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In vitro inactivation of herpes simplex virus by a biological response modifier, PSK

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Abstract

Herpes simplex virus (HSV) causes herpes genitalis, primary gingivostomatitis and recurrent herpes labialis. In order to elucidate in vivo mechanisms by which PSK, a biological response modifier, exerts a protective effect against HSV infection, we used an in vitro system to study whether PSK inactivated infectivity of HSV-type 1 (HSV-1) and HSV-type 2 (HSV-2) isolated from patients with herpes genitalis in addition to a laboratory-cultured strain of HSV type 1 (HSV-1-GC$^+$). It was found that HSV-1-GC$^+$ was inactivated by PSK in a dose dependent fashion of concentrations of PSK and virus titers. Concentrations of PSK as low as 0.31 mg/ml was shown to inactivate the infectivity of HSV-1-GC$^+$. Inactivation required at least 30 min of incubation at 37°C with maximal inactivation observed at 60 min incubation time. Similar to HSV-1-GC$^+$, clinically isolated strains of HSV-2 were inactivated by PSK although clinically isolated strains of HSV-1 were resistant to PSK, compared with HSV-2. It was also shown that PSK-treated HSV retained the ability to adsorb to the cell membrane, but did not synthesize viral protein(s). These data illustrate that there is a biological difference in the sensitivity to PSK between HSV type 1 and type 2, and also suggest that PSK could inactivate HSV in lesions at peripheral sites of recurrent herpes. © 1997 Elsevier Science B.V.

Keywords: Herpes simplex virus; Inactivation; PSK

1. Introduction

Herpes simplex virus (HSV) causes the typical fever blister or cold sore in the oropharyngeal region (Notkins et al., 1973; Lafferty et al., 1987). The virus also can produce encephalitis with severe neurological sequelae and death (Kastrukoff et al., 1987). Herpes genitalis is an infection of the genitalia caused by HSV (Lafferty et al., 1987). One of the characteristics of HSV infection is the establishment of latent infection in peripheral nerve ganglia and the central nervous

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system of man and experimental animals (Efstathiou et al., 1986). HSV can be reactivated in vivo by several procedures such as ultraviolet light irradiation (Blyth et al., 1976), neurectomy (Price and Schmitz, 1978) and irritation of epithelial surfaces (Shimizu et al., 1989).

PSK, a protein-bound polysaccharide extracted from the mycelia of *Coriolus versicolor* in Basidiomycetes, has been recognized for its antitumor activity (Tsukagoshi et al., 1984) and has the ability to induce antimicrobial activity in mice (Harada et al., 1989). It has been reported that PSK has the activity to decrease the duration and frequency of recurrent genital herpes (Kawana, 1988). We have studied the mechanisms by which PSK exerts its activity against HSV infection in a mouse model system, and reported in previous paper that PSK had a preventive effect on an acute HSV infection in mice, resulting in elongation of survival time as well as an increase in the overall survival rate (Shimizu and Monma, 1991).

In the present study, we examined whether PSK had the ability to inactivate HSV in vitro, using HSV type 1 and 2 clinically isolated from patients with genital herpes and a laboratory-cultured strain of HSV-1.

2. Materials and methods

2.1. Propagation of viruses and cells

Laboratory-cultured virus of HSV-1-GC$^+$ (Nii and Kamahora, 1961a,b) was propagated in Vero cells in our laboratory. This virus was isolated from the vesicular fluid of a patient with labial herpes in 1960 and produces large syncytial giant cells. A total of 11 isolates of HSV-1 and nine isolates of HSV-2 were isolated from female patients with genital herpes at the Hospital of Tokyo University and propagated several times in Vero cells. The viruses were stocked at -80°C until use. Vero cells were grown in Eagle's minimal essential medium (MEM) containing 10% calf serum (CS), penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (0.25 μg/ml).

2.2. Serotyping of HSV

Serotyping of HSV was carried out by microtrak herpes direct test using Syva MicroTrak® (Syva, CA). In brief, specimens from genital lesions or HSV-infected Vero cells were fixed on glass slides by acetone and then incubated with fluorescein isothiocyanate (FITC) labeled mouse monoclonal antibodies against either HSV-1 specific glycoprotein C or HSV-2 specific protein (Goldstein et al., 1983) for 30 min at room temperature. The glass slides were mounted in glycerol-PBS and read at a magnification of 200.

2.3. PSK

A 50 mg/ml preparation of PSK dissolved in sterile PBS was obtained from Kureha, Tokyo, Japan (Tsukagoshi and Ohashi, 1974). Lower concentrations were prepared by diluting the solution in Hanks' balanced salt solution (HBSS). The pH of 2.5 mg/ml of PSK dissolved in HBSS is 7.2–7.4 depending on the pH of HBSS used. Osmolarity of the 2.5 mg/ml PSK suspension is 306 ± 1 mOsm/kgH_2O which is similar to 304 ± 2 mOsm/kgH_2O of HBSS.

2.4. Inactivation of HSV by PSK

A 0.5 ml volume of HSV of different titers was mixed with an equal volume of solutions of PSK of increasing concentration and incubated at 37°C. At various time intervals, serial dilutions into MEM containing 2% of CS were made, and residual virus titers were determined by plaque-forming units (PFU)/ml in Vero cell monolayer cultures using 1% methylcellulose in MEM containing 5% CS (Shimizu et al., 1977). As control, HBSS was used instead of PSK. Preliminary data showed that residual PSK added to cell monolayer at the time of plaque assay did not cause the inactivation observed, since pretreatment of cell monolayer with 1.25–2.5 mg/ml PSK solution for 90 min, 1 day and 3 days had no effect on the plaque development and virus yield. In our assay system, more than 0.5 log difference in virus titer between PSK-treated HSV and HBSS-treated virus was considered significant. Inactivation ex-

periments were carried out several times, and similar results were obtained. Representative result was presented.

2.5. Mice and viral infection

Female Balb/c mice, 7–8 weeks of age, were obtained from the Institute for Experimental Animal, Tohoku University School of Medicine. All mice were housed and handled in accordance with Tohoku University School of Medicine guidelines. Mice were inoculated with either 0.2 ml of PSK-treated or non-treated HSV-1-GC$^+$ intraperitoneally (ip). For fatal challenge of mice with HSV-1-GC$^+$, 0.1 ml of virus with 10^5 PFU was inoculated ip. Mortality rate was determined 4 weeks after virus infection. Preliminary experiments showed that 50% lethal dose (LD_{50}) of HSV-1-GC$^+$ was 3×10^2 PFU per mouse, when the virus was inoculated ip.

2.6. Preparation of virus-adsorbed cells and immunization of mice

For adsorption of virus to cells, confluent monolayer of Vero cells (approximately 6×10^5 cells) in a six well plate was incubated for 90 min at 37°C with 1×10^4 particles of HSV-1-GC$^+$ which had been treated with 2.5 mg/ml PSK for 60 min at 37°C. As control, HBSS-treated HSV-1-GC$^+$ was used. The confluent monolayer of Vero cells was then washed five times in a total volume of 50 ml phosphate buffered saline (PBS), trypsinized and suspended with 350 μl PBS. Pooled cell suspension from two wells was heat-treated for 30 min at 56°C to kill live virus on/in the cells.

Female Balb/c mice (13 week old) were immunized with approximately 1.2×10^5 of above cells (more than 1.4×10^3 virus particles) in complete Freund's adjuvant in 0.2 ml distributed equally between axillary lymphnodes and inguinal lymphnodes. After an interval of 5 weeks, a second intraperitoneal immunization with 2.6×10^5 cells (more than 2.1×10^3 virus particles) was given.

2.7. Immunofluorescence test

HSV-1-GC$^+$-infected Vero cells on glass slides were fixed by acetone and stained with sera from mice which had been infected with HSV-1-GC$^+$ for 30 min at 37°C. After washing with PBS for 30 min at room temperature, FITC-labeled goat anti-mouse immunoglobulin (Ig) G, Fc fragment (Jackson Immunoresearch Lab., PA) was used as secondary antibody.

3. Results

3.1. Kinetics of inactivation of HSV-1-GC$^+$ by PSK

In order to determine rate of inactivation of HSV-1-GC$^+$ during in vitro exposure to PSK, approximately 3×10^4 PFU/ml of HSV-1-GC$^+$ (final titers) were treated with 2.5 mg of PSK/ml (final concentration) for up to 60 min at 37°C. As

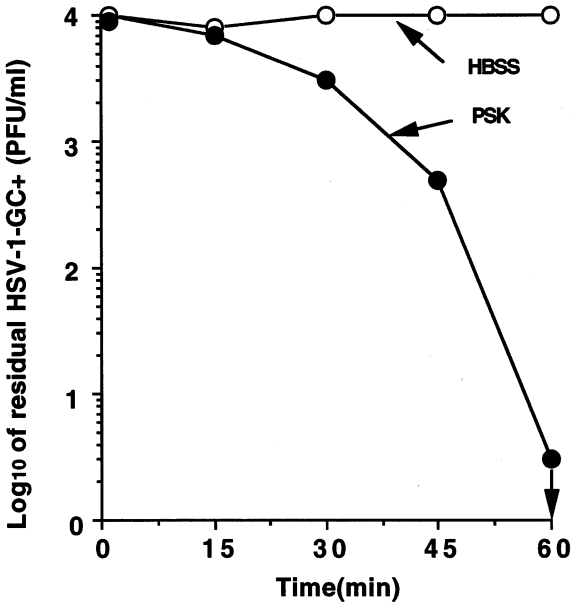

Fig. 1. Effect of exposure time on inactivation of HSV-1-GC$^+$ by PSK. 10^4 PFU/ml of virus was treated with 2.5 mg/ml of PSK for up to 60 min at 37°C. HBSS was used for control. Residual virus was assayed as described in Section 2. Arrow indicates residual virus was not detected.

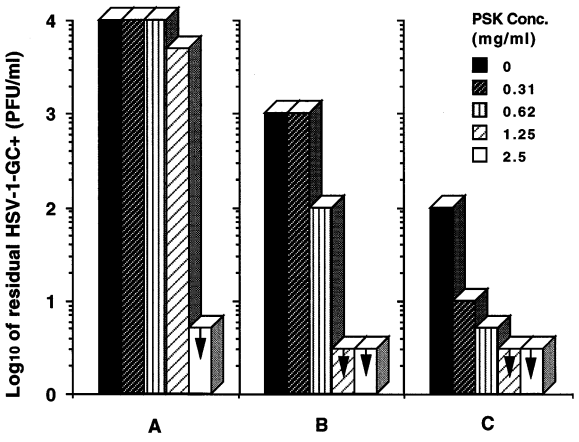

Fig. 2. Loss of infectivity of HSV-1-GC$^+$ of various titers by PSK with various concentrations. HSV-1-GC$^+$ was incubated with PSK for 60 min at 37°C. Residual virus was assayed. Arrow indicates residual virus was not detected.

shown in Fig. 1, HSV-1-GC$^+$ was inactivated with time nearly complete inactivation at 60 min.

We next determined the effect of PSK concentration on different titers of HSV-1-GC$^+$. The different infectivities of HSV-1-GC$^+$ were exposed to PSK at four different concentrations for 60 min at 37°C. When 10^4 PFU/ml of HSV-1-GC$^+$ was exposed to the concentration of 0.31–2.5 mg/ml PSK, the virus was completely inactivated at 2.5 mg/ml PSK, while there was no or little inactivation of the virus at three other concentrations of PSK (Fig. 2A). If 10^3 PFU/ml of HSV-1-GC$^+$ was exposed to these concentrations of PSK, the virus was completely inactivated at more than 1.25 mg/ml PSK and 90% of PFU of the virus was inactivated at 0.62 mg/ml PSK, while no inactivation of the virus was seen at 0.31 mg/ml (Fig. 2B). When 10^2 PFU/ml of HSV-1-GC$^+$ was exposed to 0.31–2.5 mg/ml of PSK, the virus was completely inactivated at more than 1.25 mg/ml PSK, and 95% and 90% of PFU of the virus were inactivated at 0.62 and 0.31 mg/ml PSK, respectively (Fig. 2C). Thus, the inactivation of HSV-1-GC$^+$ by PSK is dependent both on virus titer and PSK concentration.

3.2. In vivo assay of infectivity of HSV-1-GC$^+$ treated with PSK

To investigate whether PSK-treated HSV actually lost the infectivity or merely aggregated to make less infectious foci in vitro plaque assay system, HSV-1-GC$^+$ with 2×10^3 PFU and its preparation treated by 2.5 mg/ml of PSK were inoculated into Balb/c mice ip and mortality rate was determined. As shown in Table 1, mortality rate of mice inoculated with PSK-treated viruses was 0%, while mortality rates of mice inoculated with intact viruses and mice inoculated both with intact viruses and PSK were 69 and 70% respectively, suggesting that PSK had inactivated the infectivity of HSV-1-GC$^+$ rather than causing the aggregation of virus particles.

3.3. Adsorption of PSK-treated HSV to cells

The next experiment was carried out to discover whether or not PSK-treated HSV can attach to the cell membrane. Mice were immunized with Vero cells which had been incubated with PSK-treated or HBSS-treated HSV-1-GC$^+$ as described in Section 2. These mice were then challenged with fatal infection of HSV-1-GC$^+$, and mortality rate was determined. As shown in Table 2, similar to mice immunized with Vero cells incubated with HBSS-treated viruses, mortality rate of mice immunized with Vero cells incubated with PSK-treated viruses was 0%, while mortality rate of mice immunized with Vero cells

Table 1
Mortality of mice following infection with PSK-treated HSV-1-GC$^+$

Virus	Number of mice dead/tested (%)
PSK-HSV[a]	0/14 (0)
HBSS-HSV[a]	9/13 (69)
HBSS-HSV, then PSK[b]	7/10 (70)

[a] 1O^4 PFU of HSV-1-GC$^+$ were treated with either 2.5 mg/ml of PSK or HBSS as control for 60 min at 37°C. A 0.2 ml volume of each was inoculated into Balb/c mice ip. Mortality rate was determined as described in Section 2.
[b] Inoculation of HBSS-HSV was followed 90 min later by an ip inoculation of 0.1 ml of 2.5 mg/ml PSK.

Table 2
Mortality of mice immunized with PSK-treated HSV-1-GC$^+$ after fatal challenge with HSV

	Mortality rate of mice immunized with[a] (%):		
	PSK-HSV adsorbed Vero cells	HBSS-HSV adsorbed Vero cells	Vero cells alone
Challenged with HSV[b]	0 ($n=4$)	0 ($n=4$)	100 ($n=8$)

[a] Mice were immunized twice with Vero cells which had been incubated with PSK-treated or HBSS-treated HSV-1-GC$^+$ as described in Section 2. As another control, mice were immunized with Vero cells alone.
[b] These mice were then challenged with fatal infection of HSV-1-GC$^+$ with 10^5 PFU and mortality rate was determined.

alone was 100%. These data show that immunization of mice with Vero cells incubated with PSK-treated viruses conferred mice protection against fatal viral challenge, suggesting that PSK-treated viruses were able to adsorb to cell membrane.

3.4. Antigen production and surface changes in cells infected with PSK-treated HSV-1-GC$^+$

Next experiments were done to know whether infection with the PSK-treated HSV-1-GC$^+$ can induce the synthesis of viral antigens and early surface changes observed in the cells infected with intact virus. The results clearly showed that neither surface nor intracellular viral antigens, as detected by immnofluorescence tests, were observed in the cells infected with PSK-treated virus after 6 and 14 h of infection (Table 3). Neither cell fusion nor polykaryocytes occurred in the cells infected with PSK-treated virus (Table 3).

3.5. Inactivation of HSV isolated from patients with herpes genitalis

In the following experiments, isolates of both HSV-1 and HSV-2 from patients with herpes genitalis were examined for inactivation by PSK. A total of 11 isolates of HSV-1 with various titers were incubated with 1.25 and 2.5 mg/ml PSK for 60 min at 37°C and tested for titers of residual virus. As shown in Fig. 3A, all the 11 isolates of HSV-1 with titers varying from 5×10^2–3×10^3 (high titer group) lost little or no virus titers on exposure to either 1.25 or 2.5 mg/ml PSK. If, however, these isolates of HSV-1 with titers ranging from 10^2–5×10^2 PFU/ml (low titer group) were tested, as shown in Fig. 4B, five of the 11 isolates (five isolates from TKH strain to right) lost titers ranging from more than 0.5–2 logs on exposure to 2.5 mg/ml PSK, although little or no virus titers loss was observed on exposure to 1.25 mg/ml. Similarly, nine strains of HSV-2 with various titers were incubated with PSK for 60 min at 37°C and tested for the titers of residual virus. Fig. 4A shows that in high titer group of HSV-2 with titers ranging from 10^3–10^4 PFU/ml, six isolates (six isolates from TVM strain to right) lost virus titer ranging from more than 0.5–2.6 logs on exposure to 1.25 mg/ml, while, on exposure to 2.5 mg/ml PSK, all nine isolates of HSV-2 lost virus titers ranging from 3–4 logs. Furthermore, when low titers of isolates of HSV-2 (rang-

Table 3
Antigen production and surface changes in cells infected with intact and PSK-treated HSV-1-GC$^+$

	HSV[a]			
	Intact		PSK-treated	
	6 h	14 h	6h	14 h
Surface antigens	+[b]	++	−	−
Intracellular antigens	+	++	−	−
Cell fusion and polykaryocytes	−	++	−	−

[a] HSV-1-GC$^+$ with 10^4 PFU, and its preparation treated with 2.5 mg/ml of PSK were inoculated into 2×10^5 Vero cells. The infected cells were cultivated at 37°C and examined for changes and production of viral antigens 6 and 14 h after infection.
[b] Reactions were graded from − to ++ depending on the degree of reaction. ++, half of the total cells; +, less than one-tenth of the total cells were positive; −, negative.

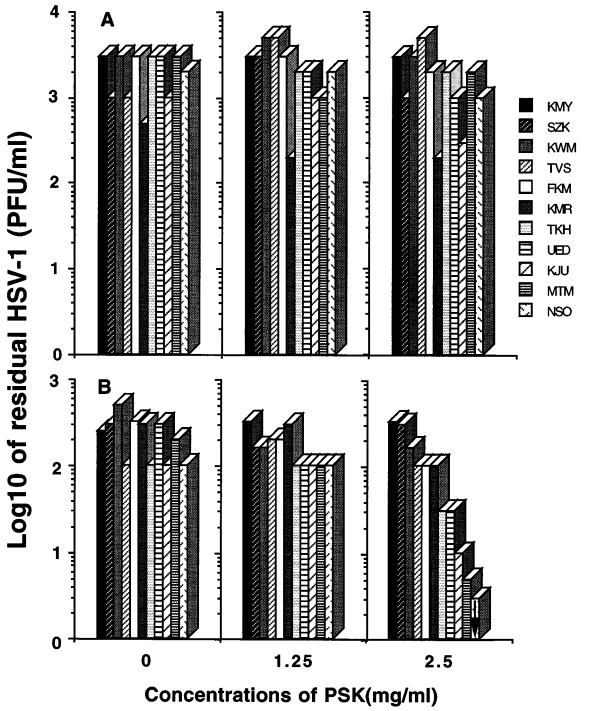

Fig. 3. Effect of PSK on infectivity of HSV-1 isolated from patients with genital herpes. A total of 11 isolates of HSV-1 were incubated with 1.25 or 2.5 mg/ml PSK for 60 min at 37°C and tested for titer of residual virus. (A) high titer group ranging from 5×10^2–3×10^3 PFU/ml and (B) low titer group ranging from 10^2–5×10^2 PFU/ml in low titer group. Arrow indicates residual virus was not detected.

ing from 10^2–3×10^2 PFU/ml) were tested, all nine isolates almost completely lost virus titers on exposure to either 1.25 or 2.5 mg/ml PSK (Fig. 4B). Thus, clinical isolates of HSV-2 tested were shown to be far more sensitive to inactivation by PSK than HSV-1 clinical isolates.

4. Discussion

In the present studies, it has been shown that PSK has the ability to inactivate HSV-1 and HSV-2 in vitro in a dose-dependent fashion. Clinical isolates of HSV-2 were shown to be far more sensitive to PSK than those of HSV-1. PSK-treated HSV-1-GC$^+$ was shown to retain the ability to adsorb to cell membrane, but not to synthesize viral protein(s).

PSK is one of a class of biological response modifiers (BRM) with unique characteristics and was widely used clinically as an oral antineoplastic agent. PSK has also been shown to have an effect on overcoming mouse cytomegalovirus in chronically or latently infected mice, presumably by activating natural killer cells and cytotoxic T-cells (Ebihara and Minamishima, 1984). In addition, PSK has been reported to be effective in decreasing the duration and frequency of recurrent genital herpes (Kawana, 1988). We have studied the mechanisms by which PSK exerts its antiviral activity in a mouse model system. In a previous paper, we found that intraperitoneal administration of PSK decreased the mortality and elongated the survival days in HSV-infected mouse experiment. We also found that PSK did

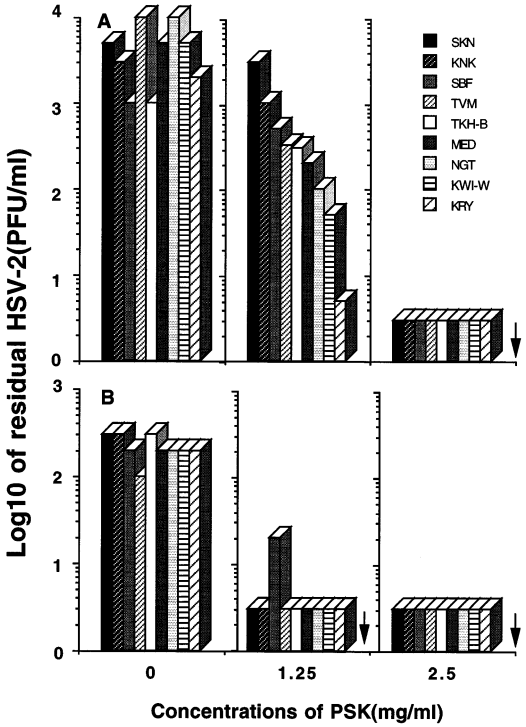

Fig. 4. Effect of PSK on infectivity of HSV-2 isolated from patients with genital herpes. A total of 9 isolates of HSV-2 were incubated with 1.25 or 2.5 mg/ml PSK for 60 min at 37°C and tested for titer of residual virus. (A) high titer group ranging from 10^3–10^4 PFU/ml and (B) low titer group ranging from 10^2–3×10^2 PFU/ml. Arrow indicates residual virus was not detected.

not suppress the reactivation of latently-infected HSV nor inhibit centipetal transport of HSV from lesions to ganglia through axon (round-trip infection), but presumably suppressed the propagation of HSV in peripheral sites such as the mucosa and the skin (Shimizu and Monma, 1991).

In the present study, we examined whether PSK directly inactivated the infectivity of HSV, and found that HSV isolated from patients with genital herpes was inactivated by PSK, although strains of HSV-1 were less sensitive to inactivation by PSK than HSV-2 strains. Patients with genital herpes from whom HSV had been isolated were treated with PSK (3 g daily in three divided doses) for 6 months to several years. Some of the orally administrated PSK was absorbed through lymphoid and sensitized lymphocytes of gut associated lymphoid tissue (GALT), and increased the developing plates of Peyer and reinforced blastoid transformation of lymphoid in GALT (Matsunaga et al., 1987). Although the concentration of PSK in bodily fluids of patients still remains undetermined, it might be possible that PSK with concentrations as low as 0.31 mg/ml is able to suppress recurrent herpes genitalis if the amount of reactivated HSV is as small as 10–100 particles. It is also suggested that PSK can be directly applied in a topical ointment for treatment of recurrent herpes.

Mechanisms by which HSV is inactivated have not been examined. However, PSK-treated HSV-1-GC$^+$ still retains the ability to adsorb to cell membrane, although cytopathic effect such as cell fusion and viral antigen(s) was not observed. These data suggest that PSK-treated HSV-1-GC$^+$ can penetrate cells but further expression of viral genomes was suppressed. Meanwhile, virion of rota virus have been shown to be destroyed in vitro by treatment with PSK, as revealed by scanning electron microscopy (Ebina et al., 1990). We do not know whether a similar effect of PSK to rota virus is operating against HSV.

In conclusion, PSK might exert its antiviral activity against HSV infection in vivo through the direct inactivation of HSV, as well as by immunological responses indirectly induced by PSK. PSK can be directly applied in a topical ointment for treatment of recurrent herpes.

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