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# Antiviral effect of the extract of culture medium of *Lentinus edodes* mycelia on the replication of herpes simplex virus type 1

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## Summary

An extract of culture medium of *Lentinus edodes* mycelia, JLS-S001, significantly blocked the release of infectious herpes simplex virus type 1 (HSV-1) from African green monkey kidney cells. The block in replication was not due to the effect of JLS-S001 on the adsorption and penetration of HSV-1 to the monkey kidney cells. This observation was supported by the fact that JLS-S001 had no significant effect on the expression of virus-specific nucleocapsid proteins in the treated cells. Furthermore, electron microscopy demonstrated the presence of nucleocapsids within the nuclei of the infected and JLS-S001-treated cells. However, the expression of glycoproteins B, C, D, E and I was reduced in the JLS-S001-treated cells. These results suggested that JLS-S001 blocked HSV-1 replication at a late stage in virus replication cycle probably in the assembly and budding of nucleocapsids and subsequent egress from the treated cells.

Anti-herpes; Phytochemical; Blocking late stage replication

## Introduction

Herpes simplex virus (HSV) causes infections ranging from asymptomatic to life-threatening diseases even in the presence of humoral and cell-mediated immunity. Herpes simplex virus infection may lead not only to symptomatic

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primary disease but also to the development of recurrent lesions following reactivation of the latent virus in the neurons of sensory ganglia (Stevens et al., 1971; Stevens et al., 1972; Bastian et al., 1972; Baringer et al., 1973). In recent years, a variety of antiviral drugs, particularly nucleoside analogs and foscarnet have been developed to treat HSV disease. However, continued research and development of new and improved therapies are important for numerous reasons, including the appearance of HSV isolates which are resistant to such existing drugs as acyclovir.

For the last several decades, a number of phytochemicals have been used to control severe infections including diseases of viral origin (Hudson, 1990). For example, alkaloids like papaverine and castanospermine have been used against human cytomegalovirus, measles virus and other viruses with membranes. Lignans like podophyllotoxin and flavonoids like chrysosplenols were active against HSV. Some other flavonoids, for example, Ro 09-410 (chalcones) has been found to have antiviral activity against *Rhinoviruses*. In addition, some plant proteins like hypericins and pokeweed antiviral protein (PAP) were found to be active against Retroviruses, poliovirus and HSV. These plant products are especially useful because of their abundance in nature and also sometimes for their low cytotoxicity. One such compound is an extract of a mycelial culture of the edible mushroom *Lentinus edodes* (LEM = *L. edodes* mycelia; Sugano et al., 1982; Sugano et al., 1985). Specific fractions of this mycelial extract, EP3 or E-P-LEM has been shown to be effective against replication and cytopathic effect induced by human immunodeficiency virus (Tochikura et al., 1988; Suzuki et al., 1989; Suzuki et al., 1990). Recently, Sorimachi et al. (1990) reported that EP3 and LS, two fractions of LEM had significant antiviral activity against HSV in vitro. However, no further details were available regarding the mechanism of action of these compounds. Analyses of the chemical structure revealed that EP3 contained a water-soluble polycarboxylated lignin as it's major component and xylose-containing polysaccharide and proteins as minor components (Suzuki et al., 1989; Suzuki et al., 1990).

In this report, we have demonstrated that JLS-S001, a subfraction of LEM, similar but not identical to EP3, significantly reduced the release of infectious HSV-1 from African green monkey kidney cells. Detailed studies suggested that JLS-S001 blocked the HSV-1 replication at a late stage in the virus replication cycle, probably in the assembly and/or budding of nucleocapsids and subsequent egress from the treated cells. The mode of action of JLS-S001 appeared to be different from that of nucleoside analogs such as acyclovir (ACV), gancyclovir (GCV) or trifluorothymidine (TFT).

## Materials and Methods

*Cell and virus*

African green monkey kidney (BS-C-1) cells were obtained from the American Type Culture Collection, Rockville, MD, and were grown in

medium 199 containing 10% heat-inactivated fetal calf serum and gentamicin (50 μg/ml). The F strain of HSV-1 was provided by B. Roizman, The University of Chicago (Chicago, IL).

*Reagents and radioisotope*

The procedure for the preparation of JLS-S001 was adapted from the method described by Sugano et al. (1982; 1985) with some modifications. In brief, *L. edodes* mycelia were cultured in a solid medium composed of sugar-cane bagasse and then the whole medium containing mycelia was extracted and filtered through a series of membrane filters. The concentrate was purified by octylcellulofine chromatography and finally freeze-dried. This purified complex contained 79% lignin (w/w), 20% carbohydrate (w/w) and 1% protein (w/w). The detailed analysis of JLS-S001 including [$^{13}$C]NMR spectrum will be published elsewhere. Reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA. Na$^{125}$I (15.5 mCi/μg of iodine) was purchased from Amersham (Arlington Heights, IL). Protein A from *Staphylococcus aureus* was purchased from Pharmacia Laboratories (Uppsala, Sweden). Protein A iodination was carried out as described by Greenwood et al. (1963). May-Grunwald stain was obtained from Aldrich Chemical Company (Milwaukee, WS) and Giemsa stain was purchased from Sigma Diagnostics (St. Louis, MO).

*Antisera*

Monoclonal antibodies to glycoproteins B, C, D, E and I were prepared in this laboratory (Koga et al., 1986; Longnecker et al., 1987; Chatterjee et al., 1989). Rabbit anti-mouse IgG was purchased from Organon Teknika (West Chester, PA). Polyclonal rabbit antiserum to HSV-1 was obtained from Lee Biomolecular Research Laboratories (San Diego, CA).

*Plaque assay for extracellular infectious particles*

To determine the effect of JLS-S001 on the release of infectious HSV-1 particles, BS-C-1 cells were infected with strain F of HSV-1 (multiplicity of infection = 5) and then treated with different concentrations of JLS-S001. One set of cells served as an untreated control. Supernatant fluids from the treated and untreated cells were collected 24 h post-infection and tested for the ability to form plaques on BS-C-1 cells. The plaques were counted by light microscopy after the cells were stained with May-Grunwald and Giemsa (Neff and Enders, 1963).

*Polyacrylamide gel electrophoresis and immunoblotting*

Untreated and JLS-S001-treated cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Chatterjee et al., 1982). The fractionated proteins were electrophoretically transferred to nitrocellulose paper and processed for immunoblotting (Towbin et al., 1979; Johnson et al., 1984; Chatterjee et al., 1985). The nitrocellulose strips were incubated at room

temperature either with mouse monoclonal antibodies against glycoproteins B, C, D, E and I or rabbit anti-HSV-1 antibody. The strips were further incubated with rabbit anti-mouse IgG for 30 min when mouse monoclonal antibodies were used. Finally, the strips were treated with [$^{125}$I]protein A for 1 h at room temperature and processed for autoradiography.

*Electron microscopy*

In brief, HSV-1-infected BS-C-1 cells grown in 60 mm culture dishes, were treated with different concentrations of JLS-S001. One set of cells served as untreated control. Samples were then thoroughly washed with phosphate-buffered saline (PBS) 24 h post-infection and fixed with 1% glutaraldehyde. Fixed cells were post-fixed with 1% osmium tetroxide and processed for electron microscopy as previously described (Chatterjee et al., 1982).

## Results

*Effect of JLS-S001 on the release of infectious HSV-1 from treated monkey kidney cells*

In a virus yield assay, JLS-S001 significantly blocked the release of infectious virus particles from treated BS-C-1 cells (Table 1). The 50% effective concentration ($EC_{50}$) against HSV-1 is 20 μg/ml. In addition, JLS-S001 also inhibited the cell fusion induced by HSV-1 in monkey kidney cells (Fig. 1). In this experiment, monkey kidney cells were infected with HSV-1 for 1 h and then treated with JLS-S001 for 18 h. Treated and untreated cells were stained with May-Grunwald-Giemsa as described before (Chatterjee and Hunter, 1979).

The block in release of virus particles and in cell-to-cell fusion was not due to toxic effects of JLS-S001 on monkey kidney cells. No significant changes were observed after JLS-S001 treatment either in cell morphology (Fig. 1B) or cell number (Table 2). In brief, BS-C-1 cells were exposed with 200, 500 and 800 μg/ml of JLS-S001 and then the number of viable cells was counted 72 h post-treatment. The result of this experiment (Table 2) demonstrated that the inhibitory concentration ($IC_{50}$) for toxicity is greater than 800 μg/ml. Thus, the selectivity index (SI) is greater than 40. The selectivity index was determined by dividing $IC_{50}$ by $EC_{50}$.

TABLE 1

Effect of JLS-S001 on the release of infectious HSV-1 from monkey kidney cells[a]

| Concentrations (μg/ml) | Plaque-forming units/ml | % Inhibition |
|---|---|---|
| 0 | $8.0 \times 10^7$ | 0 |
| 20 | $4.0 \times 10^7$ | 50.0 |
| 50 | $3.0 \times 10^7$ | 62.5 |
| 100 | $3.0 \times 10^6$ | 96.2 |
| 300 | $2.0 \times 10^4$ | 99.9 |

[a]The experimental procedure is described in the text.

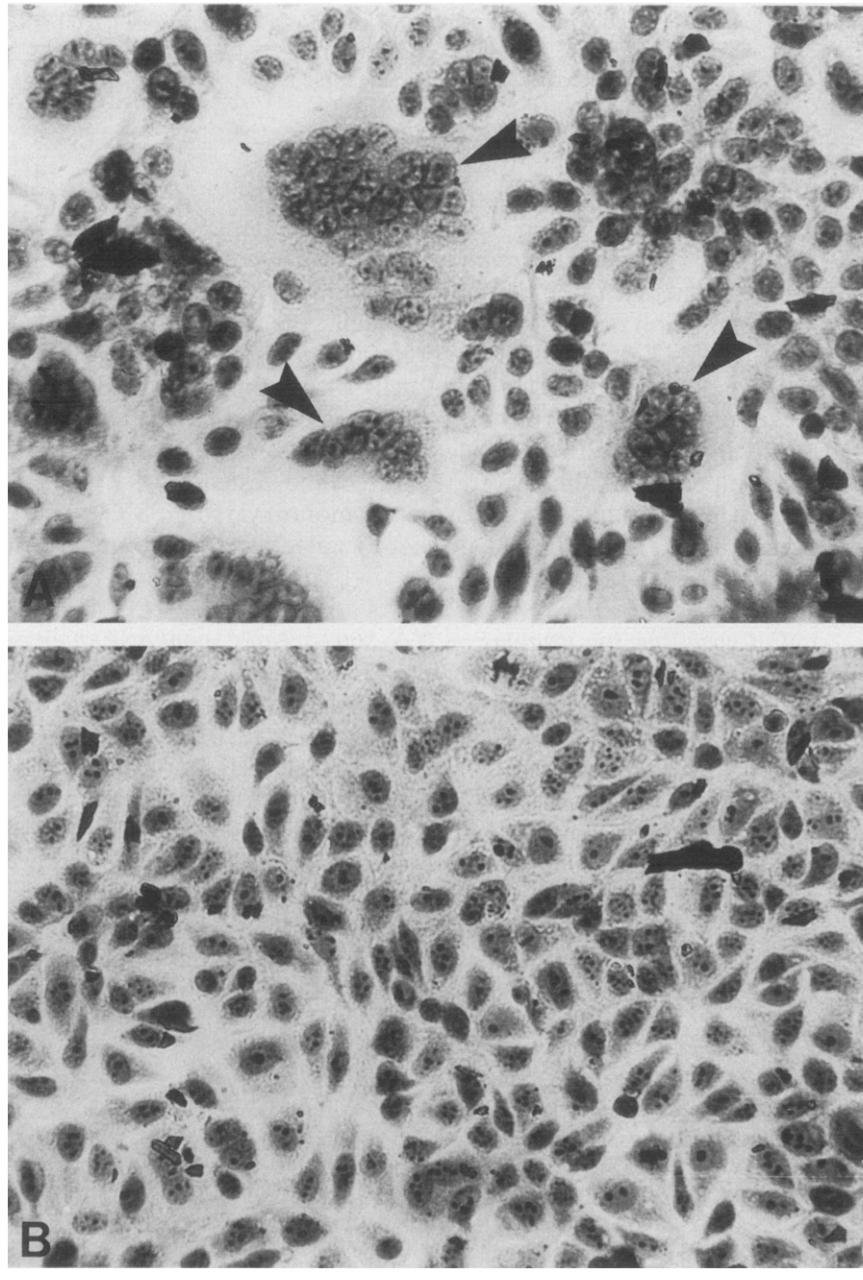



Fig. 1. Effect of JLS-S001 on the cell fusion induced by HSV-1 in monkey kidney cells. (A) HSV-1-infected cells without any JLS-S001 treatment showing several multinucleate cells (arrowheads). (B) HSV-1-infected, JLS-S001-treated (200 $\mu$g/ml) cells.

TABLE 2

Effect of JLS-S001 on the growth of BS-C-1 cells

| Concentrations (μg/ml) | Hours post-treatment | | % Survival |
|---|---|---|---|
| | 0 | 72 | |
| 0 | $1.2 \times 10^5$ | $6.4 \times 10^5$ | 100.0 |
| 200 | | $6.2 \times 10^5$ | 96.8 |
| 500 | | $5.4 \times 10^5$ | 84.3 |
| 800 | | $3.5 \times 10^5$ | 54.6 |

*Effect of JLS-S001 on the adsorption of HSV-1 to monkey kidney cells*

To determine whether the block in replication was due to the effect of JLS-S001 on the adsorption of HSV-1 to BS-C-1 cells, the following experiment was performed. Monkey kidney cells were pretreated with 100, 200 and 300 μg/ml of JLS-S001 for 3 h at 37°C. One set of cells without any JLS-S001 treatment served as an untreated control. Both the treated and untreated cells were washed and infected with HSV-1. After 90 min adsorption at room temperature, supernatant fluids were collected and tested for the presence of unadsorbed virus by adding the fluids to the monolayers of BS-C-1 cells. The results showed that JLS-S001 had no significant effect on the adsorption of HSV-1 to BS-C-1 cells (Table 3).

*Expression of virus-specific proteins in JLS-S001-treated monkey kidney cells*

To determine whether the block in virus release could be correlated with a reduction in the expression of virus-specific proteins, the following experiment was performed. Monkey kidney cells were infected with HSV-1 and then were treated with 0, 100, 200 and 300 μg/ml of JLS-S001. Cell lysates were collected 24 h post-infection and processed for polyacrylamide gel electrophoresis and immunoblotting as described in Materials and Methods. The result of this experiment demonstrated that the expression of gB, gC, gD, gI and especially gE was reduced at 100 μg/ml of JLS-S001 (Fig. 2, B,C). Treatment of cells with 200 μg/ml of JLS-S001 significantly reduced the expression of all the glycoproteins evaluated. The expression of some of the capsid proteins, however, was also reduced when the cells were exposed with 200 μg/ml of JLS-S001 (Fig. 2A).

TABLE 3

Effect of JLS-S001 on the adsorption of HSV-1 to monkey kidney cells[a]

| Concentration (μg/ml) | Residual infectivity (PFU/ml) |
|---|---|
| 0 | $4.1 \times 10^4$ |
| 100 | $4.0 \times 10^4$ |
| 200 | $3.8 \times 10^4$ |
| 300 | $4.2 \times 10^4$ |

[a]The experimental procedure is described in the text.

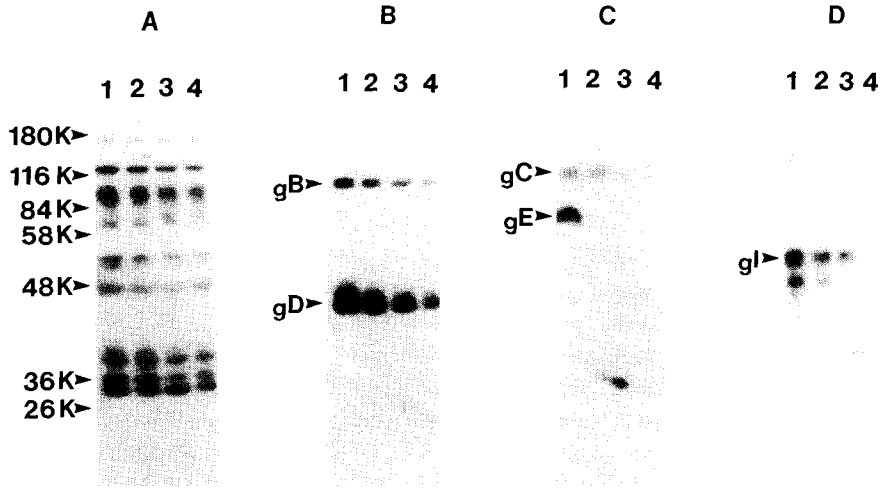



Fig. 2. Effect of JLS-S001 on the expression of HSV-1-specific proteins in treated and untreated BS-C-1 cells. (A) The nitrocellulose blot was reacted with rabbit antiserum to HSV-1. (B) The blot was reacted with monoclonal antibodies to gB and gD. (C) The blot was reacted with monoclonal antibodies to gC and gE. (D) The blot was reacted with monoclonal antibodies to gI. Lane 1, No JLS-S001; lane 2, 100 μg/ml JLS-S001; lane 3, 200 μg/ml JLS-S001; lane 4, 300 μg/ml JLS-S001.

*Assembly of virus particles in JLS-S001-treated monkey kidney cells*

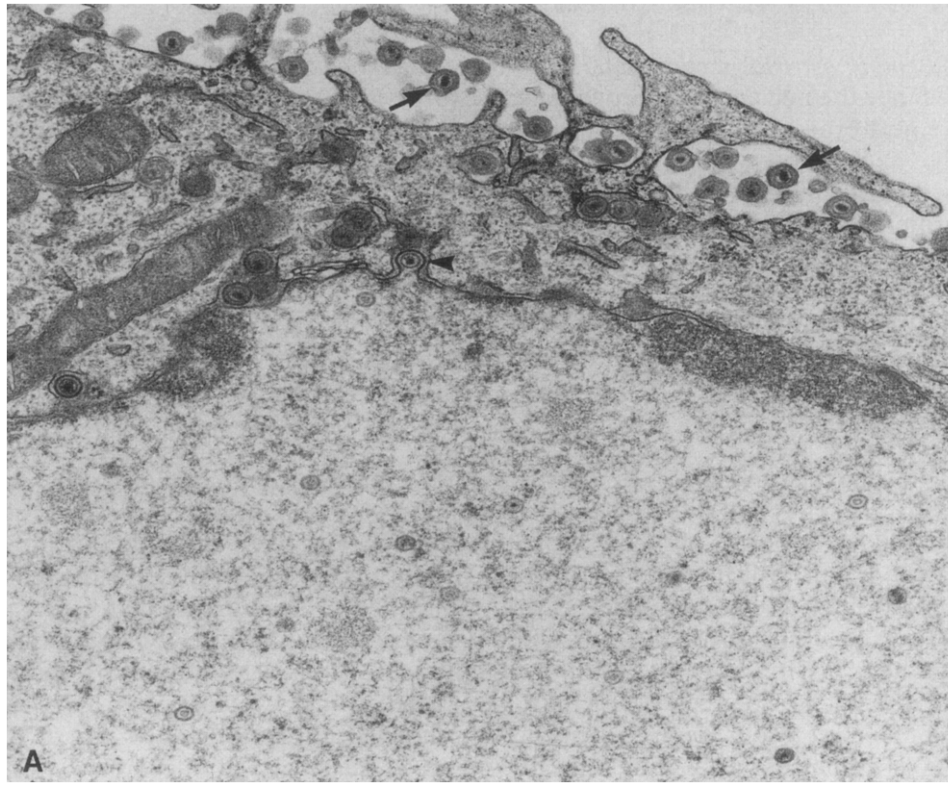
Since the nucleocapsid proteins were expressed in the JLS-S001-treated cells, we next examined the status of the intracellular virus particles. Consistent with the above observation, electron microscopy showed several nucleocapsids within the nuclei of JLS-S001-treated cells (Fig. 3B), indicating that viral capsid proteins were synthesized in the presence of JLS-S001. However, while in the untreated cells (Fig. 3A), numerous extracellular mature virus particles and budding nucleocapsids could be observed, only intranuclear nucleocapsids were seen in JLS-S001-treated cells (Fig. 3B).

## Discussion

Recently, Sorimachi et al. (1990) reported that the fractionated derivatives from an extract of the culture medium of *L. edodes* mycelia, EP3 and LS inhibited the HSV-1-induced cytopathic effect in Vero cells at a concentration of 100 μg/ml. However, no further details were available regarding the mode of action of these compounds. The viral replication cycle is divided into several stages, including, virus adsorption and penetration, uncoating, DNA synthesis, synthesis of viral proteins, assembly and budding of particles. Inhibitors of any of these stages of replication must be studied in the evaluation of antiviral agents. The results described in this communication demonstrated that JLS-S001 (similar but not identical to EP3) blocked the release of infectious HSV-1 particles from treated monkey kidney cells. We also demonstrated that JLS-

S001 significantly inhibited the cell-to-cell fusion induced by HSV-1. Cell fusion has been considered one mode by which the virus can be spread from cell-to-cell (Hoggan and Roizman, 1959; Lodmell and Notkins, 1974). More recently, Tochikura et al. (1988) reported that an extract of the culture medium of *L. edodes* mycelia, EP-LEM, also blocked the giant cell formation induced by human immunodeficiency virus.

The block in replication was not due to the effect of JLS-S001 on the adsorption and penetration of HSV-1 in the monkey kidney cells. We have, thus, ruled out the possibility that inhibition of HSV-1 replication was at the stage of virus entry (early stage) into the target cells prior to viral DNA synthesis. This observation was strengthened by the fact that JLS-S001 had no major effect on the expression of virus-specific nucleocapsid proteins in the treated cells. Furthermore, electron microscopy demonstrated the presence of nucleocapsids inside the nuclei of the infected and JLS-S001-treated cells. However, the expressions of glycoproteins B, C, D, E and I, on the other hand, were reduced. Since it has been postulated that viral glycoproteins might act as nucleation points for virus envelopment and budding, the JLS-S001-induced block in HSV-1 replication appears to be at a late stage in virus replication cycle. Immunoblot analysis of the JLS-S001-treated cells together with the



electron microscopic observations suggested that the block was probably in the assembly and/or budding of nucleocapsids from the nuclear membrane. The

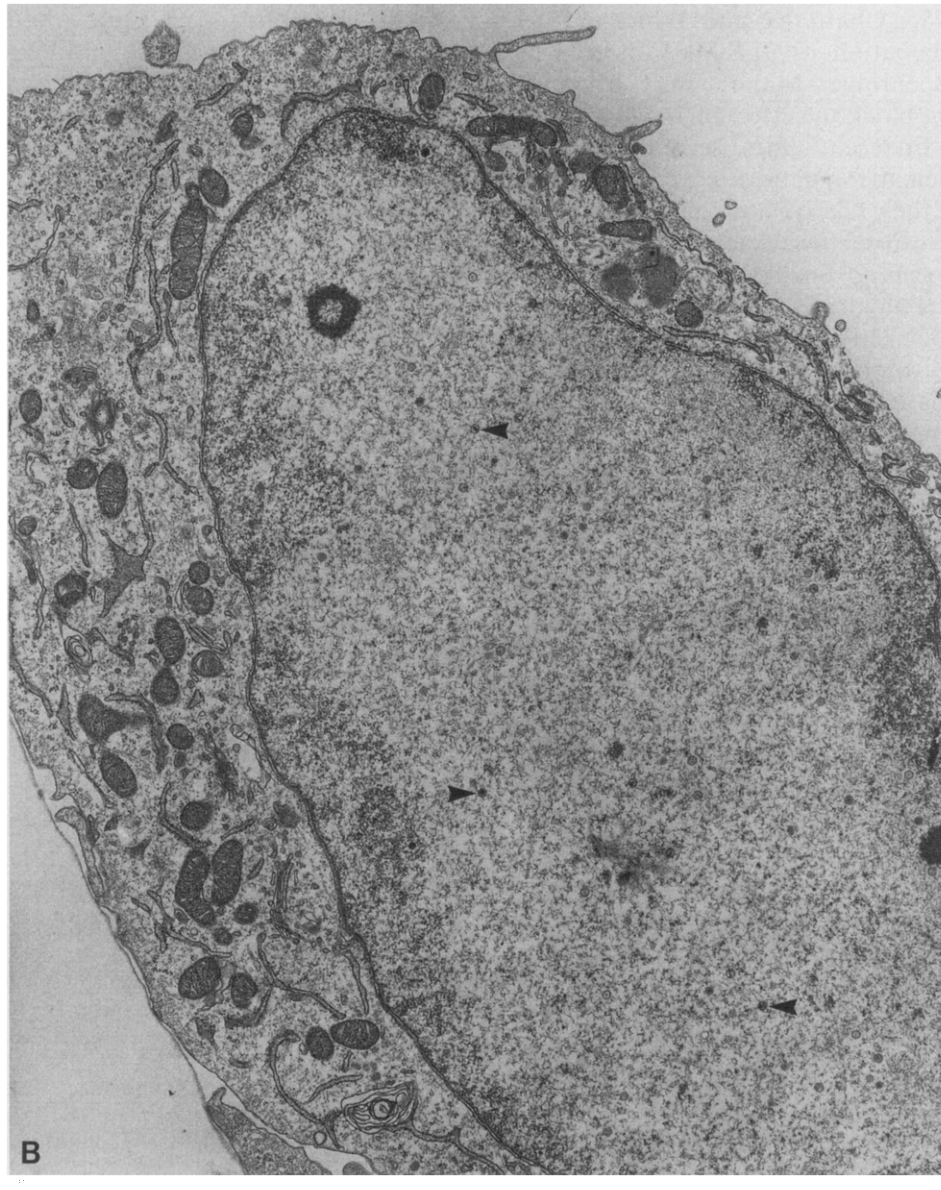



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Fig. 3. Electron microscopic observations of HSV-1-infected, JLS-S001-treated and untreated BS-C-1 cells. (A) Thin section of BS-C-1 cell infected with HSV-1 without JLS-S001 treatment, showing mature extracellular virus particles (arrows) and budding nucleocapsid (arrowhead). Magnification, × 27 700. (B) JLS-S001-treated (200 μg/ml) cell showing nucleocapsids (arrowheads). Magnification, × 13 300.

mode of action of JLS-S001 was, thus, different from that of nucleoside analogs, such as ACV, GCV or TFT and apparently similar to that of human interferons (IFNs) previously reported from this laboratory (Chatterjee et al., 1985; Chatterjee and Whitley, 1989; Chatterjee and Burns, 1990). However, incubation of JLS-S001 directly with anti-alpha IFN polyclonal antibody (Boehringer–Mannheim, Germany) prior to infection of susceptible cells did not block the effect of JLS-S001 on the replication of HSV-1 (data not shown).

In recent years, several phytochemicals have been studied extensively for their therapeutic effects. These phytochemicals are particularly useful because of their relative abundance in nature and these compounds are known to be less cytotoxic. The exact mechanism of action of JLS-S001 is unknown at present. Recently, Suzuki et al. (1990) demonstrated that digestion of proteins and carbohydrates, the two minor components of EP3, had no effect on the anti-HIV activities of this compound. However, the degradation of water-soluble polycarboxylated lignin component in EP3 reduced the anti-HIV activities of this compound (Suzuki, et al., 1990). It will be of interest in the future to pinpoint the specific factor (within lignin component) responsible for the anti-HSV-1 activities of JLS-S001 in vitro.

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