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Anti-herpetic activity exopolysaccharides produced by different species of lactic acid bacteria

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Abstract

In the present work, it was shown that, in non-toxic concentrations, all EPSs which were isolated from lactic acid bacteria have significant anti-HSV-1 activity affecting various stages of the virus reproduction. It was demonstrated that the use of ESPs leads to the normalization of the life cycle of cells infected with the herpes simplex virus to the level of uninfected cells. It was found that EPS 26a produced by *Lactobacillus sp.* possesses multiple antiviral effects as it exhibits virucidal activity, blocks adsorption, penetration of the virus into cells and the release of viruses, and reduces the infectious titer of herpes simplex virus by 97-99%, indicating its considerable anti-herpetic activity.

Indexing terms/Keywords: exopolysaccharides; herpes simplex virus type 1; cell cycle; antiviral activity.

Subject Classification: Virology

Type (Method/Approach): Experimental; *In vitro* study

Introduction

Herpes simplex virus type 1 (HSV-1) is enveloped dsDNA viruses belonging to the *Herpesviridae* family [1]. HSV infection usually causes mucocutaneous lesions that occur in oral/perioral areas, as well as on other body sites. HSV causes lifelong infection and can be reactivated by various stimuli including sunlight, fever, immunosuppression, or stress [2]. Although the disease is usually self-limited and can be treated with antivirals, severe complications can occur, particularly in neonates and immunosuppressed individuals, leading to a risk of blindness with keratoconjunctivitis, and the potentially fatal meningitis and encephalitis [3, 4]. Furthermore, in the immunocompromised host, the severe infection has been encountered and is a source of morbidity.

For a present time, no vaccine is available against HSV and there are currently no drugs that can eradicate latent HSV-infection. Although primary and recurrent infections can be controlled by nucleoside analogs such as acyclovir, penciclovir, and their prodrugs, the development of drug-resistant virus is becoming a serious problem, especially in immunocompromised patients [5]. Thus, identifying novel anti-HSV agents that act with different mechanisms is crucial for clinical management of HSV.

Herbal medicines and purified natural products provide a rich resource for novel antiviral drug development [6]. One of such natural product is exopolysaccharides (EPSs) that produces by some strains of lactic acid bacteria (LAB). LABs are gram-positive microorganisms that play an essential role in the industrial production of fermented dairy products. EPSs have been proved to show important health benefits like antioxidant, cholesterol lowering, antitumor, antiviral, and immunomodulatory activities [7-9].

The aim of the study was to evaluate antiviral activity of exopolysaccharide of lactic acid bacteria of genera *Lactobacillus*, *Leuconostoc* and *Pediococcus*.

Materials and Methods

Objects: Virus and cell culture. Herpes simplex virus type 1, strain US (HSV/US) and Syrian hamster kidney fibroblasts (BHK-21) were used through this study. The cells and virus were cultured according to standard methods.

Tested substances: The biological activaty of exopolysaccharides isolated from the lactic acid bacteria strains *Pediococcus sp*., *Leuconostoc sp.* and *Lactobacillus sp.* was studied [10]. The strains of the LAB were isolated from fermented homemade apples. Exopolysaccharides (EPSs) were isolated from the culture fluid. Antiherpetic medicament acyclovir (ACV) was used as reference compound.

Cytotoxicity assays: Cytotoxicity of EPSs was determined using a tetrazolium-based colorimetric (MTT) assay as described previously [11, 12]. Monolayers of BHK-21 cells in 96-multiwell plates were incubated with the EPSs at concentration of 370 – 1500 µg/ml for 72 h, then in a medium was added 20 µl of a 5 mg/ml solution of MTT (Sigma, USA). After 4 hours incubations, 100µl of 96% ethanol was added to the cells. The plates were detected using an automatic plate reader Multiskan FC (Thermo Scientific, USA) with a 538-nm test wavelength. The concentrations of EPSs that inhibit 50% of cell viability compared to control cells (CC_{50}) were measured.

Virucidal assay: The HSV-1 virus (multiplicity of infection of 32 PFU/ml) was mixed with an equal volume of EPSs at a concentration of 1500 µg/ml and incubated at 37°C for 3h. The monolayer of BHK-21 cells was infected by appropriate dilutions of the EPS-virus suspension of 50 µl/well. The virus was adsorbed at 37°C for 2 hours, then viral material was removed and 200 µl of a supportive medium was added. The plate was maintained at 5% CO_2 at 37°C until the appearance of a pronounced cytopathic action of the virus (3 days). The viral-containing material was selected for further study of virus titers [13].

Adsorption assay: The BHK-21 cell monolayer was incubated at 4 °C for 1 h, then infected with a suspension of HSV-1 (MOI 3.2 PFU/cell) and an equal volume of 2^X concentrations of ESPs (36-300 µg/ml) at 50 µl per well and the virus was adsorbed at 4°C for 3 hours. After adsorption, the virus-containing material was removed and 200 µl of supportive medium was added. The plate was maintained at 5% CO_2 at 37°C until the appearance of severe cytopathic action of the virus (2-3 days). The virus-containing material was taken for further study of the virus titer [14].

Viral Penetration Assay: A BHK-21 cell monolayer was prechilled at 4°C for 1 h, infected with 3.2 MOI HSV-1 and incubated at 4°C for a further 3 h. After incubation, 50 µL of 2^x concentrations of EPSs (36 - 300 µg/ml) were added and incubated at 37°C for 1 h to maximize the penetration of viruses. Then monolayer was treated with PBS (pH=3,0) for 1 min to inactivate the non-penetrated virus and washed PBS (pH=11,0) and PBS (pH=7,2). The neutral PBS was removed and the cell monolayer was covered with overlay medium. The plate was maintained at 5% CO_2 at 37°C until the appearance of severe cytopathic action of the virus (2-3 days). The virus-containing material was taken for further study of the virus titer [13, 15].

Antiviral Assay: 50 µl of virus suspension (MOI of 1.6) was added to BHK-21 cells. After 2-hour incubation, any unabsorbed virus was aspirated and 200 µl of serial two-fold EPSs-containing medium was added to each well, and incubated at 37˚C and 5% CO_2 for 2-3 days. The virus-containing material was taken for further study of the virus titer.

Yield reduction assay: The BHK-21 cells were grown in a 24-well plate to forming a 100% monolayer and infected with serial tenfold dilutions of lysates previously infected cells (not treated or treated with different concentrations of EPSs) at 0.3 ml/well, incubated for 2 hours of 5% CO_2 at 37°C. For each dilution of the virus, at least 3 wells were used. As a control, cells without adding the virus-containing materials were used. After adsorption of the virus, the overlay medium containing 1% methylcellulose, DMEM medium, and 2% fetal bovine serum was added to the cells. Cells were incubated at 37°C in 5% CO_2 during 3-5 days. Further, the coverage was removed by adding to the cell monolayer a 200 µl solution of 0.2% crystalline violet in 20% ethanol. The

titer of the virus in plaque forming units (PFU/ml) was determined by the highest dilution of the virus, in which the virus plaques were formed by the formula [16]:

The titer of virus PFU/ml = number of plaques / (dilution of the virus x volume of inoculum).

The antiviral activity of the EPSs was determined using the following formula:

% Inhibition = (1 - titer virus (experiments) / titer of virus (in control)) x100.

The reduction of the infectious titre of the virus by 99% or more compared to the control of the virus, indicates the pronounced activity of the compound against the virus, 97-98.9% - moderate action and below 97% - relative activity.

Cell Cycle Analysis: 1×10^6 cells (infected or non-infected HSV-1 and treated or not treated of the EPSs) were harvested by centrifugation at 2000 rpm for 7 min, resuspended in 96% ice-cold ethanol, and then resuspended in 300 µl solution of PBS that contained RNase (100 µg/ml) and PI (50 µg/ml), and incubated at 20°C for 1 h [17]. The cell fluorescence intensity was measured by a flow cytometer (Beckman Coulter Epics LX, USA) with laser wavelength 488 nm. Cell cycle profiles were analyzed with the program Flowing Software, version 2.5 [17].

Statistical analysis: All data were expressed as mean ± standard deviation (SD) of three replicates. P≤0,05 was considered to indicate a statistically significantly different.

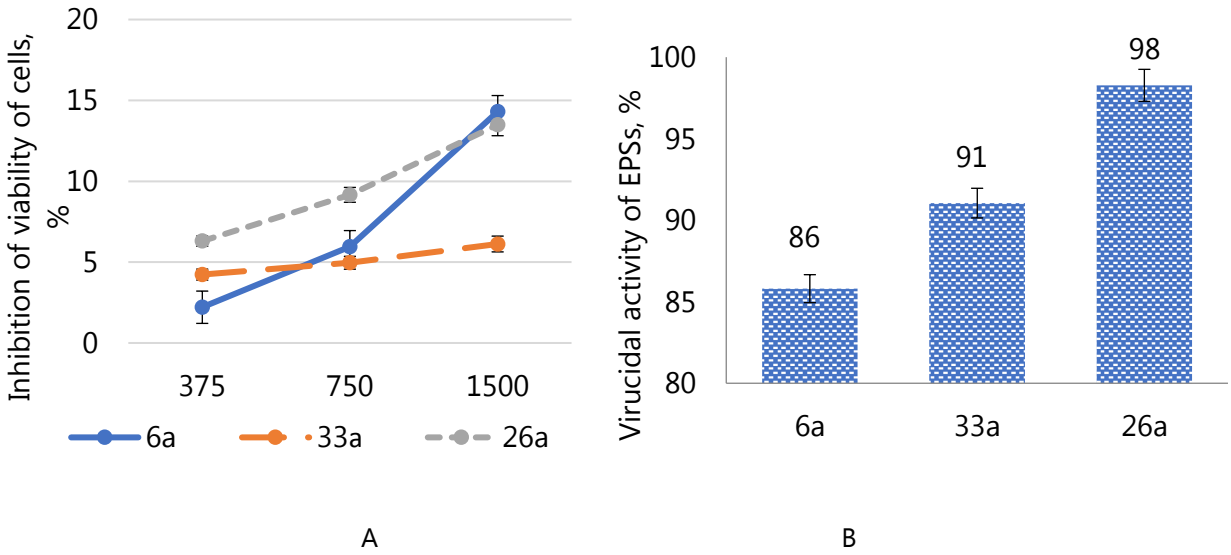
Results and Discussion

In recent years, more and more attention has been paid to the study of the biological activity of the exopolysaccharides (EPSs). In particular, it has been shown that they have immunostimulatory, antitumor and antioxidant activity. As the biological activity of the EPSs is strain-specifi, the search for new strains of producers among the LABs - representatives of the natural microbiota of fermented products remains relevant. The strains producing EPSs, especially in large numbers, are interesting from the point of view of their use both for improving the rheological properties of the product, and for the possible health effects on the human body. That's why the EPSs from strains of *Pediococcus* (6a), *Lactobacillus* (26a) and *Leuconostoc* (33a) were chosen for this study (table 1).

Table 1. Source of allocation of the strains of lactic acid bacteria and EPSs isolation

EPS	Species	Origin
6a	*Pediococcus sp.*	Sour apple
26a	*Lactobacillus sp.*	Sour apple
33a	*Leuconostoc sp.*	Sour apple

In the study of potential antiviral substances, the first step is to assess their toxicity for cell culture. The analysis of cytotoxicity on BHK-21 cells by exopolysaccharides was determined by MTT and presented in figure 1 A. All studied EPSs at higher concentration of 1500 µg/ml exhibited little cytotoxic effect, and ~85% of cells survived.



A B

Figure 1. The cytotoxic effect on BHK-21 cells (A) and virucidal activity (B) of EPSs 6a, 26a, and 33a. Each point represents the mean ± S.D. for three independent experiments. The significant difference between a test sample and control ($P < 0.05$).

It was found that only EPS 26a at concentration 750 µg/ml showed moderate virucidal activity and inhibited the reproduction of the virus by 98%, while other EPSs 6a and 33a reduced the titer of the virus by only 86 - 91% (figure 1 B). Acyclovir did not have virucidal activity (data not shown). The mechanism of it virucidal action can be realized due to the destruction of the lipid envelope of a virus and, as a consequence, prevent the attachment and penetration of HSV-1 to the cell.

Herpes virus reproduction is characterized by a complex sequence of different steps with which antiviral agents might interfere, such as adsorption, penetration, replication, and other. At the present work, the influence of EPSs 6a, 33a, and 26a at adsorption and penetration of HSV-1 to cells was studied. It was shown that EPSs 6a and 26a effectively inhibit adsorption of HSV-1 (table 2). It should be noted that the most suppression of adsorption (>99%) was observed in the minimum studied concentrations of EPSs, namely 18 and 38 µg/ml. The percentage of reduction of the virus titer by EPS 6a and 26a was in the range of 98.2 to 99.6%. The application of EPSs during penetration of the herpes virus to the cells revealed that 33a and 26a showed a lower antiviral activity and reduce the titer of the virus on 93.7 – 97.5%. Whereas EPS of 6a maximally inhibited the penetration of the HSV-1 in the range from 98.4 to 99.5% in the concentrations of 75-18 µm/ml (table 2).

Antiviral activity at the late stage of HPV-1 reproduction was shown only for EPS 26a (table 2). It was found that at the maximum tested concentration (150 µg/ml), the inhibition of infectivity of viral offspring was >99%. The received data indicate the dose-dependent effect of the EPS 26a, because, with a decrease in its concentrations, inhibition of virus reproduction was in the range of 54 to 34%.

Table 2 Effect of EPSs on infectivity of viral offspring

Compound	Concentration, μg/ml	During adsorption	During penetration	After adsorption
		Inhibition of HSV-1 reproduction,%		
6a	150	98.3	96.8	n/a*
	75	98.2	98.4	n/a
	38	99.6	99.4	n/a
	18	99.6	99.5	n/a
33a	150	53.0	97.3	n/a
	75	55.5	97.3	n/a
	38	77.0	97.1	n/a
	18	86.3	96.9	n/a
26a	150	98.6	97.5	99.2
	75	98.9	97.0	58.9
	38	99.3	96.6	49.5
	18	99.1	93.7	34.7
ACV	20.0	n/a	n/a	99.9
	4.0	n/a	n/a	99.9
	0.8	n/a	n/a	99.8

*n/a – not active

The progression of cells through the cell cycle is a complex process involving interactions between positive and negative regulators whose activities are dependent on a variety of stimulus. A lot of viruses, in particular, HSV-1, synthesized many proteins to arrested or induced changes at cell cycle distribution. In this study, we were interested in the influence of cell cycle BHK-21 with the addition of HSV-1 and different exopolysaccharides. The effect of EPSs from strains *Leuconostoc*, *Pediococcus*, *Lactobacillus* on the BHK-21 cell cycle phase distribution by flow cytometry was examined (fig. 2).

It was shown that uninfected cells have a normal DNA profile consisting of G0/G1, S, and G2/M phases. It was revealed that after 48 hours of growth 38% of BHK-21 cells remained in G1 phase, 10% were in S phase and 16% were in G2/M phase of cell cycle. Under the conditions of the EPSs treatment, the distribution of cells according to the cell cycle phases was similar to control cells but not identical. All of the exopolysaccharides do not affect on the cellular cycle of BHK-21 cells Thus, depending on the used EPSs concentrations, 34 – 39% of cells remained in G1 phase, 8 – 12% were in S phase and 15 – 18% were in G2/M phase of cell cycle. The increasing of the number of apoptotic cells in samples from 6a (4%) and the decreasing of the number of cells in S phase was observed. However, it should be noted that significant changes in the cell cycle with other EPSs were not detected. These results correlated with the study of the cytotoxicity of these exopolysaccharides and confirm that EPSs are non-toxic to BHK-21 cells.

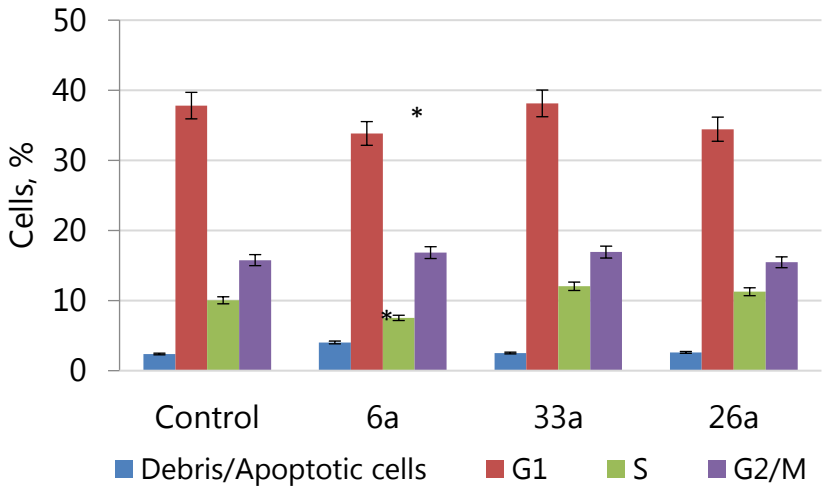


Figure 2. Influence of the different EPSs (1500 µg/ml) on the cell cycle of the BHK-21 cells. Cell cycle features of uninfected cells and treated with different EPSs were measured by flow cytometry after staining fixed cells with propidium iodide. Results corresponding to the percentage of cells in G1, S, and G2/M phases of three independent experiments are presented as mean ± S.D. * Significant difference between a test sample and control cells ($p < 0.05$).

Significant changes in the life cycle of cells BHK infected with HSV-1 were observed (figure 3). As a consequence, herpesvirus infection caused an increase in the S phase of the cell cycle. In another hand, HSV-1 caused a decreased among the number of cell in G1 phase. Our studies have shown that HSV-1 leads to the transition of cells from G1-phase to S-phase. At the same time, it is known from the literature that infection of HSV-1 leads to inhibition of cell cycle progression came from molecular studies addressing alterations in a key set of cell cycle regulatory transcription factor complexes (the E2F family) [18]. HSV-infected cells exhibited a dramatic broadening of the DNA profile where the G0/Gl, S, and G2/M peaks merged. Infection with HSV-1 has also been linked to the inhibition of cellular DNA synthesis, presumably associated with cell cycle arrest in G1/S. All this affects allows the virus to quickly and effectively replicate in the cell. There was a significant number of cells in the S (23%) and G2/M (23%) phases of the cell cycle under herpetic infection.

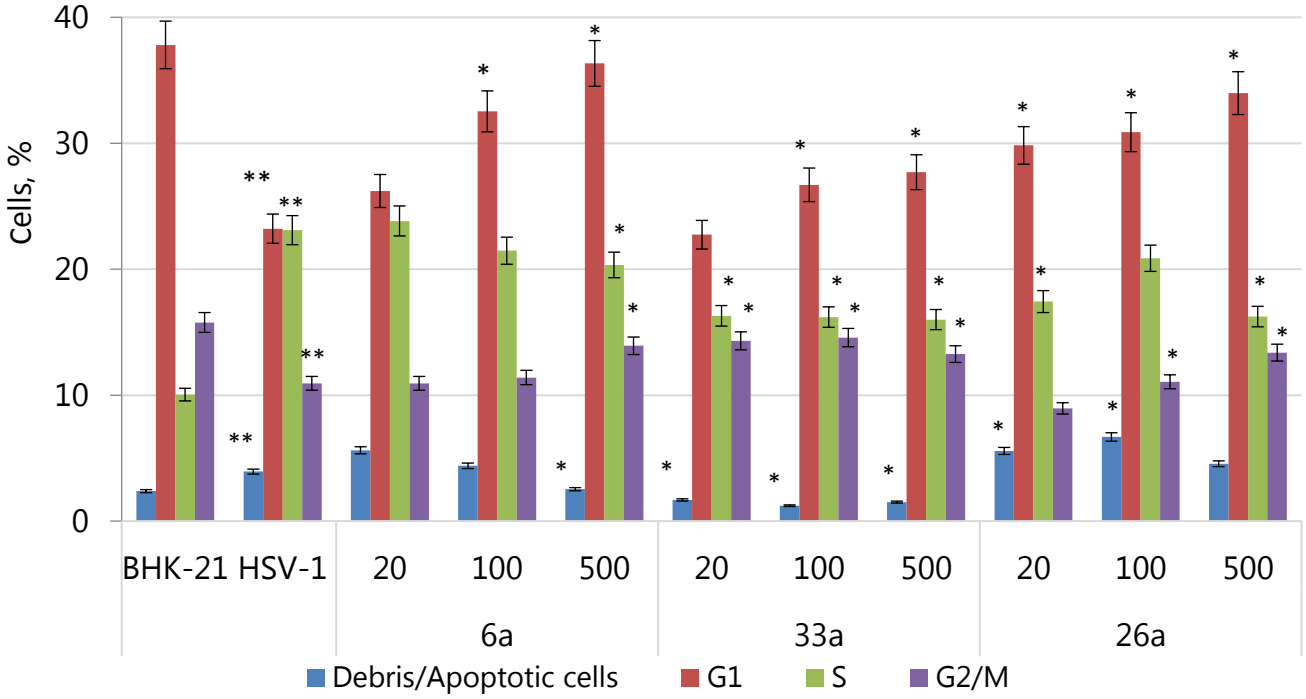


Figure 3. Effect of different EPSs on the cell cycle progression of cells infected HSV-1.

* Significant difference between test sample and control of infected cells ($p < 0.05$).

** Significant difference between a virus control and control cells ($p < 0.05$).

It was demonstrated that the use of EPSs at all studied concentration normalized the cell cycle. It has been shown that the effect of EPS 6a, 33a and 26a on the cell cycle during the infection has a dose-dependent manner since, with an increase in the concentration of EPS, a decrease in the number of cells passing from G1 to S phase was observed. All EPS at maximum studied concentration was most effective. A significant increase of cells in the G1 by 57% and G2/M by 27% phases of the cell cycle with using of EPS 6a at concentration 500 μg/ml were observed, compared with infected HSV-1 BHK cells indicates blocking reproduction of the virus in cells. Apart from the G1 and G2/M phases, it was detected the decreased number of apoptotic cell and cell in the S phase (12%). At simples with EPS 33a were shown significantly decreased cells in S-phase by 30% and G2/M phase by 21% compared with a cell infected HSV-1. In another hand, this EPS do not affect on cell number in the G1 phase. Using of EPS 26a under the conditions of herpesvirus infection decreased the number of cells in S phase by 46% compared to the infected cell. But also, was observed a significant increase of the apoptotic cell with the addition of EPS 26a at concentration 100 μg/ml and 500 μg/ml, respectively. All the results suggest that the application of exopolysaccharides leads to an inhibition of the reproduction of the herpes virus type 1 and normalization the cell cycle.

The life cycle of HSV-1 contains six basic stages: attachment, penetration (also called virus entry), uncoating, replication, assembly and release, which may be the targets for inhibiting reagents. Several possible mechanisms of influence on the development of viral infection have been shown: inhibition of adsorption and penetration of the virus into cells; blocking of late stages of virus reproduction; stimulation of the immune system of the macroorganism [19, 20]. Also, there reports that the antiviral effects of EPSs are due to their structural features such as their charge density and chain length. In fact, as demonstrated in vivo, these EPSs, such as mannan sulfate, chondroitin sulfate, heparin, are able to inhibit the attachment of the virion to the surface of the host cell [20]. Thus, our results allow suggesting that EPS from *Pediococcus sp*., *Leuconostoc sp*. and *Lactobacillus sp*. are perspective antiviral agents. EPS isolated from *Pediococcus sp.* (6a) exhibits anti-HSV-1 activity by blocking the penetration and adsorption of the virus to sensitive cells, possibly by joining the virion receptors. Another

one EPS, *Lactobacillus sp.* (26a) have high virucidal activity and can inhibit adsorption of the virus, and yield of virus particles. Also, it should be noted that all investigated EPS are able to normalize the cell cycle. One of the possible explanations for this effect may be their ability to block the early stages of viral reproduction. Because the virus does not penetrate into the cells, so there is no development of a viral infection.

Taking into account the promising anti-herpetic activity of the EPSs produced lactic acid bacteria *Leuconostoc*, *Lactobacillus* and *Pediococcus* here in reported further investigation is needed to explore the antiviral mechanism of these compounds in detail.

Conclusions

Exopolysaccharides isolated from lactic acid bacteria *Leuconostoc*, *Lactobacillus* and *Pediococcus* have been shown to exhibit significant anti-herpetic activity. It was found that EPS 6a inhibited adsorption and penetration of the virus into a cell on 98-99%. The EPS 26a have demonstrated multiple antiviral action, showing virucidal effect, decreasing adsorption of the virus to cells by 98-99%, inhibiting of the forming and exit of a complete and infectivity virus by 99%. It should be noted that all the exopolysaccharides are capable to the normalization of a life cycle of BHK-21 cells infected with herpes virus to the level of non-infected cells.

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