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Reactivatable latency of three avirulent strains of herpes simplex virus type 1 after intranasal inoculation in mice.

Yujiro Arao*

Atsushi Hatano[†]

Masao Yamada[‡]

Fumio Uno**

Shiro Nii^{††}

*Okayama University,

[†]The University of Tokushima,

[‡]Okayama University,

**Niimi Women's College,

^{††}Okayama University,

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Abstract

In order to elucidate the mechanism of latent infection of herpes simplex virus (HSV), reactivatable latency of three avirulent strains (SKO-1B, -GCr Miyama, SKa) of HSV type 1 was comparatively examined in a mouse latency model. The SKO-1B strain showed high rate of virus reactivation from explanted trigeminal ganglia without n-butyrate enhancement, while the other two strains showed a very low rate of virus reactivation in the absence of n-butyrate. In the presence of n-butyrate, however, the rate of the -GCr Miyama strain jumped to a comparable level with that of SKO-1B, although the rate of SKa remained at a low level. A more precise follow-up experiment changing the virus dose highlighted the difference of the ability to reactivate from the latent state between SKO-1B and -GCr Miyama. Virus titer in trigeminal ganglia during acute phase, infectivity to cell lines of neural origin, and susceptibility to acyclovir and phosphonoacetate were assayed to know the reasons for the variation in the ability of reactivatable latency among these strains. It was concluded that the reduced infectivity to neural cells, and limited ability of reactivatable latency shown by the SKa strain could mainly be attributed to the deficiency of thymidine kinase activity.

KEYWORDS: herpes simplex virus type 1, neurovirulence, latency, reactivation, n-butyrate

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Reactivable Latency of Three Avirulent Strains of Herpes Simplex Virus Type 1 after Intranasal Inoculation in Mice

Yujiro Arao*, Atsushi Hatano^a, Masao Yamada, Fumio Uno^b and Shiro Nii

Department of Virology, Okayama University Medical School, Okayama 700, Japan

In order to elucidate the mechanism of latent infection of herpes simplex virus (HSV), reactivable latency of three avirulent strains (SKO-1B, -GCr Miyama, SKa) of HSV type 1 was comparatively examined in a mouse latency model. The SKO-1B strain showed high rate of virus reactivation from explanted trigeminal ganglia without n-butyrate enhancement, while the other two strains showed a very low rate of virus reactivation in the absence of n-butyrate. In the presence of n-butyrate, however, the rate of the -GCr Miyama strain jumped to a comparable level with that of SKO-1B, although the rate of SKa remained at a low level. A more precise follow-up experiment changing the virus dose highlighted the difference of the ability to reactivate from the latent state between SKO-1B and -GCr Miyama. Virus titer in trigeminal ganglia during acute phase, infectivity to cell lines of neural origin, and susceptibility to acyclovir and phosphonoacetate were assayed to know the reasons for the variation in the ability of reactivable latency among these strains. It was concluded that the reduced infectivity to neural cells, and limited ability of reactivable latency shown by the SKa strain could mainly be attributed to the deficiency of thymidine kinase activity.

Key words : herpes simplex virus type 1, neurovirulence, latency, reactivation, n-butyrate

The most striking characteristics of herpes simplex virus (HSV) is the ability to cause latent infection in the sensory ganglia of humans after acute infection. Reactivation of the latent virus causes the recurrence of herpetic lesions. To elucidate the mechanism of the latent infection, several latency models using experimental animals

were developed. We recently established a mouse latency model (1). In this system, viruses introduced by intranasal inoculation infect mucosal epithelial cells, travel through trigeminal nerve and establish latent infection in the trigeminal ganglia (TG). Because no spontaneous recurrence was observed in mice, reactivation was artificially induced by excising TG and explanting them onto Vero cells. Sodium n-butyrate which was added to the explant cultures had a strong effect to enhance viral reactivation (2).

Comparison of the ability of reactivable

* To whom correspondence should be addressed.

^a: Present address: Division of Clinical Otolaryngology, University Hospital, the University of Tokushima, School of Medicine, Tokushima 770, Japan

^b: Present address: Niimi Women's College, Niimi 718, Japan

latency among strains of HSV is the first step to investigate the viral factors which are essential for latent infection. Most fresh isolates and some laboratory strains of HSV, however, have strong neurovirulence (3,4) and kill mice during the acute phase of infection. Animal death during the acute phase of infection makes it difficult to prepare enough number of animals for the comparison. Therefore, we have proposed to use rather avirulent laboratory strains for investigation of HSV latency (1).

In the present study, three avirulent strains (SKO-1B, -GCr Miyama, and SKa) of HSV type 1 (HSV-1) were compared for their ability to induce reactivatable latency, and viral factors responsible for the difference in this ability among these strains were also studied.

Materials and Methods

Cells. Vero cells derived from African green monkey kidney, MGC cells originated from human glioblastoma and IMR-32 cells originated from human neuroblastoma were grown in Eagle's minimum essential medium (MEM) supplemented with 5 % newborn calf serum (NCS), 10 % NCS and 10 % fetal calf serum, respectively.

Viruses. Three avirulent strains, SKO-1B (5, 6), -GCr Miyama (7) and SKa (8), of HSV-1 were used. The 50 % lethal doses of these strains after intranasal inoculation of 5 week-old ICR mice were more than $10^{6.5}$ PFU per mouse (5,6, unpublished data). Preparation of virus samples and plaque assay for infectivity were performed as described previously (9, 10).

Animals. 5 week-old ICR outbred mice were purchased from Charles River Breeding Laboratories.

Chemicals. Sodium n-butyrate, acyclovir (ACV) and phosphonoacetate (PAA) were all purchased from Sigma Chemical Company, St. Louis, Mo, USA.

Establishment and detection of latent infection. Intranasal inoculation of mice was performed as described previously (5, 6). Virus titer of the inoculum used in each experiment is described in the text. Detection of reactivation in explanted TG, and enhancement of reactivation by 2mM sodium n-butyrate were done as described previously (1). Briefly, explant cultures of TG were maintained with and without n-butyrate enhancement,

and the appearance of specific cytopathic effect caused by reactivated HSV-1 was observed. The rate of virus reactivation in explant cultures of TG was calculated using the following formula;

$$\begin{aligned} & \text{Rate of reactivation} \\ &= \left[\frac{\text{(number of TG yielding virus)}}{\text{(total number of TG tested)}} \right] \times 100 \end{aligned}$$

Virus titers in TG during the acute phase of infection were assayed as described elsewhere (5, 6).

Susceptibility to two antiviral drugs. Appropriate amounts of viruses were plated on Vero cell monolayers under 2 % methylcellulose overlay containing $1 \mu\text{M}$, $20 \mu\text{M}$ ACV and $100 \mu\text{g/ml}$ PAA (11, 12) and cultures were incubated at 37°C for 3 to 5 days. Resulting plaques were counted and the efficiency of plating (EOP) was calculated by the following equation;

$$\text{EOP} = \frac{\text{(PFU in the presence of the compound)}}{\text{(PFU in the absence of the compound)}}$$

Viral infectivity assay on monolayers of cell lines originated from neural tissues. Aliquots of the virus samples were inoculated onto MGC, IMR-32, and Vero cell monolayers and these cultures were incubated under γ -globulin overlay at 37°C for 3 to 5 days. Vero cells were used as controls. Viral infectivity titer was indicated as a ratio of number of plaques produced on monolayers of each neural cell line to that produced on Vero cell monolayers.

Results

Virus reactivation from explanted TG of mice inoculated with three avirulent strains of HSV-1 was compared (Table 1). More than 83 % of mice inoculated with each strain at a dose of $10^{6.5}$ PFU/mouse survived the acute phase of infection. Among these strains examined, SKO-1B

Table 1 Rate of virus reactivation from explanted trigeminal ganglia of mice inoculated with avirulent strains of HSV-1

Strains	Rate of virus reactivation	
	Without butyrate	With butyrate
SKO-1B	56 (18/32) ^a	61 (20/33)
-GCr Miyama	8 (3/38)	50 (19/38)
SKa	6 (2/36)	3 (1/40)

a: Values in parentheses indicate the ratio of number of trigeminal ganglia yielding virus to total number of trigeminal ganglia tested.

showed a high rate of virus reactivation even without n-butyrate induction, while the other two strains showed a low rate of reactivation without n-butyrate enhancement. With n-butyrate enhancement, however, the rate of reactivation of the -GCr Miyama strain was almost comparable to that of SKO-1B, although the rate of reactivation of SKa was still low.

In the second experiment, differences in the ability of reactivable latency between SKO-1B and -GCr Miyama were further examined by changing the dose of the inoculum from $10^{2.5}$ to $10^{7.5}$ PFU/mouse (Table 2). The reactivation rate of the SKO-1B strain with n-butyrate treatment jumped up from low level (0-5%) to high level (54-79%) between $10^{3.5}$ and $10^{4.5}$ PFU/mouse, while the rate of the -GCr Miyama jumped up between $10^{5.5}$ and $10^{6.5}$, showing the minimum dose of the -GCr Miyama strain required for establishment of latent infection, which was reactivable with n-butyrate enhancement, was about 100 times higher than that of SKO-1B. The reactivation rate of the -GCr Miyama strain without n-butyrate enhancement remained at a low level even at the highest dose, while those of SKO-1B increased in a dose-dependent manner.

Viral titers in TG of mice inoculated with $10^{6.5}$ PFU/mouse of each of these three strains were examined at 1, 4 and 7 days after infection. As shown in Table 3, viral titer in TG during acute

phase was in the order of SKO-1B > -GCr Miyama > SKa.

Infectivity of the three avirulent strains to glioblastoma MGC cells and neuroblastoma IMR-32 cells *in vitro* was examined. As shown in Table 4, infectivity of -GCr Miyama to MGC cells was comparable to that of SKO-1B, while infectivity of -GCr Miyama to IMR-32 cells was about one fourth of that shown by SKO-1B. On the other hand, infectivity of SKa to MGC and IMR-32 cells was approximately 10 and 100 fold lower than that of SKO-1B, respectively.

Among several enzymes which were coded by HSV, deficiency of thymidine kinase and ribonucleotide reductase was reported to limit the ability of the reactivable latency. Instead of measuring the enzyme activity of these strains, simple biological assays to test the susceptibility to certain amount of ACV and PAA were performed because the responses of thymidine kinase and ribonucleotide reductase deficient variants of the compounds were well characterized (11,12). As shown in Table 5, resistance to $20 \mu\text{M}$ ACV and sensitivity to $100 \mu\text{g/ml}$ PAA showed that the

Table 2 Rate of virus reactivation of the SKO-1B and -GCr Miyama strains of HSV-1 at various doses of virus inoculation

Virus titers inoculated (PFU/mouse)	Rate of virus reactivation			
	SKO-1B		-GCr Miyama	
	Without butyrate	With butyrate	Without butyrate	With butyrate
$10^{2.5}$	0 (0/20) ^a	0 (0/19)	NT ^b	NT
$10^{3.5}$	6 (1/16)	5 (1/19)	NT	NT
$10^{4.5}$	23 (7/30)	54 (15/28)	0 (0/34)	0 (0/39)
$10^{5.5}$	30 (3/10)	79 (11/14)	6 (1/16)	6 (1/16)
$10^{6.5}$	56 (18/32)	61 (20/33)	8 (3/38)	50 (19/38)
$10^{7.5}$	NT	NT	6 (1/18)	44 (7/16)

^a: Values in parentheses indicate the ratio of number of trigeminal ganglia yielding virus to total number of trigeminal ganglia tested. ^b: NT, not tested.

Table 3 Virus titers in trigeminal ganglia of mice during the acute phase of HSV-1 infection

Strains	Virus titers in trigeminal ganglia (PFU/ganglion)		
	1 day ^a	4 days ^a	7 days ^a
SKO-1B	50	740	ND ^b
-GCr Miyama	ND	2.5	ND
SKa	ND	ND	ND

^a: Post-infection ^b: ND, Not detected.

Table 4 Infectivity of three avirulent strains of HSV-1 to glioblastoma and neuroblastoma cell lines

Strains	Relative infectivity ^a	
	MGC/Vero	IMR-32/Vero
SKO-1B	0.35	0.95
-GCr Miyama	0.39	0.25
SKa	0.032	0.0079

^a: The average number of values in two experiments was indicated.

Table 5 Susceptibility of three avirulent strains of HSV-1 to acyclovir and phosphonoacetate

Strains	Efficiency of plating ^a		
	1 μ M ACV	20 μ M ACV	100 μ g/ml PAA
SKO-1 B	0.81	0.0024	0.00029
-GCr Miyama	0.89	0.0021	0.00015
SKa	0.88	0.13	0.00015

a: The average number of values in two experiments was indicated.

SKa strain was deficient in thymidine kinase activity. Ribonucleotide reductase activities of these strains were judged to be normal because none of these strains showed hypersensitivity to 1 μ M ACV.

Discussion

In the present study, use of avirulent strains facilitated the precise assessment of the ability of reactivatable latency which is inherent to each strain. Among the three avirulent strains, SKa had a very limited ability of reactivatable latency, as indicated by low rate of virus reactivation even with n-butyrate enhancement. -GCr Miyama also had the limited ability of reactivatable latency in contrast to SKO-1B, although the limit was overcome by treating explanted TG with n-butyrate. Examination of reactivation rates after changing the virus dose clearly showed the difference in the ability to reactivate from latent infection between these strains.

Several causes which explain the limited ability of reactivatable latency have been reported (13-17). It was reported that the thymidine kinase negative or deficient mutants could establish latent infection in mice but could not reactivate from the latent state (13,14). Deficiency of HSV in thymidine kinase activity was also reported to result in its reduced infectivity to neuroblastoma cells (13). The SKa strain, which was originally described as a highly attenuated strain to newborn mice (8), have similar characteristics to the thymidine kinase negative and deficient mutants.

In fact, reduced thymidine kinase activity of the SKa strain was shown by the pattern of susceptibility to two antiviral drugs, although the actual enzyme activity was not assayed. Therefore, the limited activity of reactivatable latency and restricted growth in the cell lines of neural origin shown by the SKa strain could be mainly attributed to its deficiency in thymidine kinase.

Leib *et al.* (17) investigated the role of three immediate early regulatory proteins (ICP4, ICP27 and ICP0) in the establishment and reactivation of ganglionic latency in a mouse model. Three ICP0 deletion mutants, which could replicate in the eye and ganglion, varied in their ability to establish and reactivate from the latent state. Limited rate of virus reactivation shown by one of these mutants was circumvented by dimethyl sulfoxide which was known to affect gene expression. In the present study, low rate of reactivation shown by the -GCr Miyama strain was circumvented by n-butyrate which was also reported to increase the expression of immediate-early genes in neuroblastoma cell line (18,19). This relationship presents the possibility that mutation in the ICP0 gene of the -GCr Miyama strain might play a major role in limiting reactivatable latency.

In conclusion, we described the reactivatable latency and related biological characteristics shown by three avirulent strains. Further investigations on the possible mutations of these strains are necessary to define the viral factors which are essential to each step of latent infection, *i.e.* establishment, maintenance, and reactivation.

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