Differential localization of neurons susceptible to enterovirus 71 and poliovirus type 1 in the central nervous system of cynomolgus monkeys after intravenous inoculation

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Poliovirus and enterovirus 71 (EV71) are both neurotropic enteroviruses that cause serious neurological diseases, such as poliomyelitis and encephalitis. The neurovirulence of EV71 in cynomolgus monkeys was demonstrated previously by intraspinal inoculation. In this study, an improved simian model of EV71 infection was established by using intravenous inoculation, which revealed clinical and neuropathological similarities between this model and human cases of encephalitis. Experimental EV71 infection induced direct neurological manifestations, such as tremor, ataxia and brain oedema, but not non-neurological complications, such as pulmonary oedema and cardiac failure. Using this model of EV71 infection, the neurotropic characteristics of the prototype strains of EV71 and poliovirus type 1 (PV1) were compared. Three monkeys were inoculated intravenously with $10^5$–$10^5$ TCID$_{50}$ EV71 and all developed neurological disease signs within 4–6 days of inoculation. However, after inoculation with $10^5$–$10^5$ TCID$_{50}$ PV1 strain OM1 (PV1-OM1), the major manifestation was flaccid paralysis, starting from the lower limbs 6–9 days post-inoculation. Histopathological and virological analyses of moribund monkeys revealed that disseminated EV71 infection was characterized by severe panencephalitis involving both the pyramidal and extrapyramidal systems. In contrast, the lesions induced by PV1-OM1 were mainly restricted to the pyramidal tract, particularly the spinal motor neurons, thalamus and motor cortex. In conclusion, neuropathological involvement in this model correlated well with the apparent differences in neurological disease induced by EV71 and PV1-OM1. Thus, intravenous inoculation with EV71 is an excellent model to study the neuropathology of EV71 and to evaluate candidate vaccines and potential antiviral agents.

INTRODUCTION

Poliovirus and enterovirus 71 (EV71) are both neurotropic human enteroviruses. Based on VP1 sequences that encode the capsid protein, EV71 has been reclassified into the cluster A enteroviruses; this group includes coxsackievirus A16 (CA16) and coxsackievirus A10, which cause hand, foot and mouth disease (HFMD) and herpangina (Pulli et al., 1995; Oberste et al., 1999). By contrast, poliovirus is related to the cluster C enteroviruses. Moreover, clinical and neuropathogenic features that are associated with apparent EV71 and poliovirus infections do not appear to be identical. The major neurological manifestation of severe poliovirus infection is paralytic poliomyelitis. In contrast, recent and historical EV71 epidemics throughout the world have demonstrated a wider variety of clinical manifestations, ranging from the mild HFMD to fatal encephalitis and acute flaccid paralysis (Schmidt et al., 1974; Chumakov et al., 1979; Melnick, 1984; Ho et al., 1999; Chan et al., 2000).

The global poliomyelitis eradication programme has resulted in a remarkable decrease in the circulation of wild-type poliovirus throughout the world, although the World Health Organization (WHO) missed the initial target year of 2000 (WHO, 2002). Interruption of circulation of indigenous wild-type polioviruses has been certified in three individual WHO regions: in the Americas region in 1994, in the Western Pacific region in 2000 and in the European
region in 2002 (WHO, 2002). In the Western Pacific region, the last indigenous poliomyelitis case caused by wild-type poliovirus was identified in Cambodia in 1997.

The recent re-emergence of EV71 epidemics that are associated with severe neurological diseases appears to be synchronized with the decline of wild-type polioviruses in this region. In Malaysia in 1997 and in Taiwan in 1998, there were two outbreaks of HFMD, mainly in young children, which were characteristically associated with severe neurological complications that caused several fatalities (Ho et al., 1999; Chan et al., 2000). Thereafter, further EV71 epidemics with severe neurological diseases have been reported in Western Australia in 1999 (McMinn et al., 2001), in Japan in 1997 (Komatsu et al., 1999) and 2000 (Fujimoto et al., 2002) and in Taiwan in 2000 (Wang et al., 2002). Despite epidemiological and virological studies of recent EV71 epidemics, the neuropathological characteristics of EV71 have been reported only in some cases (Wong et al., 1999; Wong, 2000) and its pathogenesis remains unclarified, due to the lack of adequate experimental systems.

To characterize the neuropathogenic features of EV71 experimentally, we applied an intraspinal inoculation system of EV71 in cynomolgus monkeys in a previous study and described, to some extent, a similarity in neurological manifestations of humans and the animal model (Nagata et al., 2002). In addition, wild-type strains, even those that were isolated from patients with HFMD, showed neurovirulence after inoculation into the monkey spinal cord. To avoid the possibility of direct invasion of viruses from the initial inoculation site in the lumbar cord to the central nervous system (CNS), we modified the site of inoculation from an intraspinal to an intravenous route in the present study. The neuropathological findings showed more a reliable distribution of lesions and viral antigens in the CNS in terms of their resemblance to natural EV71 infections in humans. This model thus allowed us to investigate the spread of EV71 in the CNS after artificial viremia. Therefore, this well-established animal model was used to compare the neuropathological features of two neurotropic enteroviruses, poliovirus type 1 (PV1) and EV71.

METHODS

Virus. A variant of the prototype strain of EV71, BrCr/tr, was used in experiments. The BrCr strain, which was originally isolated from a patient with aseptic meningitis in California, USA, in 1970 (Schmidt et al., 1974), was maintained in cynomolgus monkey kidney (CMK) or Vero cells (Hagiwara et al., 1978, 1982). The temperature-sensitive (BrCr/ts) and temperature-resistant (BrCr/tr) variants of the BrCr strain had previously been cloned by picking large and small viral plaques, respectively, in CMK cells. The BrCr/tr variant was further plaque-subcloned in Vero cells from the heterogeneous virus mixture in the original BrCr/ts virus stock. After two rounds of plaque purification, the temperature-resistant phenotype of BrCr/tr was confirmed by titration at 35°C and 39.5°C in Vero cells. The virus stock for inoculation was prepared in Vero cells with three-round passages after plaque purification and the temperature-resistant phenotype was confirmed as described previously (Nagata et al., 2002). PV1 strain OM1 (PV1-OM1) was prepared in HEp-2 cells after transfection of in vitro-transcribed RNA that was derived from a DNA clone based on the neurovirulent Mahoney strain of PV1 (Shiroki et al., 1995). The high neurovirulence of the Mahoney strain has been demonstrated in cynomolgus monkeys (Bodian & Howe, 1941).

Virus titration. Viral infectivity titres were determined by a microtitration assay as described previously (Nagata et al., 2002) and expressed as TCID₅₀. BrCr/tr and OM1 strains were titrated in Vero and HEp-2 cells, respectively. For plaque purification of the EV71 variants, a plaque assay was performed as described previously (Nagata et al., 2002), but using Vero instead of CMK cells.

Temperature-resistant phenotype. Logarithmic differences of viral infectivity titres (TCID₅₀) in Vero cells at 35-0 and 39.5°C were determined. A logarithmic difference of <1.5 was considered to be indicative of a temperature-resistant phenotype.

Animals and housing. Eight adult female cynomolgus monkeys (Macaca fascicularis; age range, 6–17 years) that had been bred in captivity at the Tsukuba Primate Center, National Institute of Infectious Diseases, Japan, were used and confirmed to be seronegative for EV71 and PV1. These monkeys also tested negative for tuberculosis and simian immunodeficiency virus. All animal procedures, including virus inoculation, were approved by the Committees on Biosafety and Animal Handling and Ethical Regulation of the National Institute of Infectious Diseases, Japan.

Antibody. Rabbit antisera that recognize the capsid of EV71 and PV1 were used; their immunohistochemical application has been reported previously (Nagata et al., 2001, 2002).

Virus inoculation. After anaesthesia of three monkeys with ketalar and xylazine, 100 μl virus solution containing 10⁻⁷ TCID₅₀ EV71 BrCr/tr or PV1-OM1 was inoculated into the right tibial vein. In addition, two monkeys were inoculated with BrCr/tr into the spinal cord at the level of L3–L5 in accordance with the WHO method for neurovirulence testing for oral poliovirus vaccine (WHO, 1990). The needle position in the spinal column at inoculation was confirmed by observation of twitching of one or both hind limbs. After inoculation, monkeys were observed daily for 10 days for clinical manifestations, and for neurological signs in particular. The monkeys were sacrificed under deep anaesthesia on day 10 post-inoculation (p.i.). Monkeys that died or became moribund before day 10 p.i. were autopsied or sacrificed under deep anaesthesia using ketalar. After autopsy or sacrifice, the brain and spinal cord tissues were excised for histological and virological analyses.

Histology. The brain and spinal cord were fixed in 10% formalin in PBS and embedded in paraffin. Histological examination of the CNS was performed on the frontal, motor, sensory, parietal, temporal and occipital cortices, as well as the hippocampus, basal ganglia, claustrum, thalamus, subthalamus, substantia nigra, brainstem and spinal cord. The spinal cord was sectioned horizontally 10 times at the cervical level and 12 times at the lumbar level. Paraffin sections, 6 μm in thickness, were stained with haematoxylin and eosin (H&E) and with Luxol-fast blue/cresyl violet (Klüver–Barrera method).

Immunohistochemistry. Immunohistochemical detection of the capsid polypeptides of EV71 and PV1 was performed on paraffin-embedded sections according to previously described methods (Nagata et al., 2001, 2002). Briefly, sections were deparaffinized with xylene and rehydrated in ethanol. They were then treated with 0.25% trypsin solution and 0.5% CaCl₂ in PBS for 30 min and incubated in 1% hydrogen peroxide in methanol to block endogenous peroxidase activity. Thereafter, normal goat serum was
applied to the sections for 5 min, followed by rabbit antibody against the capsid polypeptides of EV71 and PV1 and incubated overnight at 4 °C. After three washes in PBS, the sections were incubated with biotin-conjugated anti-rabbit IgG for 30 min at 37 °C, followed by streptavidin–peroxidase. The peroxidase activity was developed in diaminobenzidene with hydrogen peroxide. The nuclei were counterstained by using methyl green.

**Virus titration.** Tissue homogenates (10%, w/v) prepared in Eagle’s minimal essential medium containing antibiotics were clarified by centrifugation at 10,000 r.p.m. in a TMA-11 rotor (Tomy Seiko) for 20 min. Virus infectivity titres in supernatants were determined in Vero cells by using the microtitration assay described above.

**RESULTS**

**Differences in clinical manifestations after intraspinal and intravenous inoculation of EV71**

Initially, we compared the outcome of EV71 infection in cynomolgus monkeys after intraspinal inoculation (n = 2) and intravenous inoculation (n = 3) with the BrCr/tr strain (Fig. 1a). The two intraspinally inoculated monkeys died 3–5 days earlier, mainly due to flaccid paralysis. By contrast, the three intravenously inoculated monkeys exhibited extrapyramidal signs, including tremor and ataxia. Thus, intraspinally inoculated monkeys developed principally pyramidal signs. No skin lesions, exanthema or pulmonary oedema were observed.

**Fig. 1.** Time-course of clinical observations in monkeys after inoculation with EV71 BrCr/tr or PV1-OM1. (a) Monkeys inoculated with EV71 BrCr/tr. Two animals (4404 and 4406) were inoculated intraspinally (i.s.p.) and three animals (4410, 4411 and 4412) were inoculated intravenously (i.v.). (b) Monkeys inoculated intravenously with PV1-OM1. †, Animal died.

**Fig. 2.** Virus infectivity titres in tissues of monkeys inoculated intravenously with EV71 BrCr/tr (a) or PV1-OM1 (b). CC, Cervical cord; DG, dorsal root ganglion; LC, lumbar cord; LN, lymph node; TG, trigeminal ganglion.
Differences in clinical outcome after intravenous inoculation of EV71 and PV1

To demonstrate the differences in clinical manifestations of monkeys that were inoculated with EV71 BrCr/tr from those inoculated with PV1, we inoculated a virulent strain of PV1-OM1 into three monkeys by the intravenous route. Two of three monkeys developed flaccid paralysis starting in the left limb (contra-lateral side of virus inoculation) on days 6–9 p.i. (Fig. 1b). The remaining monkey showed no neurological signs. Thus, EV71 caused both pyramidal and extrapyramidal signs, whereas PV1 caused only pyramidal involvement.

Virus replication in the CNS of monkeys inoculated intravenously with EV71 or PV1

High viral titres were detected in the cerebral cortex, spinal cord and brainstem of monkeys inoculated with EV71 BrCr/tr (Fig. 2a). EV71 could not be isolated from the trigeminal and dorsal root ganglia, cervical lymph nodes or tonsils. By contrast, PV1 was isolated from the entire CNS, including trigeminal and dorsal root ganglia, as well as from non-CNS sites, including cervical lymph nodes and tonsils (Fig. 2b). Neither virus was isolated from the heart, liver, spleen, kidney, mesenteric lymph node, Peyer’s patch, sciatic nerve or bone marrow of EV71- and PV1-infected monkeys.

Localization of virus-infected lesions in the CNS

We plotted (i) the distribution of neurons that were positive for viral antigen; (ii) neuronal damage, such as degeneration, necrosis and neuronophagia; and (iii) inflammatory changes such as gliosis, vascular cuffing, neutrophilic infiltration and spongiosis in the parenchyma and subarachnoid infiltration (Fig. 3). Neurons that were susceptible to EV71 tended to localize in both the pyramidal and extrapyramidal systems, but those that were susceptible to PV1 were confined mainly to the pyramidal system.

Monkeys inoculated with EV71. Typical changes consisted of diffuse panencephalomyelitis (Fig. 4b, e and h), particularly in the cerebellar nuclei and pontine vestibular

Fig. 3. Distribution of virus-induced lesions in infected monkeys. Rows from top to bottom show the cerebrum and diencephalon, midbrain, pons and cerebellum, medulla oblongata and spinal cord, respectively. Results are shown for six monkeys, each inoculated intravenously with either EV71BrCr/tr (a) or PV1-OM1 (b). △, Virus antigen; ●, neuronal damage; grey areas, inflammation.
nuclei (Fig. 5b and e). In the spinal cord, not only were the motor neurons damaged, but also other neurons in the posterior horn and intermediate zone. However, some motor neurons remained undamaged (Fig. 6c and d). Viral antigen was detected in the neurons of the thalamus, pons and spinal cord of two monkeys (Fig. 3a). One surviving monkey (no. 4411) had no viral antigen-positive cells, but showed histological changes, such as neuronal loss and perivascular cuffing. In addition, we could not detect any extraneural lesions, such as pulmonary oedema, in any of the three monkeys.

Monkeys inoculated with PV1. Neuronal damage and loss with neuronophagia and granulocytic infiltration...
were observed principally in the pyramidal tract, particularly in the motor neuron of the spinal cord and motor cortices (Fig. 4c, f and j). The lumbar and sacral motor neurons were more damaged than the cervical and thoracic motor neurons (Fig. 6e and f). Viral antigen was detected in the motor neurons of the spinal cord and pontine nucleus neurons (Fig. 3b). In addition, some cells in the cerebellar dentate nuclei, red nuclei, thalamic nuclei and motor cortex were positive in one monkey.

Fig. 5. Cerebellar and brainstem changes in control (uninoculated) monkeys (a, d) and in monkeys inoculated intravenously with EV71 BrCr/tr (b, e) or PV1-OM1 (c, f). Prominent neuronal degeneration and necrosis (arrowheads) were observed in the dentate nuclei of monkey no. 4410, which was inoculated with EV71 (b), but not in monkey no. 3881, which was inoculated with PV1 (c). Similar neurological changes were also observed in the vestibular nuclei of pons of monkey no. 4410, which was inoculated with EV71 (e). Mononuclear cell infiltration (asterisks) was observed in these areas in monkey no. 4410, which was inoculated with EV71 (b and e). Sections were stained with H&E. Magnification, ×100.

Comparison of the lumbar spinal cord of monkeys inoculated with EV71 and PV1. To further clarify the differential neuropathogenicity of EV71 and PV1, we examined the same region of the lumbar cord. Monkeys inoculated with EV71 showed some damaged motor neurons in the anterior horn, but had remaining intact neurons (Fig. 6c and d), whereas monkeys inoculated with EV71 showed total loss or damage of motor neurons in the anterior horn (Fig. 6e and f).

Fig. 6. Histology of the lumbar part of the spinal cord of control (uninoculated) monkeys (a, b) and monkeys inoculated intravenously with (c, d) EV71 BrCr/tr (no. 4412) or (e, f) PV1-OM1 (no. 3881), stained by using the Klüver–Barrera method. Parts (b), (d) and (f) show higher magnifications of the boxes in the anterior horns of (a), (c) and (e), respectively. Normal motor neurons (b) (arrowheads) contained dark (Nissl) granules. In the monkey that was inoculated with EV71, some motor neurons (d) (arrowheads) lost these dark granules, particularly around the eccentrically localized nuclei (central chromatolysis), and other motor neurons disappeared with neuronophagia (asterisks), although a few intact neurons remained. In contrast, almost all motor neurons were destroyed or disappeared (asterisks) in the monkey that was inoculated with PV1 (e and f). Magnification, ×20 (a, c, e); ×400 (b, d, f).
Neurotropism of enterovirus 71 and poliovirus
DISCUSSION

In this study, we have demonstrated the susceptibility of cynomolgus monkeys to intravenous administration of PV1 and EV71 and the remarkable neuron-specific tropism of both viruses. However, histopathological and virological analyses of both PV1 and EV71 infections indicated distinct differences in the distribution and intensity of virus-induced lesions in the CNS. Monkeys inoculated with EV71 showed a widespread distribution of virus-induced lesions, not only in the pyramidal tract, but also in the extrapyramidal tract of the CNS. In contrast, it is well-known that poliovirus-related lesions are localized predominantly in the pyramidal tract, particularly in the motor neurons in the spinal anterior horn, which mimics the major CNS lesions in human paralytic poliomyelitis cases and has been confirmed in monkey experiments (Bodian & Howe, 1940). This CNS tropism can be explained by the expression pattern of the human poliovirus receptor (hPVR) in the CNS (Mendelsohn et al., 1989; Koike et al., 1990).

During recent EV71 epidemics in the Western Pacific region, children with mild brainstem encephalitis commonly showed extrapyramidal tract manifestations, such as tremor, ataxia and myoclonus (Chang et al., 1999; Chan et al., 2000; Liu et al., 2000; McMinn et al., 2001). The characteristic change in the monkeys that were inoculated intravenously with EV71 was panencephalitis with severe oedema and involvement of cerebellar nuclei. This major target of EV71 in the CNS was different from that of poliovirus and was consistent with its clinical manifestation by involving the cerebellar nuclei in all monkeys with EV71, whereas only one moribund monkey with PV1 showed involvement of these nuclei. Moreover, as shown in Fig. 6, the motor neurons in the anterior horn of the lumbosacral spinal cord were infected in both types of infection, but in monkeys that were inoculated with EV71, these cells were not damaged in a uniform pattern, as they were in monkeys that were infected with poliovirus. Total loss of motor neurons in the anterior horn was the cause of flaccid paralysis and partial loss might be associated with incomplete paralysis (paresis).

Studies using the transgenic murine cell lines L20B and Lz, which express the hPVR on the cell surface, support the replication of poliovirus and group A coxsackieviruses in culture (Pipkin et al., 1993; Hovi & Stenvik, 1994; Wood & Hull, 1999; Nadkarni & Deshpande, 2003). However, the prototype strain of EV71 and some other EV71 field isolates failed to replicate in L20B and Lz cells (data not shown). This suggests different receptor usage between polioviruses and EV71 and is compatible with our pathological analysis.

The intraspinal inoculation method is well-established for the qualified in vivo neurovirulence assay for live, attenuated poliovirus vaccine strains (WHO, 1990). After intraspinal inoculation, poliovirus is thought to spread from the initial inoculation site to other CNS tissues in a cell-to-cell fashion (Ponnuraj et al., 1998). Apparent differences in clinical manifestations and pathological changes in monkeys infected with EV71 by intraspinal and intravenous inoculations are, in part, explained by possible direct virus invasion in the inoculation site.

After initial replication, poliovirus may be transmitted to the CNS through the haematogenous pathway during viraemia or through the peripheral nerves. Except for some clinical and experimental evidence of provoked poliomyelitis, the former pathway is generally supported by efficient protection using neutralizing antibodies against poliomyelitis. The present intravenous model is similar to the viraemia that is observed in natural EV71 infections and this model is applicable to the evaluation of efficacy of preventative and therapeutic candidate vaccines and promising antivirals, in contrast to the intraspinal model.

During recent large HFMD epidemics, particularly in Malaysia and Taiwan, pulmonary oedema was one of the major clinical manifestations and was frequently observed in fatal brainstem encephalitis (Lum et al., 1998; Chang et al., 1998; Ho et al., 1999). However, although pulmonary oedema was rarely described in previous EV71 epidemics, >26% of patients with severe EV71 infection developed pulmonary oedema in the 1997 epidemics in Taiwan. In both intravenous and intraspinal inoculation models, no monkey developed pulmonary oedema in this or a previous study (Nagata et al., 2002). No EV71-induced involvement or viral antigen in the lungs was detected in monkeys or in the human post-mortem examinations. Similar pulmonary oedema and brainstem encephalitis were described in patients with bulbular poliomyelitis (Baker, 1949). In human cases with bulbular poliomyelitis, poliovirus involvement in the medullary ventral reticular formation of the medulla was considered to play an important role in the pathogenesis of pulmonary oedema (Baker, 1949; Chang et al., 1998; Lum et al., 1998). Apparent differences regarding pulmonary oedema in recent human cases with brainstem encephalitis and experimentally infected monkeys with brainstem involvement remain to be clarified.

Cluster A enteroviruses, including EV71 and CA16, are known to cause flaccid paralysis in suckling mice. However, this EV71-induced paralysis in suckling mice was caused primarily by skeletal muscle infection in our study (data not shown). In this respect, besides the monkey model presented here, no alternative in vivo method has so far been established for the identification and characterization of EV71-specific neurovirulence.

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