Mechanism of Pruritus and Peracute Death in Mice Induced by Pseudorabies Virus (PRV) Infection

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ABSTRACT. Mechanisms of postinfectious pruritus and peracute death in mice by pseudorabies virus (PRV) were investigated by inoculating the Yamagata-S81 strain of PRV peripherally or intracerebrally into 4-week-old ICR and BALB/c mice. Clinical signs developed most rapidly in mice inoculated intracerebrally, with intermediate speed in mice inoculated intracutaneously, and slowly in mice inoculated subcutaneously. Since intracutaneously inoculated mice showed an acute reaction and this is considered a peripheral route, the distribution of viral antigens in the nervous system of intracutaneously inoculated mice was examined immunohistologically. Viral antigens were mainly detected along the trigeminal and the oculomotor nerves, but neither necrosis nor an inflammatory response was observed in these areas. The infectious virus was efficiently recovered from the viral antigen-positive tissues. In the pruritic skin lesions, viral antigens were not observed. These findings indicate that the main route of viral spread in intracutaneously inoculated mice is the trigeminal and oculomotor nerves and that the virus in the trigeminal nerve may trigger pruritus.—KEY WORDS: pruritus, pseudorabies virus (PRV), trigeminal nerve.


Pseudorabies virus (PRV) is a worldwide infection spread by swine which are considered the natural host of this virus [13, 16]. In neonatal swine, PRV causes an acute neurologic disease with a high fatality rate, whereas in adult swine PRV induces respiratory disease with a relatively low mortality rate. Besides swine, many other species of animals such as cattle [12], dogs, cats, and rodents regarded as non-natural host can be infected by PRV either naturally or experimentally, and they sometimes develop severe pruritus followed by peracute death. In dogs experimentally infected with PRV, cardiac lesions consisting of inflammation of the coronary vessels, nerves, and ganglia cause arrhythmias which may lead to sudden death [14]. In rats and mice, PRV infection also induces acute or peracute death associated with severe pruritus [2, 4, 5, 10]. The clinical features in these animals indicate that peracute death is due to the neural spread of the virus.

Herpes simplex virus (HSV), which has a tropism to the sympathetic nervous system [8] and the trigeminal nervous system [9, 20-22], has been shown to be transported transneuronally in the retrograde direction [8, 9, 20-22]. PRV is also considered to have a tropism to the nervous system, and has been used as potential transneuronal tracers [11, 17-19]. In rats inoculated with PRV into the anterior chamber, the viral antigen-positive cells were located in the trigeminal nerve and its spinal nucleus consisted of only the oralis nucleus, but not in the other trigeminal nuclei in the central nervous system (CNS) [11]. On the other hand, the presence of PRV antigens in the sympathetic nervous systems was also reported for mice inoculated into the adrenal gland [18, 19] or for rats inoculated into the anterior chamber [17]. In spite of these detailed studies on the route of virus transmission in PRV-infected animals, the mechanisms of pruritus and peracute death associated with PRV infection have not been understood.

In the present study, we investigated histochemically the patterns of neuronal spread of virus in mice following peripheral inoculation, and attempted to find the specific areas which are involved in pruritus or in the peracute death.

MATERIALS AND METHODS

Virus: The Yamagata-S81 strain of PRV isolated from a pig in Japan [15] was inoculated to CPK cells cloned from the SK-H cell line of swine kidney origin [6], and the cells were incubated at 37°C using maintenance medium which consists of Eagle’s minimum essential medium (E-MEM) with 2% calf serum. When over 90% of the infected monolayers showed a cytopathic effect, the cultures were frozen-thawed and centrifuged at 4°C for 10 min at 2,000 rpm. The supernatant was stored at −80°C as a stock virus. The 50% tissue culture infectious dose (TCID50) of the stock virus was 10^3.5 ml.

Mice: Female ICR mice aged 4 weeks (Charles River Japan Inc., Kanagawa) were used to determine the mortality rate, and male BALB/c mice at 4- to 5-week old were used for immunohistopathological and virological examinations.

Determination of the fatality rate: A total of 50 mice were anesthetized with pentobarbital sodium and inoculated subcutaneously (s.c.) into the abdominal skin with 25 μl of the stock virus at titers of 10^2, 10^3, 10^4, or 10^5 TCID50. The lethality was expressed as the dose of virus
resulting in the death of 50% of the animals (LD₅₀).

**Virus inoculation:** Fifteen groups of 10 BALB/c mice each were inoculated with PRV at 3 different doses by 5 different routes, i.e., intracerebrally (i.c.), subcutaneously in the forelimb (s.c. fl), subcutaneously in the hindlimb (s.c. hl), intravenously into the tail vein (i.v.), and intraocularly into the vitreous body (i.o.). The inoculation volume was 25 µl for the i.c. and s.c. routes, 100 µl for the i.v. route, and 1 µl for the i.o. route. As control, 1 µl of phosphate buffered saline (PBS) was inoculated i.o.

**Isolation of virus:** Nervous tissue was collected from 2–3 mice inoculated i.o. with PRV at titer of 10⁹.⁵ TCID₅₀ at 12, 36, and 48 hours post inoculation (hpi) and at the moribund stage, and the virus content was titrated by a plaque assay. The brain and trigeminal nerve ganglia were divided into the following 6 regions: the right cerebrum and basal ganglia, the left cerebrum and basal ganglia, the cerebellum, the mesencephalon with the mesons and medulla oblongata, the right trigeminal nerve ganglia, and the left trigeminal nerve ganglia. Each tissue sample was immediately stored at −80°C after collection. The tissues were homogenized to give a 10% v/w suspension in E-MEM containing 2% calf serum, and then centrifuged at 3,600 rpm for 10 min at 4°C. One-tenth ml of the supernatant were inoculated into the wells of 6-well plastic dishes containing Vero cells. After adsorption of the virus for 1 hr, the dishes were overlaid 0.9% agar medium supplemented with 1% calf serum, and incubated for 2 days, and the plaques were stained by 0.01% neutral red. Virus titers were expressed as plaque-forming units (PFU).

**Histopathological examination:** The brains, trigeminal nerve ganglia, and eyeballs of mice were fixed in 10% neutral buffered formalin for one week, processed by standard histopathological methods, and embedded in paraffin. All tissues were cut in 4 µm section, deparaffinized and stained with hematoxylin and eosin (HE).

**Detection of viral antigens in paraffin sections:** Paraffin sections were placed on glass slides coated with neoprene in 0.1% toluene (Ohken Shoji Co. Ltd., Tokyo) and dried for three days. Subsequently, the sections were deparaffinized, treated with 3% H₂O₂ in methyaltedcohol for 10 min and 3% bovine serum albumin in PBS for 10 min, and then incubated at 37°C for 1 hr with polyclonal rabbit antiserum to PRV diluted 1:1000 in PBS. In order to check the specifity of this antisera, normal tissues were incubated with rabbit preimmune serum. The sections were reacted sequentially with biotinylated anti-rabbit IgG (Amersham Japan Co. Ltd.), streptavidin-biotinylated-horseradish peroxidase complex (ABC complex) solution (Amersham Japan Co. Ltd.), and 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution [7].

**RESULTS**

**Clinical findings:** In the first experiment, 200 mice were inoculated s.c. to determine the fatality rate. At 2 days post inoculation (dpi), they started to show slight bradykinesia and roughening of the fur. Thereafter, frequent scratching mainly at the virus-inoculated skin on the abdomen and hyperkinesia were observed. Specific neurological signs were rarely seen. Once clinical signs appeared, all the mice died within 24 hr. On the other hand, all the mice which showed no clinical signs survived. The fatality rate depended on the inoculated dose and fatality rates of 100% and 0.01% were seen at 10⁹.⁰ TCID₅₀, and 10⁻¹¹ TCID₅₀. The 50% lethal dose (LD₅₀) was 10⁻⁹.⁵ TCID₅₀.

In the second experiment, 15 groups of 10 mice each were inoculated i.c., s.c., i.v., or i.o. with PRV at titers of 10⁹.⁰, 10⁶.⁵, and 10⁻¹¹ TCID₅₀, respectively, and the clinical signs were observed. Although a variety of clinical signs were observed in each inoculated group, the common clinical sign was pruritus. In the group of i.c.-inoculated mice, 3–4 out of 10 mice showed slight hyperkinesia and rolling but the other mice remained normal. Two mice with rolling suddenly started to run while squeaking loudly, developed convulsions, and soon died. In the groups inoculated by the other routes, hyperkinesia and rolling were moderate.

The mean incubation period and time of death were compared among the different groups. As shown in Table 1, the most rapid appearance of clinical signs and death was observed in the i.c. group, the speed was intermediate in the i.o. group and the onset was slow in the s.c. and i.v. groups. The fatality rate at a viral dose of 10⁻².⁵ TCID₅₀ was 100% in the i.c. group, 100% in the i.o. group, 94% in

<table>
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<th>Route</th>
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<th>Fatality rate (%)</th>
<th>Incubation period (hr)</th>
<th>Mean time of death (hr)</th>
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<td>59.3±4.7</td>
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<td>50</td>
<td>76.0±18.9</td>
<td>90.0±14.8</td>
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*Intracerebral inoculation. **Intravenous inoculation. ***Subcutaneous inoculation in the fore limb. ****Subcutaneous inoculation in the hind limb. *****Intracutaneous inoculation into the vitreous body. *P<0.001 for comparison with each other routes inoculated at the same dose. **P<0.05 for comparison with i.v. route inoculated at the same dose, by Student's two tailed test. ***ND: Not done.
the i.v. group, and 90% in the s.c. group. At a dose of $10^{1.1}$ TCID$_{50}$, the fatality rate was 90% in the i.c. group, 50% in the i.v. group, 20% in the i.o. group, and 10% in the s.c. group. The LD$_{50}$ of i.o., s.c. fl., s.c. hl., and i.v. were $10^{1.1}$, $10^{2.3}$, $10^{1.7}$ and $10^{1.9}$ TCID$_{50}$, respectively.

Virus isolation from nervous tissue: The mice inoculated i.o. were chosen for virus isolation, since i.o. inoculation caused peripheral infection resulting in peracute death along with neurological signs similar to those seen with i.c. inoculation. The results of virus isolation from the various parts of the brain and the trigeminal nerve ganglia are shown in Table 2.

At 12 hpi, no virus was isolated from any of the neural tissues. Infectious virus was first isolated from the ipsilateral trigeminal nerve ganglia at 24 hpi and thereafter consistently from the same tissue. The highest titer of virus was found 58 hpi at the moribund stage. Infectious virus was first isolated from the contralateral trigeminal nerve ganglia at 36 to 48 hpi, and the virus titer was always lower than the ipsilateral one.

In the brain, a relatively high titer of virus was recovered from the brainstem at the moribund stage, but the titer was lower than that in the trigeminal nerve ganglia at the same stage. Only low titers of virus were recovered from the cerebrum and cerebellum at the later stages, with the titer from the ipsilateral cerebrum being slightly higher than that from the cerebellum.

On the other hand, infectious virus was not isolated from any nervous tissues of mice surviving the i.o. infection.

Histopathological findings and distribution of virus antigen

—Trigeminal nerve ganglia—: At 12 hr intervals after i.o. inoculation with $10^{5.5}$ TCID$_{50}$ of PRV, mice were killed for histopathological examination and for the detection of viral antigens. The histopathological changes observed in HE-stained sections were very mild at any time.

Even in the ipsilateral trigeminal nerve ganglia at the moribund stage, only slight vacuolation and mild shrinkage of the ganglion cells were observed. No inflammatory reaction including perivascular cellular infiltrates was observed at any stage. Viral antigens stained by the ABC method were not observed before 12 hpi in bilateral trigeminal nerve ganglia. The first appearance of viral antigens was noted in a few ipsilateral ganglion cells at 24 hpi, and then the antigens tended to spread gradually to the surrounding cells. Viral antigen-positive cells were divided into the following three types according to the pattern of antigen distribution (Fig. 1): (a) localization of antigens only in the nucleus, (b) localization in the

Table 2. Isolation of PRV at different times from nervous tissues

<table>
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<tr>
<th>hpi*</th>
<th>Cerebrum R</th>
<th>Cerebrum L</th>
<th>Cerebellum R</th>
<th>Cerebellum L</th>
<th>Brain stem R</th>
<th>Brain stem L</th>
<th>Trigeminal nerve R</th>
<th>Trigeminal nerve L</th>
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<td>8.2</td>
<td>6.7</td>
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* Hours post inoculation. **R: right L: left.

Fig. 1. Ipsilateral trigeminal ganglion. Three types of localization of viral antigens are observed: (a) only in the nucleus, (b) only in the cytoplasm, and (c) in both the nucleus and the cytoplasm. Viral antigens were detected using anti-PRV rabbit serum and ABC method in i.o.-inoculated mice at 53 hpi (× 218).
Fig. 2. Distribution of PRV antigens in the brains of 2 i.o.-inoculated mice at the moribund stage. Black dots represent viral antigens detected by the ABC method (the grade of positivity is expressed as +, ++, or +++). (A) Serial coronal section. (B) Serial horizontal section. The key is given below.

Fig. 2-A. a. dorsomedial hypothalamic nucleus. b. thalamic nucleus. b'. subparafascicular thalamic nucleus. b''. centrum medianum-parafascicular thalamic nucleus. b'''. central gray thalamic nucleus. c. dorsal anterior mammillary nucleus. d. Darkschwitsch nucleus. e. interstitial nucleus of Cajal. f. Edinger-Westphal nucleus. g. Forel nucleus. h. mesencephalic trigeminal nucleus. i. suprageniculate pontine nucleus. j. facial nucleus. j'. facial nucleus. j''. accessory facial nucleus. k. raphe nucleus. k'. raphe magnus nucleus. k''. raphe pallidum nucleus. l. olivary nucleus. l'. inferior olivary nucleus. l''. dorsal olivary nucleus. m. spinal trigeminal nucleus. m'. caudalis nucleus. m''. interpolaris nucleus. n. nucleus of the medulla oblongata. n'. lateral nucleus of the medulla oblongata. n''. central gray matter of the medulla oblongata. o. solitary nucleus.

Fig. 2-B. a. spinal trigeminal nucleus. a'. oralis nucleus. a''. interpolaris nucleus. a'''. caudalis nucleus. b. raphe magnus nucleus. c. olivary nucleus. c'. superior olivary nucleus (lateral part). c''. medial accessory olivary nucleus. d. lateral hypothalamic nucleus. e. motor trigeminal nucleus. f. nucleus of the optic tract. f'. basal nucleus of the optic tract. f''. medial nucleus of the optic tract. g. Edinger-Westphal nucleus. h. red nucleus. i. Darkschwitsch nucleus. j. mesencephalic trigeminal nucleus. k. dorsal tegmental nucleus. l. oculomotor nucleus. m. lateral habenular nucleus. n. lateral thalamic nucleus.

Fig. 3. Ipsilateral spinal nuclei and spinal tract of the trigeminal nerve in an i.o.-inoculated mouse at 49 hpi. Nerve cells are in the upper part and nerve fibers in the lower part. PRV antigens detected by the ABC method are observed in both areas (× 109).
cytoplasm, and (c) antigens in both the nucleus and the cytoplasm.

The ganglionic cells on the ipsilateral side had more viral antigens than those on the contralateral side even at the moribund stage. These results suggest that PRV spreads to the brain mainly via the ipsilateral trigeminal nerves.

—Brain—: Mice were killed at the moribund stage (49 to 58 hpi) after i.o. inoculation and the brains cut in the horizontal planes were examined. Neither inflammation nor necrosis was observed in any part of the brain, and slight vacuolation was limited to the cytoplasm of cells in the spinal nuclei of the trigeminal nerve. In contrast, viral antigens were distributed in a wide area of the brain, as shown in Fig. 2. The trigeminal nerve, its bilateral tracts, and the associated nuclei in the brainstem were most severely affected by PRV. Thus, positive staining for viral antigens was detected in the ipsilateral spinal nuclei such as the oralis, interpolaris, and caudalis nuclei, the motor nucleus, and the mesencephalic nucleus of the trigeminal

Fig. 4. Edinger-Westphal nuclei and the Forel, Cajal, and Darkschwich nuclei of the mesencephalon in an i.o.-inoculated mouse at 58 hpi. (A) Abundant viral antigens detected by the ABC method are located in the center of the figure (× 44). (B) Viral antigens in large neural cells (× 218).
nerve (Fig. 3). In the spinal nuclei, viral antigens were found only in the ipsilateral side, whereas in the motor and mesencephalic nuclei, the antigens were detected bilaterally. Viral antigens in the spinal nuclei were more abundant than the other nuclei, and detected not only in neuronal cells but also in glial cells.

Besides the trigeminal circuit, a small amount of viral antigen was present in the pons and medulla oblongata, being found bilaterally in the raphe nuclei, the olivary nuclei, and the suprageniculate pontine nuclei, and ipsilaterally in the facial nerve nuclei.

In the mesencephalon, viral antigens were found ipsilaterally in the dorsal tegmental nucleus and bilaterally in the nucleus of Darkschewitsch, the interstitial nucleus of Cajal, the Edinger-Westphal nuclei (E-W nuclei, equivalent to the oculomotor accessory nuclei), the oculomotor nerve nuclei and the nucleus of Forel (Fig. 4). Viral antigens were much more abundant in the area around the E-W nuclei.

In the diencephalon, antigens were present ipsilaterally in the lateral habenular nucleus, and the lateral nucleus of the thalamus, and bilaterally in the lateral or dorsomedial nuclei of the hypothalamus and the nuclei associated with the optic tract. Viral antigens were not detected in the cortex of the cerebrum or cerebellum, nor in the olfactory bulb and the optic nerve near the eyeball.

Thus, the distribution of viral antigens in the brain was mainly limited to the brainstem regions related to the trigeminal nerve and to the sites in the mesencephalon related to the oculomotor nerve.

—Peripheral tissue—: Mice were killed at the moribund stage (49 to 58 hpi.) after i.o. inoculation, and their peripheral tissues were examined. The most severe lesions were observed in the inoculated eye. They consisted of keratinous precipitates in the cornea, infiltration of inflammatory cells in the iris and ciliary body, lens edema, vacuolation and severe degeneration of the retinal ganglionic cell layer, and necrosis in the inner nuclear layer. Viral antigens were localized in the corneal endothelium, iris, ciliary body, and retina especially the ganglionic cell layer, inner nuclear layer, and pigment epithelium (Fig. 5). Thus, the localization of antigens paralleled that of the histopathological changes in the eyeball. Neither lesions nor viral antigens were seen in the contralateral eyes or in the eyes of control mice inoculated with PBS.

There were also severe lesions in the skin close to the inoculation site. The facial and cervical lesions became raw due to scratching by the mice. Despite the presence of these pruritic lesion, viral antigens were not detected.

**DISCUSSION**

Several clinical signs including neurological ones were observed in mice inoculated with PRV by various routes. The most common clinical sign was pruritus, except in i.e.-inoculated mice which showed only slight hyperkinesia and rolling. The major clinical signs in mice inoculated i.o. were pruritus followed by peracute death, and the mechanisms causing these phenomena were investigated in detail.

The pruritic lesions of the skin were localized in the head and neck, and seemed to be closely related to the distribution of the trigeminal nerve, although the lesions sometimes extended beyond the exact distribution of the trigeminal nerve. Despite the absence of viral antigens in the skin lesions, such antigens were found in the ganglion.
cells and in the central nuclei of the trigeminal nerve. These results suggest that the virus is transported retrogradely from the eye to the brainstem via the trigeminal pathway so that the virus reaches the spinal nuclei of this nerve and then spreads to the facial nerve. The stimulation of the trigeminal nerve caused by the virus might cause pruritus in the facial skin since this nerve is considered to transmit pain, temperature, and position sensation from the head and face. The distribution of viral antigen shown in this study also suggests involvement of the trigeminal nerve in the pathogenesis of pruritus. A correlation between the pruritic lesions and the distribution of virus particles in the trigeminal nerve ganglia was also suggested by previous electron microscopic and electrophysiological examinations [3].

Viral antigens in the brain seemed to be localized in two distinct sites, the structures associated with the trigeminal nerve in the brainstem and those associated with the oculomotor nerve in the mesencephalon. The detection of a large amount of viral antigen in the spinal nuclei of the trigeminal nerve in the brainstem indicates that these sites serve as the main route of viral spread. The raphe nuclei in the medulla oblongata were also viral antigen-positive and they are indirectly connected with the trigeminal nerve in the posterior horn of the upper spinal cord. Viral antigens were abundant in the autonomic nucleus of the E-W nuclei, which is located at the upper end of the oculomotor nuclear tract, and in the interstitial nucleus of Cajal or nucleus of Darkschewitsch, which are located near the E-W nuclei and are considered to be secondary oculomotor nuclei. It is known that preganglionic neurons from the E-W nuclei reach to the ciliary ganglion, whereas the postganglionic neurons innervate the cornea, iris, choroid, and constrictor pupillae in the eyeball. Thus, the presence of viral antigen-positive cells in the oculomotor-associated nuclei indicates that the virus also spreads through the oculomotor nerve. In addition, the presence of antigens in the E-W nuclei suggests that the parasympathetic nervous system is also involved in the spread of the virus. A small number of antigen-positive cells were detected in the ipsilateral habenular nuclei related to the limbic system and in the nuclei associated with the optic tract. However, the amount of viral antigen in these nuclei was extremely less than that in the trigeminal and E-W nuclei. This suggests that neither the limbic system nor the optic tract is an important route for viral spread in the CNS, even in the case of virus inoculation into the eyeball.

From the known anatomy of the innervation of eye, the following two routes of viral spread from the eye to the nerves may be suggested. The virus may pass into the first ophthalmic division of the trigeminal nerve, and thus reaches the iris and cornea. Alternatively, the virus may enter the oculomotor nerves in the muscles of the eyeball and subsequently reaches the nuclei associated with the oculomotor nerve, especially the E-W nuclei related to the parasympathetic nerve.

The neural spread of PRV has been reported in rats [11]. In their study, viral antigens were found in the first ophthalmic division of the ipsilateral trigeminal nerve as early as 16 hpi after inoculation into the anterior chamber. By 36 hpi, and also found in the root of the trigeminal nerve and in its spinal nuclei. Though the appearance of antigens in the trigeminal nerve or the brainstem was earlier than that in our experiment, the localization of the virus corresponded to our results.

PRV seems to grow efficiently in both the retinal ganglion cells and the ganglion cells of the trigeminal nerve. Viral antigens could not be observed in the optic nerve, although they were found abundantly in the trigeminal tract. The reason why the virus does not spread in the retinal ganglion cells in contrast to its efficient spread through the ganglion cells of the trigeminal nerve is unknown at present.

This study showed a clear-cut correlation between virus isolation and the distribution of viral antigens in the CNS. A large amount of infectious virus was isolated from the brainstem, in a result in agreement with the extensive distribution of viral antigens in the same area. It is thus conceivable that the brainstem, particularly the pons and mesencephalon, plays an important role in the clinical signs of PRV infection.

At present, the mechanisms underlying peracute death due to PRV infection are unknown. It has been claimed that peracute death may be caused by respiratory paralysis [3]. However, we found virus neither in the spheconuclei nor in the respiratory center in the medulla oblongata. The large amount of viral antigens in the spinal nuclei of the trigeminal nerve probably only trigger the pruritic lesions, and is not likely to have a direct role in causing death. One possibility is that mild lesions such as slight edema of the nerve cells may provide evidence for a disturbance of homeostasis that causes peracute death, since such lesions are present close to the viral antigen-positive regions. Another possibility is a sympathetic disorder, since a large number of antigens as well as a marked vacuolation were found in the ganglion cells of the superior cervical ganglion (data not shown). Since this ganglion is important in the sympathetic nervous system, the virus may impair sympathetic functions and thus causes peracute death.

In mice with acute encephalitis induced by HSV inoculation either intranasally or intracoronally, virus was shown to spread via the olfactory pathways into the CNS [1, 20]. Both HSV and PRV induce a neurological disease with a peracute nature and invade the CNS via the cranial nerves. A comparison of these two viruses may thus give some clues to the mechanism of peracute death.

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References


