Echinacea purpurea Polysaccharide Reduces the Latency Rate in Herpes Simplex Virus Type-1 Infections

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Abstract

Objective: During the latency period of herpes simplex virus type-1 (HSV-1), the virus can occasionally reactivate, travel back to the eye and cause recurrent ocular disease. As this condition arises from the ability of HSV-1 to produce a dormant infection, effective medication to prevent the virus enter a latent state should prevent it. In this study, we applied *Echinacea* polysaccharide (EP) fraction as prophylactic mediator for latency prevention. ***Methods:*** In order to investigate the protective properties of EP, we evaluated its immunostimulatory functions on different immune aspects that play important roles in latency prevention (particularly IFN-γ as one of the main indicators of cellular immunity and latency). Finally, we assessed establishment of latency by detection of thymidine kinase gene in trigeminal ganglia of BALB/c mice. ***Results:*** We demonstrated that EP promotes immune response, leading to a reduced latency rate, and it has a promising effect on latency prevention. ***Conclusion:*** EP was able to exert an antiviral action on the development of recurrent HSV-1 disease when supplied prior to infection.

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Introduction

Herpes simplex virus (HSV) infections are extremely common in humans and can occur at diverse sites with a wide range of symptoms, which range in severity from unapparent to life-threatening encephalitis [1]. After the initial infection, HSV replicates in the skin or mucosal epithelium, then infects sensory nerves and is transported to the involved sensory ganglia. Here HSV establishes a life-long latent infection that is punctuated by periodic reactivations [1, 2].

Following ocular infection, HSV-1 latency is established in neurons of the trigeminal ganglion. Reactivated HSV-1 can travel back to the eye via axonal transport and be detected by culturing tear films (virus shedding). The scarring that occurs as a result of HSV ocular recurrence is a major cause of corneal blindness [3].

Despite of the seriousness of recurrent ocular herpes, there is no effective treatment for preventing the establishment of a latent infection and its consequent recurrences. Hence, development of medication for both prevention and treatment of HSV infections has been the focus of research, as this is a cost-effective alternative to chemical antiviral drugs [4, 5].

Recently, an increased interest has been observed in natural medicines that have immunotropic activity and

which can increase cellular and humoral immunity against pathogens [6]. *Echinacea purpurea* extract has been used in prophylaxis and therapy of various viral infections, mainly respiratory tract infections in animals and humans [7].

Echinacea is perhaps best known for its reputed immunostimulating properties [8]. In vitro studies have shown that *Echinacea* acts directly on a number of cell types, including natural killer cells [9], polymorph nuclear leukocytes [10] and macrophages [11].

Pharmacological investigations have shown immunomodulatory activities of cichoric acid, alkamides and polysaccharides from *E. purpurea* [12]. It appears that the immunostimulating effects of *Echinacea* result from polysaccharides surrounding tissue cells which provide protection from pathogenic invasions [12].

E. purpurea polysaccharides (EPs) possess strong macrophage-activating properties. Although they also have some effect on B cell proliferation, their main targets seem to be macrophages [13].

Intravenous treatment of mice with EP significantly increased the survival rate in mice injected with lethal doses of *Candida albicans* or *Listeria monocytogenes* [14].

The immune-stimulating properties of the polysaccharide fractions from other *Echinacea* species *(E. angustifolia)* and other plants *(Platycodon grandiflorum* [15] and safflower petals) have also been proved [16].

In a previous study, we showed that EPs had a role in reducing severe acute ocular disease of HSV-1 in a mouse model [17]. In this study, EPs with in vivo anti-HSV-1 activity were examined for their prophylactic effects on latency establishment of HSV-1 in mice.

Materials and Methods

Virus

We extracted a virulent strain from a patient with signs of herpetic encephalitis and confirmed this as being HSV-1 by using a HSV-1-specific monoclonal antibody [18]. In this study we used the nonvirulent KOS strain of HSV-1 as a positive control. Stock viruses were grown and assayed on Vero cells in minimum essential medium containing 5% fetal bovine serum (Gibco BRL, Gaithersburg, Md., USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, Mo., USA).

Extract Characterization

EP fraction isolated from *E. purpurea* aerial parts was supplied by Zardband Pharmaceutical Company (batch E.PC04-L002-82) at a concentration of 20 mg/ml. Polysaccharides were precipitated to create a polysaccharide-enriched extract which was controlled for quality using thin-layer chromatography and densitometry (data not shown).

Polysaccharides have a molecular weight of 1.52×10^5 Da and consist of rhamnose, arabinose, galactose and galacturonic acid in a ratio of 0.5/3.5/2.5/11.25.

Before use in experiments, EP was diluted in media and filter sterilized with 0.22 μm syringe filters.

Mice

Female BALB/c mice, 4 to 5 weeks old, were purchased from the Razi Vaccine and Serum Research Institute of Iran. Animals were handled in accordance with our institutional guidelines and were kept in animal housing under standard conditions. They had an average weight of 20 g. The mice were separated into 3 experimental groups (10 mice per group) and the first group was intraperitoneally inoculated twice with 100 μl of inoculums containing 10^5 pfu of live KOS strain of HSV-1. The second group of mice, the negative control, was inoculated in the same manner with PBS. The last group of mice was inoculated with 100 μg of EP extract. All of the inoculations were performed on days 0 and 21.

Delayed-Type Hypersensitivity

Delayed-type hypersensitivity (DTH) response to HSV-1 was tested 2 weeks after the last immunization in control and test groups. UV-inactivated HSV-1 (KOS) with a titer of 10^5 pfu in 5 μl of minimum essential medium (prior to UV inactivation) was subcutaneously injected into the right footpad as a test, and mock antigen (Vero cell extract) was injected into the left footpad as a negative control. The footpad thickness was measured in a blinded fashion with a Mauser dial caliper 24, 48 and 72 h later. Results were expressed as a mean increase in footpad thickness at different hours following footpad injection over the pre-challenge thickness. Three mice per group were used, and the values obtained were analyzed for statistical significance.

The results were calculated according to the following formula:

Right footpad challenged with antigen – left footpad challenged with saline × 100/left footpad challenged with saline.

Splenocyte Proliferation

Splenocyte proliferation was measured 2 weeks after the last inoculation for all the test and control groups. A final concentration of 2.5×10^5 spleen cells/ml were exposed to 10 μg/well phytohemaglutinin (positive control) and inactivated HSV (specific antigen) for 5 days in a humidified tissue culture incubator (37°, 5% CO_2) [19].

The optimal concentrations of antigens or mitogens used to stimulate cells were determined in preliminary studies: inactivated virus as an antigen in 3 MOI. After incubation, 20 μl of MTT solution (5 mg/ml) was added to each well, and incubated again for 6 h. The crystals produced were dissolved in DMSO (Merck, Darmstadt, Germany), and optical density was measured (by the ELISA reader; Labsystem Multiskan MS) at a wavelength of 540 nm. All assays were performed in triplicate. Results were expressed as stimulation index which was defined as the ratio of the mean absorbance of antigen-stimulated cells to the mean of unstimulated ones.

IFN-γ Assay

One of the immune parameters commonly used to assess the immunological impact is the production of IFN-γ. IFN-γ protein level in supernatants of stimulated spleen cells was measured by

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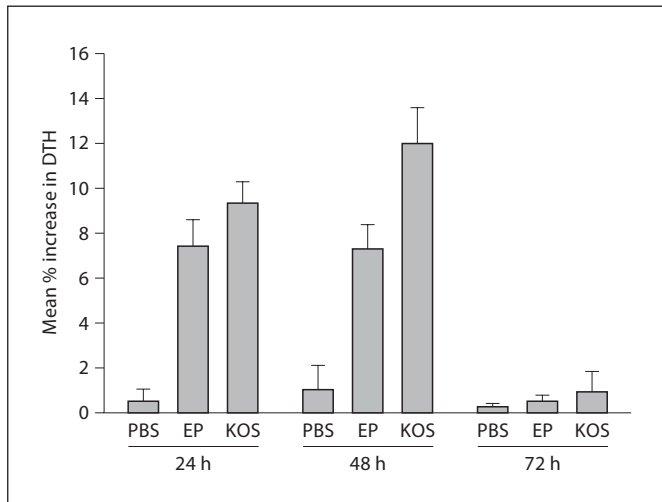
Fig. 1. Delayed type hypersensitivity. The effect of KOS and EP on the mean percent of DTH responses in BALB/c mice against inactivated HSV virus. Each bar represents the mean percent of DTH ± standard error (error bar) for 3 mice per group. KOS and EP (Extract) groups are significantly different from the values vs. negative control group (p < 0.05 by the Student's t test).

ELISA using a quantitative sandwich enzyme immunoassay technique (Quantikine Kits; R&D Systems, Minneapolis, Minn., USA). The test was carried out according to the manufacturer's instructions. Supernatants were harvested from wells for each group at 32 h post re-stimulation and they were diluted 1:2 with the calibrator diluents before assaying to bring IFN-γ levels within the range of the standards. Standards, controls and samples were assayed at a wavelength of 450 and 550 nm for wavelength correction. Optical density (OD) was determined as the difference in OD between the 2 wavelengths, and the intensity of color reaction product was quantitated on a microplate reader (ELx800; BioTek Instruments, Winooski, Vt., USA).

A range of IFN-γ dilutions was used to generate a standard curve to determine IFN-γ concentration in the sample supernatant. The assay was performed in triplicate. The sensitivity of the tests was less than 2 pg/ml of IFN- γ.

Ocular Challenge
Three weeks after the last immunization, all mice were anesthetized by intraperitoneal injection of 100 mg/kg ketamine (Parke-Davis, Pontypool, UK) and 10 mg/kg xylazine (Bayer, Bury St. Edmunds, UK) and then challenged with lethal HSV-1. Both corneas were lightly scarified using a 26 gauge needle. Then they were inoculated with 2 MLD50 (50% mouse lethal dose) of wild-type virus in a volume of 25 μl PBS ($5 \times 10^{5.5}$ pfu/eye). The lids were held closed and gently rubbed for 30 s. All of the mice were monitored for 28 days after challenge.

DNA Extraction from Trigeminal Ganglia
At 28 days after challenge, mice were sacrificed and trigeminal ganglia were removed and rapidly frozen in liquid nitrogen. DNA was extracted from the tissue. Briefly, frozen tissue was chopped into small pieces and then suspended in 3 ml of DNA extraction buffer [10 mM Tris (pH 7. 5), 25 mM EDTA, 10 mM NaCl], 100 μl of 20% sodium dodecyl sulfate and 100 μg of proteinase K (20 mg/ml) were added, and the treated tissue was digested at 56° overnight. The DNA solution was purified 3 times with phenol-chloroform and once more with chloroform and then precipitated with ethanol overnight. After centrifugation, it was finally dissolved in 20 μl of double distilled water [20].

Latency Assay
In order to detect the presence of HSV-1 TK DNA in the ganglia, PCR was used to amplify 398 bp of the HSV-1TK gene with the primers for TK and adipsin gene (as an internal control) with a 176 bp PCR product described previously [20].

Statistical Analysis
ANOVA test, at p ≤ 0.05, was used for comparison of the stimulation index of spleen splenocytes proliferation and DTH in animal treated with EP and KOS. Student's t test, at p < 0.05, was used for comparison of IFN-γ production. Statistical differences in mouse survival among groups were analyzed using the Kaplan-Meier test.

Results

Delayed-Type Hypersensitivity
In order to assess the effect of EP and KOS on the DTH responses, 3 mice/group were used. The results indicated a significant increase of DTH response after 24 and 48 h in both EP and KOS treated groups compared to the negative control ones. At 72 h after inoculation of antigen, both KOS and EP groups showed no significant differences in DTH responses compared to the controls (fig. 1).

Splenocyte Proliferation
In order to evaluate the effect of EP and KOS treatment on the proliferation of T lymphocytes, 3 mice/group were used. The mice were inoculated with KOS virus, PBS or treated with EP. The animals were sacrificed and the spleen cells were harvested. The splenocytes were activated in vitro by UV-inactivated HSV-1 KOS and evaluated by MTT methods. A significant increase (p ≤ 0.05) in the splenocyte proliferation was evident in KOS and EP treated groups over those of negative control ones, following stimulation of the spleen cells with UV-inactivated virus, as shown in table 1.

IFN-γ Assay
As shown in figure 2, the spleen cells of EP-treated mice produced more IFN-γ in the culture than did the cells from untreated mice. Values are averaged and statistical analysis was carried out by a Student's t test.
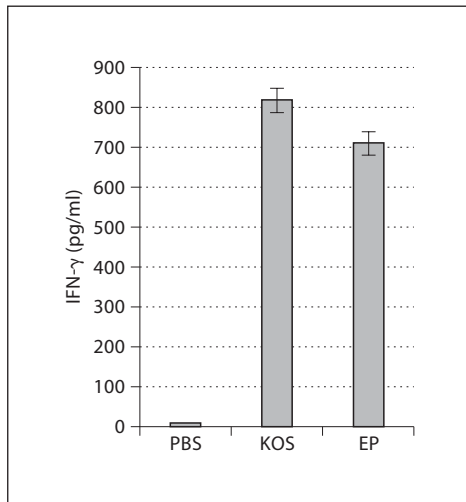
Fig. 2. The effects of EP extract on mouse IFN-γ production. BALB/c mice (3 mice/treatment group) were treated as described in 'Materials and Methods'. Spleen cells of these mice were cultured for 32 h and the culture medium analyzed for IFN-γ by ELISA. Values are averaged and statistical analysis was done by a Student's t test.

Table 1. Spleen lymphocyte stimulation index

Treatment (n = 3/group)	Stimulation index $\pm$ SD
KOS (positive control)	1.99 ± 0.025[a]
EP	2.36 ± 0.033[a]
PBS (negative control)	0.98 ± 0.030

[a] $p < 0.05$ vs. PBS control (ANOVA).

Table 2. PCR results on extracted DNA from trigeminal ganglia of mice (χ^2 p=0.05) showed significant difference between EP and PBS groups

Inoculum	Mice, n	Survival to 28 days after challenge	PCR-positive
EP	10	9	4
HSV-1 KOS	10	10	0
PBS	10	1	10

Survival Analysis

In order to evaluate the ability of KOS and EP treatment to protect of mice following ocular HSV-1 challenge, 10 mice from each group were used. Three weeks after the last immunization, the mice were challenged bi-ocularly with $5 \times 10^{5.5}$ pfu/eye [2 MLD_{50} (50% mouse lethal dose)] of wild-type strain of HSV-1. After lethal challenge, the mice were monitored for death. Ten mice out of 10 survived from the lethal challenge in KOS positive controls, while in the EP treated group only 1 death out of 10 was observed. In contrast, 9 out of 10 PBS-inoculated mice died following lethal challenge. The difference between the test group and mock inoculated group were statistically significant using Kaplan-Meier test (p $\leq$ 0.05). The results indicated that the EP provided significant protection against ocular challenge and enhanced the survival time of HSV-1-infected mice [21] (fig. 3).

EP Effect on Establishment of Latent Infection

The surviving mice challenged with 2 MLD_{50} HSV-1 per eye were killed 28 days after infection. Trigeminal ganglia were removed and analyzed individually for the presence of latent HSV-1 gene. Latent genomes were detected by PCR using oligonucleotide primers to the HSV-1 TK gene. The results indicated that significant protection (χ^2 p = 0.05) against establishment of latency in EP groups had developed, as shown in table 2.

Discussion

HSV infection is widespread. It is estimated that 70–90% of people over the age of 18 have antibodies to HSV-1 and/or HSV-2 and carry the latent virus, with approximately 25% showing clinical symptoms [3]. During the life of a latently infected individual, the virus can occasionally reactivate, travel back to the eye and cause recurrent disease. A major cause of corneal scarring is that induced by HSV-1 after reactivation from latency [3, 4].

In order to break the vicious circle of the latency, recurrence and renewed latency of the virus, it is essential to induce a protective immune response for clearance of the virus itself, since little or even no latency can then reoccur in the case of primary or recurrent infection. The most important solution is therefore an immunostimulatory substance, whereby the virus is killed [22, 23].

EP, which inhibits HSV-1 multiplication in vitro [24], was studied in a murine experimental model. Adult BALB/c mice were inoculated with HSV-1 at their corneas after abrasion. EP was administered twice as prophylaxis. Our findings implied that the treatment with EP as an immunostimulator, before lethal challenge, enhances the efficient immune responses leading to reduced viral multiplication in the eye. Subsequently, EP significantly affected and diminished the establishment

Fig. 3. Cumulative survival percent (median time of survival) of mice following various treatments. The figure shows that 90% of mice from EP group (9/10 mice) survived longer than 7 days after lethal challenge. This is compared to the negative control which shows that only 10% (1/10) survived longer than 16 days after lethal challenge. The difference of survival time percent between test and control groups with Kaplan-Meier test ($p \leq 0.05$) has been approved.
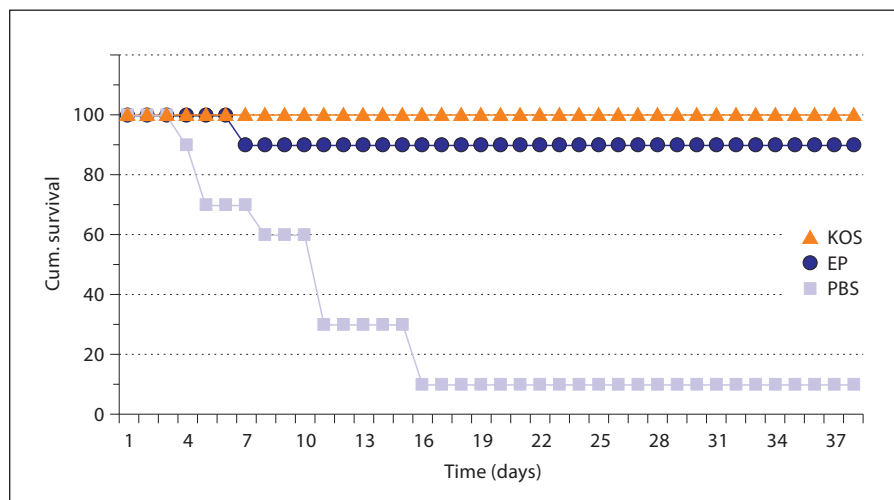
of a latent infection. As described before, we also confirmed in vivo the anti-herpetic effect of EP and showed that it significantly reduced the incidence and the severity of ocular disease with respect to untreated infected mice [17].

To our knowledge, in this study for the first time we report that EP does induce T cell proliferation. This has been attributed to macrophage activation to stimulate IFN-γ production in association with the secondary activation of T lymphocytes. The great production of IFN-γ by spleen cells from EP-treated mice suggested that injection of EP would induce an appropriate Th1 immune response [22]. DTH and splenocyte proliferation results also confirmed immunity.

On the other hand, it has been shown that IFN-γ is one of the major factors in latency prevention [20, 25, 26].

Although there is ongoing controversy in the literature as to whether the immune-stimulating properties of *Echinacea* are attributable to its polysaccharide component, or whether it is the alkylamides that are responsible, this study once again verified the immunostimulatory effects of the polysaccharide component [11].

Numbers of active polysaccharides have been isolated from *E. purpurea*. Frequently, these include a 45-kDa polysaccharide fraction isolated from cultivated plants as well as 2 neutral fucogalactoxyloglucans (10 and 25 kDa) and a 75-kDa acid arabinogalactan from large-scale plant cell culture. It has been suggested that they synergize to mediate a protective effect [27].

When injected into mice, arabinogalactan was found to activate macrophages and was associated with an increased production of TNF-α, IL-1 and interferons [24].

Therefore, macrophage responses and consequent IFN-γ induction to these polysaccharides can reduce latency rates.

In comparison to the KOS group (as a positive control group), EP induced IFN-γ with insignificant difference. Because of complete latency protection of KOS mice, it seems that EP has high prospective ability for improvement of its efficiency against latency in further studies.

In conclusion, EP proved to exert an antiviral action on the development of recurrent HSV-1 disease when supplied prior to infection.

Acknowledgments

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