml. On the other hand, F particles were less active in inducing IF, similar to the results obtained in Table 1.

For the protective tests on WEE virus infection *in vivo*, 3-week-old male mice of the JCL-ICR strain (Clea Japan Inc., Tokyo) were used. Mice were treated with S or F particles by intraperitoneal injection in the amounts of 0.2 to 0.3 ml, 5 hours before intraperitoneal inoculation of 10^1 to 10^3 LD_{50} of WEE virus. Mice were observed for 20 days after virus infection. Protective effects were evaluated by percent survival and relative survival times. As shown in Table 3, 60 per cent of the infected mice survived when the mice were treated with 1.5 mg/kg of S particles 5 hours before virus challenge with 10^2 LD_{50} of WEE virus. None of the untreated mice or mice treated with 0.30 mg/kg survived, although the survival time was somewhat longer in the treated groups (4.0 days) than in the control (2.9 days). The protective effect was still evident with a higher virus challenge (10^3 LD_{50}) where 40 per cent of the treated mice survived. On the other hand, only 20 per cent of infected mice survived when considerably higher doses (30.0 mg/kg) of F particles were administered 5 hours prior to virus challenges of 10^1 and 10^2 LD_{50}. None of the treated or control mice infected with 10^3 LD_{50} of WEE virus survived. No toxic effects were observed from the doses of S or F particles used.

Table 2. *Interferon induction in RK-13 cells by S or F particles and the RNA extracted from S*[a]

Inducer (μg/ml)	IF titer (units/ml)
S 10.0	80
RNA 1.0	640
F 10.0	40
No inducer	<5

[a] RK-13 cell cultures were exposed to each inducer and samples of culture fluid were harvested after incubation at 37° C for 18 to 24 hours. IF titer was determined by 50 per cent plaque reduction method in RK cells using VSV for challenge

Maximum IF titers in the blood of mice treated with S or F ranged from 20 to 80 units/ml after 5 to 24 hours when IF was assayed by VSV inhibition in mouse L cells. The IF titer then declined rapidly. On the other hand, WEE viremia in the treated mice was markedly suppressed.

The present report shows that both S particles and RNA extracted from S particles are most active in inducing IF and inhibiting VSV growth in rabbit kidney cell cultures. In addition, a single administration of S particles prior to virus challenge reduced significantly the mortality of mice infected with WEE virus. This protective effect can be explained at least in part by the antiviral activity of IF induced by the ds-RNA contained in S particles.

Although the virus or virus-like particles have been detected in numerous fungal species by electron microscopy, only a few viruses have been described in detail (8). INOUE (4) first observed in 1970 three kinds of virus-like particles in *L. edodes*. Recently, USHIYAMA *et al.* (16) have also purified polyhedral virus-like particles of approximately 25, 30 and 39 nm in diameter from both fruiting bodies and mycelia of *L. edodes*. In addition, they have demonstrated that 39 nm particles contain a ds-RNA, suggesting that ds-RNA present in the Shiitake mushroom extract, as reported by ISHIDA *et al.* (5, 15), may be derived from these 39 nm virus-like particles. It is of interest that F particles have shown a lower inhibitory effect against viral infections in both cell cultures and mice, because these particles may be composed of only protein without nucleic acid (17). However, at present, the relationship between S and F particles which are usually detected together in *L. edodes* remains unresolved.

Table 3. *Effect of prophylactic treatment with S and F particles on western equine encephalitis virus infection in mice*

Treatment	WEE virus challenge (Log_{10} LD_{50})	No. of surviving mice / Total	Mean survival time (days) of dying animals ± standard deviation
S (1.5 mg/kg)	1	6/10	6.0±1.0
	2	6/10	5.5±1.9
	3	4/10	8.0±1.6
None	1	0/10	4.0±0.8
	2	0/10	2.9±0.7
	3	0/10	2.7±0.7
F (30 mg/kg)	1	2/10	4.8±0.9
	2	2/10	3.9±1.9
	3	0/10	4.0±0.8
None	1	0/10	2.8±0.9
	2	0/10	2.5±0.7
	3	0/10	2.3±0.5

Mice were given an intraperitoneal dose of S or F particles, and 5 hours later were inoculated intraperitoneally with 10^1, 10^2 or 10^3 LD_{50} of WEE virus

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