Mouse Model of Bell’s Palsy Induced by Reactivation of Herpes Simplex Virus Type 1

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Abstract. In order to investigate the mechanism of Bell’s palsy, we developed an animal model of facial nerve paralysis induced by the reactivation of herpes simplex virus type 1 (HSV-1). Eight weeks after recovery from facial nerve paralysis caused by inoculation with HSV-1, the mice were treated with auricular skin scratch at the site of the previous inoculation, or with intraperitoneal injection of anti-CD3 monoclonal antibody (mAb), or combination of both procedures. No mice developed facial nerve paralysis when they were treated with either auricular scratch or mAb injection alone. In contrast, 20% of mice developed facial nerve paralysis with the combined treatment. With one exception, no mouse treated with either auricular scratch or mAb injection showed HSV-1 DNA in their facial nerve tissue, whereas 4 out of 6 mice receiving both treatments showed HSV-1 DNA on day 10 after treatment. Histopathological findings showed neuronal degeneration in the geniculate ganglion and demyelination of the facial motor nerve in paralyzed mice. These findings suggest that a combination of stimuli, local skin irritation, and general immunosuppression is essential for successfully inducing facial nerve paralysis in mice with latent HSV-1 infection.

Key Words: Facial nerve paralysis; Herpes simplex virus; Immunosuppression; Latent infection; Skin irritation.

INTRODUCTION

Bell’s palsy is defined as an idiopathic peripheral facial nerve paralysis of sudden onset, and is considered the most common cause of facial nerve paralysis. Recently, herpes simplex virus type 1 (HSV-1) DNA was detected in clinical specimens, endoneural fluid, and saliva, from Bell’s palsy patients (1, 2). These studies suggested that the majority of Bell’s palsy cases might be caused by reactivation of latent HSV-1 infection in the geniculate ganglion. Since HSV-1 is highly neurotropic, it travels retrogradely along sensory nerves to the sensory ganglia where latency is established. HSV-1-specific latency-associated transcripts were detected in the geniculate ganglia of unselected cadavers at autopsy (3). These findings provided the first support for the idea that latent HSV-1 is present not only in the trigeminal ganglia, but also in the geniculate ganglia.

Although HSV-1 is a causative agent of the paralysis, the precise mechanism of the facial nerve paralysis is still unknown. Due to difficulty in obtaining the geniculate ganglia from patients, a model of Bell’s palsy is necessary in order to investigate its pathogenesis. In 1994, we succeeded in producing facial nerve paralysis in animals by inoculating HSV-1 into the auricle of mice (4, 5). The paralysis was transient and healed completely within 7 days. In this study, we tried to produce a mouse model of facial nerve paralysis induced by the reactivation of latently infected HSV-1. To our knowledge, this is the first report of an animal model of Bell’s palsy induced by reactivation of HSV-1.

Animals

Four-week-old specific pathogen-free female Balb/c A/Jcl mice (16±18 g) were purchased from Clea Japan, Inc. (Tokyo, Japan). All mice were maintained in our Laboratory Animal Center and cared for in compliance with the Guide for Animal Experimentation at Ehime University School of Medicine.

Virus and Virus Inoculation

The KOS strain of HSV-1 was prepared in Vero cells and plaque-titrated at 6.7 × 10^6 plaque-forming units (PFU) per milliliter. Virus was inoculated following the procedure described by Sugita et al (4), which is a modification of a previously published method (6). Following anesthesia with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), the posterior surface of the right auricle was scratched 20 times with a 27-gauge needle, and then 25 μl of virus solution (1.7 × 10^6 PFU) was placed on the scratched area. The animal was returned to its cage after completing this procedure.

Monoclonal Antibody

145.2C11 hybridoma that produced anti-CD3 mAb was purchased from the American Type Culture Collection (Rockville, MD). Hybridoma cells were cultured in Dulbecco’s Modified Eagle Medium (Gibco BRL, Grand Island, NY) containing 10% fetal calf serum (FCS).
Co-Cultivation of Geniculate Ganglia

Three weeks after virus inoculation, 8 mice were killed to evaluate the establishment of latent HSV-1 infection in the geniculate ganglion. The bilateral geniculate ganglia were excised under microscopy, minced, and planted on Vero cells cultured in a 24-well microplate with 1.5 ml Minimum Essential Medium (MEM) with 5% FCS. The culture medium was exchanged every other day. The cell monolayers were observed for cytopathic effects (CPE) for 4 wk. A culture was determined to be HSV-1-positive when at least 1 focus of CPE appeared in the well.

Evaluation of Facial Nerve Paralysis

The general condition of the mice, blink reflex, and vibrissae movement were carefully observed daily after the virus inoculation. The blink reflex was evoked twice by blowing air onto the eye through an 18-gauge needle with a 5-ml syringe. The degree of blink reflex was scored on a 0 to 2 scale: 0, no difference between right and left side; 1, the blink reflex was delayed compared with the healthy side; 2, the blink reflex disappeared completely. Vibrissae movement was observed for 30 seconds (s) and scored on a 0 to 2 scale: 0, no difference between right and left sides; 1, the vibrissae movement was weaker than on the healthy side; 2, the vibrissae movement disappeared completely. The total score was defined as the sum of the blink reflex and vibrissae movement scores. When the total score was 3 or 4 points, we determined that facial nerve paralysis was present.

Induction of Facial Nerve Paralysis

Only mice that developed a transient facial nerve paralysis after the primary infection were used for the following experiments. Eight weeks after recovery from the primary paralysis, the mice were subjected to 1 of the following 3 procedures; the surface of the right auricle where the HSV-1 was previously inoculated was scratched with a 27-gauge needle, 50–200 µg of 2C11 (anti-CD3 mAb) was injected intraperitoneally, or both procedures were combined. After treatment, the mice were observed daily for the appearance of facial nerve paralysis and change in general condition. Neither recurrent dermatitis nor facial paralysis occurred in any of the mice until these treatments were performed.

Flow Cytometry

Blood (100 µl) taken from a tail vein was immediately poured into a microtube containing 50 µl of 0.1 M EDTA (pH 7.4) solution. The tube was centrifuged and the cells were washed once with phosphate-buffered saline (PBS) (20 mM phosphate buffer, pH 7.4, 130 mM NaCl). Then, the sample was incubated with phycoerythrin-conjugated anti-CD3 monoclonal antibody (CALTAG, Burlingame, CA) for 30 min on ice to detect T cells. After washing with PBS, the cells were hemolyzed with Lysing Reagent (PharMingen, San Diego, CA). Stained cells were analyzed using a FACScan analyzer (Becton-Dickinson, Mansfield, MA).

Blood (20 µl) taken from the tail vein was used to measure the number (mm⁻³) of white blood cells (WBC) using a Microcell Counter (CC-130A, Sysmex, Kobe, Japan). The lymphocyte ratio was obtained by counting the numbers of lymphocytes, granulocytes, and monocytes by flow cytometry. The number of T cells was calculated using T cells (mm⁻³) = WBC number (mm⁻³) × lymphocyte ratio × CD3-positive cells (%) /100. The number of CD3-positive cells (%) was expressed as a percentage of the total lymphocytes.

Extraction and PCR of HSV-1 DNA from the Intratemporal Portion of the Facial Nerve

Ten days after the auricular scratch or administration of mAb, the mice were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg) and killed by transcardial perfusion with 50 ml PBS. Then, the intratemporal portions of both facial nerves including the geniculate ganglia were dissected under a microscope. The facial nerve was cut with scissors and placed in a sample tube containing 100 µg TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). After adding 20 µg of proteinase K, the tissue was digested at 56°C for 120 min. Following heat inactivation of the proteinase K, 2 µg of plasmid DNA carrier (Bluescript, KS) was added to the sample and the DNA was extracted and then dissolved in 10 µl of TE. Synthetic primers encoding part of the virion glycoprotein C (UL44: 5'-CCACCAGGCGCAGGTGATC-3') and the hydrophobic N terminus located on the HSV-1 gene (UL45: 5'-GCCGACCGCCTGCTGCTGCT-3') were used for PCR amplification under previously described conditions (1, 7). Briefly, 4 µl of the extracted DNA and the 2 primers (25 pmol each) were denatured for 7 min at 95°C in 20 µl PCR buffer. One microliter of AmpliTaq DNA polymerase (2.5 U; Perkin-Elmer, Branchburg, NJ) was added and the mixture was overlaid with mineral oil at 95°C. Thirty-five cycles of amplification (80 s at 95°C; 90 s at 60°C; 60 s at 73°C) were performed and the sample was incubated at 73°C for 15 min for a final extension. DNA purified from HSV-1-infected Vero cells was used as a positive control. Distilled water was used instead of extracted DNA as a negative control. Five microliters of the PCR product were separated by electrophoresis in Tris-borate-EDTA buffer (2.0% agarose gel containing 0.5 mg/ml ethidium bromide).
TABLE 1
Presence of a Cytopathic Effect in Cultures of Geniculate Ganglia Co-Cultivated with Vero Cells on the Third Week after Virus Inoculation

<table>
<thead>
<tr>
<th>Presence</th>
<th>Right (inoculated) side</th>
<th>Left (inoculated) side</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paralysis (+)</td>
<td>4/4 (100%)</td>
<td>3/4 (75%)</td>
</tr>
<tr>
<td>Paralysis (-)</td>
<td>4/4 (100%)</td>
<td>3/4 (75%)</td>
</tr>
<tr>
<td>Control</td>
<td>0/2 (0%)</td>
<td>0/2 (0%)</td>
</tr>
</tbody>
</table>

Paralysis: facial nerve paralysis caused by the primary infection of HSV-1. (+) = present; (-) = absent.

Results

Latent HSV-1 Infection in the Geniculate Ganglion

Of the 122 mice inoculated in the auricle with HSV-1, 71 mice (58.2%) developed unilateral facial nerve paralysis exclusively on the inoculated side on the seventh or eighth day after virus inoculation. The paralysis persisted for 7 to 9 days and recovered spontaneously in all cases. On the third week after virus inoculation, latent HSV-1 infection in the right geniculate ganglion was proven by co-cultivation of virus in all 8 mice tested, regardless of the appearance of facial nerve paralysis (Table 1). With the exception of 1 mouse showing facial nerve paralysis, all mice were also latently infected in their left geniculate ganglion.

Effects of 2C11 mAb Injection on T Cell Number in Peripheral Blood

Cell-mediated immunity plays a major role in host defenses against HSV-1 (8). Thus, it was expected that T cell depletion would lead to reactivation of a latent HSV-1 infection. We first evaluated whether injection of 2C11 mAb depleted the peripheral T cells. Monoclonal antibody (2C11) is a competent anti-CD3 antibody and its effects were evaluated by examining peripheral blood (Fig. 1). On the day after a 100-μg mAb injection, T cells were virtually depleted from the peripheral blood and the depletion of T cells persisted for 10 days. T cells reap-peared on the 14th day after mAb injection and returned to normal within 60 days.

Facial Nerve Paralysis Induced by Auricular Scratch and T Lymphocyte Depletion

Eight weeks after recovery from the facial nerve paralysis brought about by HSV-1 inoculation, mice were divided into 3 treatment groups to induce reactivation: auricular scratch (Group A), intraperitoneal injection of 100 μg mAb (Group B), or both (Group C), as shown in Table 2. Ten mice were used in each group. In Group A, all mice began to show erythema on the right auricle on the day after it was scratched. The erythema continued...
incidence of facial nerve paralysis and mortality in different groups:

**TABLE 2**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Auricular scratch</th>
<th>mAb injection</th>
<th>Facial nerve paralysis</th>
<th>Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>-</td>
<td>0/10 (0%)</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
<td>+</td>
<td>0/10 (0%)</td>
<td>2/10 (20%)</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>+</td>
<td>2/10 (20%)</td>
<td>2/10 (20%)</td>
</tr>
</tbody>
</table>

A: auricular scratch alone, B: mAb injection alone, C: combination of both treatments.

**TABLE 3**

<table>
<thead>
<tr>
<th>Dose</th>
<th>Facial Nerve Paralysis</th>
<th>Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µg (n = 20)</td>
<td>3 (15%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>100 µg (n = 20)</td>
<td>3 (15%)</td>
<td>3 (15%)</td>
</tr>
<tr>
<td>200 µg (n = 20)</td>
<td>1 (15%)</td>
<td>12 (60%)</td>
</tr>
</tbody>
</table>

**TABLE 4**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Auricular scratch</th>
<th>mAb injection</th>
<th>Right (inoculated) side</th>
<th>Left (uninoculated) side</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>-</td>
<td>0/6 (0%)</td>
<td>0/6 (0%)</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
<td>+</td>
<td>1/6 (16.7%)</td>
<td>0/6 (0%)</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>+</td>
<td>4/6 (66.7%)</td>
<td>1/6 (16.7%)</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>0/3 (0%)</td>
<td>0/3 (0%)</td>
</tr>
</tbody>
</table>

A: auricular scratch alone, B: mAb injection alone, C: combination of both treatments, Control: no treatment.

A: auricular scratch alone, B: mAb injection alone, C: combination of both treatments.

for 6 to 9 days (average 7.5 days). No mice developed facial nerve paralysis. In Group B, although all mice were debilitated the day after mAb injection, no living mice developed facial nerve paralysis. Two mice (20%) in Group B died of limb paralysis on days 2 and 3 after mAb injection. Two mice (20%) in Group C developed unilateral facial nerve paralysis on days 5 and 7 after treatment (Table 2). In 1 mouse, the blink reflex disappeared on the fifth day and weakness of vibrissae movements was seen on the sixth day (score 3). The paralysis persisted for 7 days and then recovered completely. In the other mouse, the blink reflex disappeared on the seventh day and vibrissae movements weakened on the eighth day (score 3). The paralysis persisted for 5 days. Two mice (20%) in Group C also died of limb paralysis, 2 and 3 days after mAb injection.

**Dose of mAb and the Incidence of Facial Nerve Paralysis**

To determine the optimal dose of mAb, 3 different doses of mAb were injected in combination with the auricular scratch treatment. The results are summarized in Table 3. The incidence of facial paralysis was 15% in both groups of mice given 50 or 100 µg mAb, whereas it was 5% in the group of mice injected with 200 µg. Mortality was 5%, 25%, and 60% in the group of mice injected with 50, 100, and 200 µg mAb, respectively.

**Detection of HSV-1 DNA**

The intratemporal portion of the facial nerve was examined for HSV-1 DNA in another 18 mice. These mice were divided into 3 groups according to the method used to reactivate HSV-1. The mice were killed 10 days after the reactivation procedure. As a control, 3 mice that recovered from the paralysis produced by primary HSV-1 infection were used. Intratemporal facial nerve tissue including the geniculate ganglia was examined to detect HSV-1 DNA (Table 4). No HSV-1 DNA was detected in either control or Group A mice. HSV-1 DNA was detected from the right facial nerve in 1 of 6 mice in Group B, whereas it was detected in 4 of 6 mice in Group C. Figure 2 shows typical patterns seen in the PCR assay. In panel C, the bands of approximately 150 and 280 bp that appear in mice 1, 2, and 3 might be PCR artifacts.

**Histopathologic Findings**

Two of 3 paralyzed mice in Table 3 were used for the histopathologic study. Electron microscopic findings indicate that the HSV-1 particles replicated in geniculate ganglion neurons on the eighth day after the reactivation procedures predominantly (Fig. 3a). These infected neurons had vacuolar changes in the cytoplasm that were exclusively brought about by replicating virus. Replicating viruses were found only on the rough endoplasmic reticulum. A Schwann cell of the intratemporal portion of the facial motor nerve shows vacuolar changes in the cytoplasm, which resulted in demyelination (Fig. 3b). These findings were found only on the paralyzed side.

**DISCUSSION**

Successful experimental reactivation of HSV-1 has been brought about in sensory nerve areas, such as the skin and cornea, using various stimuli, such as skin irritation (9, 10), ultraviolet (UV) light irradiation (11–13), and the administration of immunosuppressive agents (14, 15). However, nerve degeneration induced by HSV-1 reactivation in a motor nerve has not been reported. We
believe this study is the first report of the induction of facial paralysis by a reactivation mechanism. Histopathological study of the intratemporal portion of the facial nerve showed that the virus replicated profusely in the neurons of the geniculate ganglion, and that the motor fibers of the facial nerve underwent demyelination in paralyzed mice. Facial nerve consists of motor and sensory fibers. Sensorial neurons of facial nerve, innervating the posterior auricle, are located in the geniculate ganglion. Therefore, it is considered that auricular scratch in combination with immunosuppression promotes the reactivation of the latently infected HSV-1 in the geniculate ganglion. Replicated and released viruses must infect Schwann cells adjacent to the facial motor nerve, causing demyelination. Subsequently, vigorous destruction of the facial nerve, which induces an inflammatory reaction in the narrow facial canal in the temporal bone, may exacerbate the paralysis.
Skin irritation is believed to be an appropriate stimulus to induce reactivation. Hill et al (9) reported that erythema occurred on the skin surface of the ear after stripping cellophane tape from the previously inoculated site. In their study, about 30% of mice showed recurrent erythema, which continued for 3.5 days on average. Erythema is thought to be the result of reactivation of latent HSV-1. In Group A in our study, erythema persisted for 7.5 days on average. This suggests that scratching the skin with a needle is a more powerful stimulus than stripping cellophane tape. In spite of the appearance of auricular erythema, HSV-1 DNA was not detected in the geniculate ganglion of the mice treated with auricular scratch alone. We concluded that a skin scratch alone was sufficient to induce a skin lesion, but not to evoke facial nerve paralysis.

Cell-mediated immunity plays a major role in host defense against herpes virus (8, 16, 17). According to Shimeld et al, both CD4$^+$ and CD8$^+$ T cells accumulate in the trigeminal ganglion after UV-irradiation of the cornea in mice with latent HSV-1 infection (18). These cells are thought to inhibit the spread of reactivated HSV-1. Therefore, T cell-depletion was expected to enhance HSV-1 reactivation. T cells in the peripheral blood were virtually completely depleted for 10 days after injection of anti-CD3 mAb, as indicated by Hirsch et al (19). However, no facial nerve paralysis occurred by the deletion of T cells in our mouse model. In the PCR assay, 1 of 6 mice showed HSV-1 DNA in the facial nerve on the 10th day after mAb injection. In contrast, 4 of 6 mice treated with both auricular scratch and mAb injection showed HSV-1 DNA. It was of interest that the incidence of HSV-1 DNA detection corresponded with that of facial nerve paralysis, although our PCR conditions were not quantitative. The rate of incidence (15%) of the development of facial paralysis was the same in the groups of mice injected with 50 and 100 μg mAb in combination with auricular scratch. Increasing the dose of mAb injected to 200 μg per mouse increased mortality (60%) rather than increasing the incidence of facial paralysis. Thus, a combination of different kinds of stimulation, local skin irritation and general immunosuppression seems to be essential for successfully inducing facial paralysis in mice latently infected with HSV-1.
Aviel et al (20) reported that peripheral T lymphocytes were significantly decreased in patients with Bell’s palsy in the early stage of the disease. Schielke et al (21) found 7 cases (4.1%) of facial nerve paralysis in 170 human immunodeficiency virus (HIV)-infected patients. Overall, the incidence of Bell’s palsy in HIV patients is higher than that in the general population in which the annual estimated incidence of Bell’s palsy is 20 to 30 patients per 100,000 population (22). These lines of evidence support the idea that synergistic stresses including immunosuppression and physical stimulation, such as dental treatment (23), are required for the induction of Bell’s palsy due to herpes virus reactivation, as suggested by this study.

ACKNOWLEDGMENTS

We thank Kenji Kameda for excellent technical support with the FACS analysis. We also thank Professor Naoaki Yanagihara for helpful discussion.

REFERENCES


Received July 13, 2000
Revision received November 29, 2000 and March 2, 2001
Accepted March 8, 2001