NOTE *Virology*

Analysis of the Mechanism of Reactivation of Latently Infecting Pseudorabies Virus by Acetylcholine

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ABSTRACT. In this study, the effect of cholinergic or adrenergic inhibitors on the reactivation of latent Pseudorabies virus (PRV) was analyzed to clarify the mechanism of the reactivation of latent PRV by acetylcholine. For acetylcholine inhibition, latently infected mice were injected with scopolamine or succynilcholine before acetylcholine stimulation. For sympathetic blocking, mice were preinjected intraperitoneally with phenoxybenzamine or propranolol. The signals to both acetylcholine receptors had no relationship to the reactivation of latent PRV, and both sympathetic blockers showed inhibition of PRV reactivation by acetylcholine. In our reactivation model, a large amount of acetylcholine may stimulate the sympathetic nerve system in some way to reactivate the virus.
KEY WORDS: acetylcholine, latent infection, mechanism, murine model, Pseudorabies virus.

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The establishment of latent infections is an extremely interesting feature of all herpes viruses. The viruses of alphaherpesviridae, one of the three subfamilies of herpesviridae, persist in an inactive state, primarily in neural tissues and mainly within the neurons of ganglia, for varying durations [1, 18] and avoid the host's immune responses. Such latent viruses are often reactivated by stress [11, 12, 17]. Herpes virus infection thus results in a long-term course of recurrent disease. Animal models provide experimental systems for elucidating the molecular mechanisms of latent viral reactivation.

Pseudorabies virus (PRV), a member of the alphaherpesviridae, causes Aujezskey's disease (AD). Piglets infected with PRV die of acute symptoms within a few days. In contrast, the clinical signs in adults include coughing, sneezing, lethargy, nervousness, uncoordinated movements and abortion of infected pregnant sows. The virus becomes localized in the trigeminal ganglia of infected pigs and establishes a latent infection there [2, 3, 14, 15]. Latently infecting viruses may be reactivated by stress, such as transportation, change of food and/or several diseases [23, 24]. Complete clearance of AD is difficult once it invades a farm, since it is almost impossible to distinguish latently infected pigs from uninfected pigs from their outward appearance.

We previously reported that latent PRV infection in swine can be reactivated by treatment with acetylcholine both *in vivo* and *in vitro* [19]. We have also established a PRV latent infection model in mice with the wild PRV YS-81 strain [20]. The mice were pre-treated with anti-PRV swine serum and then challenged with YS-81 based on a procedure reported by Osorio and Rock [13]. Almost all the mice survived, and PRV was detected and reactivated in the trigeminal ganglia (TGs) of the mice. PRV was reactivated in latently infected mice *in vivo* by stimulation with acetylcholine or dexamethazone [21].

The effect of acetylcholine on the reactivation of latent PRV is still unknown. Although we analyzed the kinetics of various immunological cytokines in a previous report and Sainz *et al*. reported the relationship between stress-associated immunofactors and HSV infection [16], cytokines related to stress could not reactivate latent PRV *in vitro* or *in vivo* [22]. Stress is initiated by many factors, and we hypothesize that acetylcholine might reactivate latent PRV by activating some of these factors. On the other hand, there is possibility that acetylcholine may work directly without intermediating factors. We therefore need to confirm whether acetylcholine reactivates latent infecting PRV directly or indirectly. In this study, the effect of cholinergic or adrenergic inhibitors on the reactivation of latent PRV was analyzed to clarify the mechanism of reactivating latent virus by acetylcholine.

BALB/C mice were purchased from Charles River Japan, Inc. (Yokohama, Japan) and used as the latent infection model. The animal experiments were approved by the Committee on Animal Experiments of Oita University and undertaken in accordance with the Guidelines for Animal Experimentation, Oita University.

Acetylcholine chloride (ACH) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Scopolamine hydrochloride (SCO), a cholinergic muscarinic inhibitor, was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, U.S.A.). Succynilcholine chloride (SUC), a cholinergic nicotinic inhibitor, was purchased from Tokyo Kasei (Tokyo, Japan). Phenoxybenzamine hydrochloride (PBZ), an alpha-adrenergic blocker, and propranolol hydrochloride (PRL), a

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Table 1. Effects of competitors of ACH receptors on PRV reactivation

Pre-treated		Virus excretion			Total		χ^2 value
		Day 1	Day 2	Day 3	Positive	Negative	
Scopolamine	1 mg/kg	0	10	0	10	20	0.573137916
Succinylcholine	1 mg/kg	2	10	0	12	18	0.273321894
PBS		2	6	0	8	22	

Data on virus excretion are shown as the number of mice that showed virus presence in nasal swabs by PCR. The "total" means the total number of mice that were positive or negative for virus presence in nasal swabs from Day 1 to Day 3. The chi square value compared the group inoculated with a inhibitor of ACH receptor and the negative control group inoculated with PBS.

Table 2. Effects of sympathetic blockers on PRV reactivation with ACH

Pre-treated		Virus excretion			Total		χ^2 value
		Day 1	Day 2	Day 3	Positive	Negative	
Phenoxybenzamine	1 mg/kg	0	4	4	8	22	0.000107511
Proplanolol	1 mg/kg	0	8	0	8	22	0.000107511
PBS		0	10	4	14	16	

Data on virus excretion are shown as the number of mice that showed virus presence in nasal swabs by PCR. The "total" means the total number of mice that were positive or negative for virus presence in nasal swabs from Day 1 to Day 3. The chi square value compared the group inoculated with a sympathetic blocker and the negative control group inoculated with PBS.

beta-adrenergic blocker, were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany).

PRV wild-type strain YS-81 was grown in porcine kidney cells, PK-15, and the virus titer was assayed in cloned PK cells (CPK) [6]. Cells were grown in Eagle's minimum essential medium (MEM) containing 5% fetal bovine serum, 1.5% $NaHCO_3$ and 0.1% each of penicillin G potassium, streptomycin sulphate and kanamycin sulphate.

Five-week-old mice were passively immunized by intraperitoneal (i.p.) inoculation of 0.25 m*l* anti-PRV swine serum. The final neutralization titer of this serum was 1:128. Thirty min later, the pre-immunized animals were infected i.p. with 100 lethal dose, 50% (LD_{50}) of YS-81. Mice surviving the challenge were kept for 2 months and used as latently infected (LI) mice. The presence of PRV DNA in the TGs of these LI mice was confirmed [20] after the mice were euthanized.

For ACH inhibition, latently infected mice were preinjected i.p. with SCO or SUC, 1 mg/kg, before ACH stimulation. For the sympathetic block, latently infected mice were preinjected i.p. with PBZ or PRL, 1 mg/kg, before ACH stimulation. The dose of these inhibitors was determined as the maximum concentration that was prepared as much as possible to satisfy the inhibiting effect completely. The animals inoculated with chemicals at this dose showed no side effects in this study. The latently infected mice were injected i.p. with 2.73 mg ACH. During the study, nasal swabs were harvested as previously described [5]. The presence of latent PRV DNA was assessed in nasal swab specimens by polymerase chain reaction (PCR) amplification of a 531-bp target sequence contained in the gene encoding PRV glycoprotein G (gG), following the method described in our previous report [22].

The significance of differences in the number of positive or negative in virus DNA detection from nasal swab specimens was analyzed by the chi square test.

To identify the direct activity of ACH for the reactivation of latent infecting PRV, LI mice were pretreated with inhibitors against ACH receptors and then injected with ACH to reactivate the virus. The nasal swab specimens were harvested, and viral DNA in swabs was detected by PCR. All groups showed PRV excretion by stimulating with ACH. However, the number of mice which showed viral excretion after pretreatment with an ACH inhibitor, SCO, a inhibitor of the muscarinic receptor, or SUC, a inhibitor of the nicotinic receptor, slightly increased, and the inhibitors showed no inhibition of virus reactivation (Table 1). A significant difference was not seen between mice prepared with ACH inhibitors and positive control mice ($P>0.05$). These results mean that the signals to both ACH receptors have no relationship to the reactivation of latent PRV.

To determine the effects of the sympathetic pathway in reactivating latent virus in ACH injection, sympathetic blockers, PBZ as an alpha-adrenergic blocker or PRL as a beta-adrenergic blocker, were pre-administered to LI mice to analyze their effects on latent PRV reactivation by ACH stimulation. The number of mice that showed viral excretion in pretreatment with both adrenergic blockers decreased significantly ($P<0.01$), as shown in Table 2. This means that both blockers showed inhibition of PRV reactivation by ACH, and it was shown that ACH reactivates latent virus through the sympathetic pathway.

The rabbit eye model has been extensively studied with respect to the pathogenesis of herpes simplex virus. In this model, latent infection of the trigeminal ganglia can be established with virus strains, such as the 17syn+ or McKrae

strain, and the latent virus can be reactivated with epinephrine [4, 7–9]. On the other hand, our reports stated that acetylcholine, a cholinergic effecter that shows opposite effects to epinephrine, reactivated latent infecting PRV in pigs [19] and mice [20, 21]. First, we supposed that different secondary factors might exist in the reactivation pathway of latent PRV by ACH because epinephrine has a completely opposite effect against ACH and must not react by the same pathway. We hypothesized that inoculated ACH might induce reactivation of latent PRV through cholinergic receptors and tried to confirm that by using ACH inhibitors. However, our hypothesis was rejected in this study. Therefore, what is the role of ACH in the reactivation of latent virus? In our reactivation model, a large amount of ACH was inoculated, close to the LD_{50}. We therefore hypothesized that such an amount of ACH may stimulate the sympathetic nerve system in some way and then reactivate the virus. In order to confirm this hypothesis, the effect of sympathetic blockers on the reactivation of latent PRV was analyzed by stimulating with ACH. PBZ as an alpha-adrenergic blocker and PRL as a beta-adrenergic blocker were used, and both blockers inhibited the reactivation of latent PRV by ACH. This means that ACH reactivates latent virus through a sympathetic pathway. Although there has been no report that PBZ inhibited the reactivation of latent herpesvirus, Kaufman *et al.* reported that PRL suppressed HSV-1 ocular recurrences in hyperthermia [10]. As we hypothesized, there is a possibility that a large amount of ACH may stimulate the sympathetic pathway in some way to compensate and latent virus is reactivated. We currently do not know the precise mechanism of reactivation of this latent virus. Continued research will eventually prove the pathway of the reactivation of latent PRV in detail.

In conclusion, to clarify the mechanism of reactivating latent Pseudorabies virus by acetylcholine, the effect of cholinergic or adrenergic inhibitors on the reactivation of latent PRV was analyzed. Neither the muscarinic nor nicotinic effect of ACH was related to the reactivation of latent PRV. Alpha- and beta-adrenergic blockers had a sympathetic effect in inhibiting the reactivation of latent infecting virus. There is the possibility that a large amount of ACH reactivates latent PRV by stimulating the sympathetic nerve system indirectly.

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