Effect of Mild Stress in Mice Latently Infected Pseudorabies Virus

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Abstract: Stress is one of the important factors that induces reactivation of pseudorabies virus (PrV) in latently infected pigs. We established a murine model of latent PrV infection and examined the effects of mild stress treatment in order to demonstrate that this model simulates natural infection in the pig. Latently infected mice excreted PrV from the nasal cavity under stress treatments consisting of restraint, exposure to cold or transport. Similar reactions have been observed upon treatment with acetylcholine and dexamethasone. The present findings demonstrate that these kinds of mild stress reactivate the virus in murine latent infection models in a manner similar to the induction of latent infection in pigs in the field.

Key words: acetylcholine, latency, pseudorabies virus, reactivation, stress

Introduction

The establishment of a latent infection model is an extremely interesting feature of all herpesviruses. The viruses of *alphaherpesvirinae*, one of the three sub-families of *Herpesviridae*, persist in an inactive state, primarily in neural tissues, mainly within the ganglial neurons, for varying durations [1, 12] and avoid the host’s immune responses. Such latent viruses are often reactivated by stress [6, 7, 11]. Herpesvirus infection thus results in a long-term course of recurrent disease. Animal models have provided experimental approaches to understanding the molecular mechanisms of latent viral reactivation.

Pseudorabies virus (PrV), a member of the *alphaherpesvirinae*, causes Aujezsky’s disease (AD). Piglets infected with PrV die within a few days with acute symptoms. In contrast, the clinical signs of adults include coughing, sneezing, dullness, nervous signs, uncoordinated movements, and abortions in infected pregnant sows. The virus localizes in trigeminal ganglia (TGs) and establishes a latent infection [2, 3, 9, 11]. Such viruses are reactivated by stress due to transportation, change in diet, and several diseases [16, 17]. The complete eradication of AD is difficult once it invades farms, since to distinguishing latently infected pigs from uninfected pigs is almost impossible by clinical observation.

We have established a PrV latent infection model in mice using the wild type PrV strain, YS-81 [14]. Based on a procedure reported by Osorio and Rock [8], mice were pre-treated with anti-PrV swine serum and then challenged with YS-81. Almost all the mice survived, and PrV was detected and reactivated from their TGs. We also reactivated PrV from latently infected (LI) mice *in vivo* by stimulation with ACH or dexamethasone.
(DEX) [15]. However, whether these reactivations reflect the process under natural stresses is unknown.

Thus, the purpose of the present study was to investigate the effect of relevant stressors in LI mice. In accordance with a previous report [4], three stress treatments, restraint, cold stress and transport stress were investigated. During and after stress treatment, PrV excretion and antibody against PrV in serum was analyzed.

### Materials and Methods

#### Animals

BALB/c mice used for the latent infection experiments were purchased from Charles River Japan, Inc. The experimental procedure was inspected and approved by the Animal Experiments Committee of Oita Medical University, and the experiments proceeded in accordance with the Guidelines for Animal Experimentation, Oita Medical University.

#### Chemicals

Acetylcholine chloride (ACH) was purchased from Wako Pure Chemical Industries, Ltd, Japan.

#### Cells and viruses

The wild type PrV strain, YS-81, was grown in porcine kidney cells (PK-15), and the virus titer was assayed in cloned PK cells (CPK) [5]. Cells were grown in Eagle’s minimum essential medium (MEM) containing 5% fetal bovine serum, 1.5% NaHCO₃, and 0.1% each of penicillin G potassium, streptomycin sulphate, and kanamycin sulphate. CPK cells were also used for virus isolation in the ACH reactivating test.

#### Animal inoculation

Six-week-old mice were passively immunized by i.p. inoculation of 0.25 ml of anti-PrV swine serum. The neutralization titer of this serum was 1:128. Thirty minutes later, the pre-immunized animals were infected i.p. with 100 lethal dose 50s (LD₅₀s) of YS-81. Mice surviving the challenge were maintained for 2 months and used as latently infected (LI) mice. The presence of PrV DNA in the TGs of these LI mice was confirmed as previously described [14] after euthanasia.

#### Stress treatment

Three types of stress, restraint, cold stress and transport stress, were examined. Each stress was applied for 2 h/day for 3 days. Restraint stress was applied by pulling a mouse into a 50 ml centrifuge tube with ventilation holes. Cold stress consisted of placing the mice in a cold room at 4°C. Transport stress involved placing the cage on a reciprocal shaker and shaking at 100 strokes/min. Nasal swab specimens were harvested from individual mice daily for 7 days and serum samples were obtained on day 7. Four mice were used for restraint stress and 3 mice each were used for cold and transport stress. As a control group, 3 mice were injected i.p. with 2.73 mg ACH daily for 3 days.

#### Harvest of nasal swab

LI mice were anesthetised with nembutal (Dainippon Pharmaceutical Co.LTD., Japan), and 100 µl of MEM injected into one nasal cavity. The wash was then harvested from the other cavity and mouth using a swab (MENTIP, Japan) and immersed in 100 µl of MEM and stored at −80°C until the virus isolation test.

#### Virus isolation test

Following euthanasia the TGs were minced using a cell strainer (FALCON, USA). TG cells were harvested, washed once with phosphate-buffered saline, inoculated onto monolayers of CPK cells in 96-well plates, and co-cultured in the presence of ACH, at a final concentration of 10⁻⁴ M. Cultures were incubated at 37°C in a 5% CO₂ atmosphere and monitored daily for the presence of PrV cytopathic effect (CPE). The specificity of CPE was confirmed by immunostaining with anti-PrV swine serum.

#### Neutralizing test

Seven days after the first treatment, serum samples were harvested from all LI mice and utilized for neutralizing tests. CPK cells were cultured in 96-well tissue culture microplates. Serial 2-fold dilutions of each serum sample were mixed with the YS-81 strain of PrV (200 TCID₅₀/ml) and incubated at 37°C for 1 h. As a virus control, the virus solution was incubated in growth medium without serum. Each mixture was added to the cells in the culture plates, followed by incubation at 37°C in 5% CO₂. When CPE appeared in the virus control wells, all wells were examined. The reciprocal
of the highest dilution that inhibited CPE was regarded as the neutralizing titer.

**Results**

**Latent infection**

Thirty mice were challenged with YS-81 after priming and then observed for the following 2 months. Three mice died in the first week after challenge. The remaining 27 mice survived for 2 months and were used as the LI mice in the activation test. TGs samples were harvested from these LI mice after euthanasia and analyzed for the existence of latent infecting virus. All 27 LI mice were latently infected by YS-81.

**Effects of stress**

For all three stress types, after each 2-h stress treatment, the mice’s behavior was subdued, but normalized within a few hours. No other side effects were observed in the test period.

**Excretion of PrV**

In the test period, nasal swab specimens were harvested and used to detect PrV. Virus was detected in specimens from days 3, 4 and 5 from the start of stress treatment (Table 1). No significant differences in virus detection were observed either between ACH treatment and the stress treatments, or among the three stress treatments.

**Anti-PrV antibody in serum**

On day 7 after stress treatment, all mice were euthanatized and serum samples were harvested to determine anti-PrV antibody titer. As shown in Table 2, an increase in antibody titer was observed in mice stimulated by ACH, whereas antibodies were not detected in most of the mice treated with stress. In particular, cold stress did not induce any detectable antibodies whatsoever.

**Discussion**

The three stress treatments administered in the present study induced the same side effect in the mice as ACH has previously been documented to cause, namely mild depression. This is in contrast to pigs, in which ACH induces several side effects including cramping, excess saliva secretion and stomach pains [13].

At the point of virus reactivation, PrV was detected from nasal swab specimens on days 3, 4 and 5 after the start of stress treatment. ACH has been shown to have similar activity [14], indicating that the three stress treatments reactivate latent PrV in LI mice in a manner similar to ACH.

We detected very little antibody after the stress treat-
ments. In our previous studies, the anti-PrV neutralizing titer by ACH stimulation was lower than that of DEX [15]. The three types of stress in the present study yielded similar titers to ACH, suggesting that the pathway of PrV reactivation by ACH is similar to that of natural stress. One possible explanation for the lack of antibody detection is the relatively short 7 day test period.

With regard to side-effect, latent PrV reactivation and neutralizing titer, ACH stimulation is closer to natural stress treatments than other stressors, such as adrenal hormone, DEX, stimulation which has been utilized as stressor in various experiments. Therefore, the ACH stimulation mouse model is the most appropriate for research into PrV latent infection or maybe for investigation of other stress-induced diseases. There is no direct evidence yet as to whether ACH follows the same mechanism of natural stress, however further analysis should make this clear.

In the present latent infection, 10% of infected mice died during the establishment of latency. While not an abnormal rate compared with our previous studies [14, 15], the reason for the deaths is unknown and obviously as yet unknown critical requirements must be met to more successfully establish latent infection in mice and reduce animal loss.

References