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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 $10^{5.5}$ $TCID_{50}$ EV71 and all developed neurological disease signs within 4–6 days of inoculation. However, after inoculation with $10^{5.5}$ $TCID_{50}$ 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 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 viraemia. 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 35·0 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 $TCID_{50}$. 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 ($TCID_{50}$) 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 ketaral and xylazine, 100 µl virus solution containing $10^{5·5}$ $TCID_{50}$ 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 ketaral. 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% $CaCl_2$ in PBS for 30 min and incubated in 1% hydrogen peroxide in methanol to block endogenous peroxidase activity. Thereafter, normal goat serum was

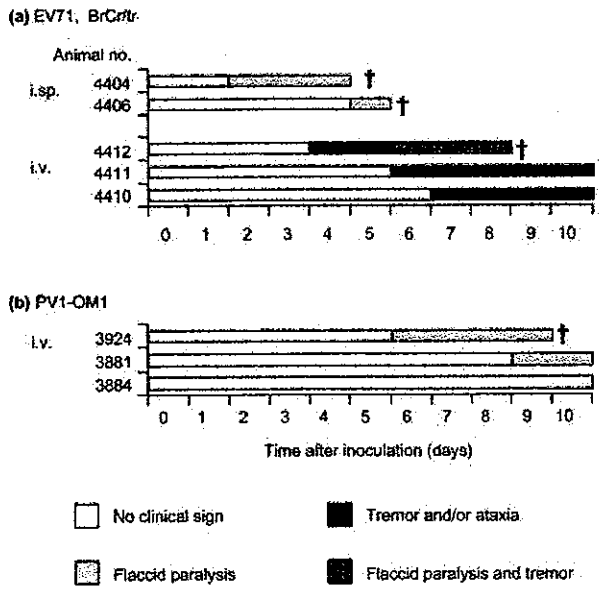


Fig. 1. Time-course of clinical observations in monkeys after inoculation with EV71 BrCr/tr or PV1-OM1. (a) Monkeys inoculated with EV71 BrCr/tr. Two animals (4404 and 4406) were inoculated intraspinally (i.sp.) and three animals (4410, 4411 and 4412) were inoculated intravenously (i.v.). (b) Monkeys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

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.

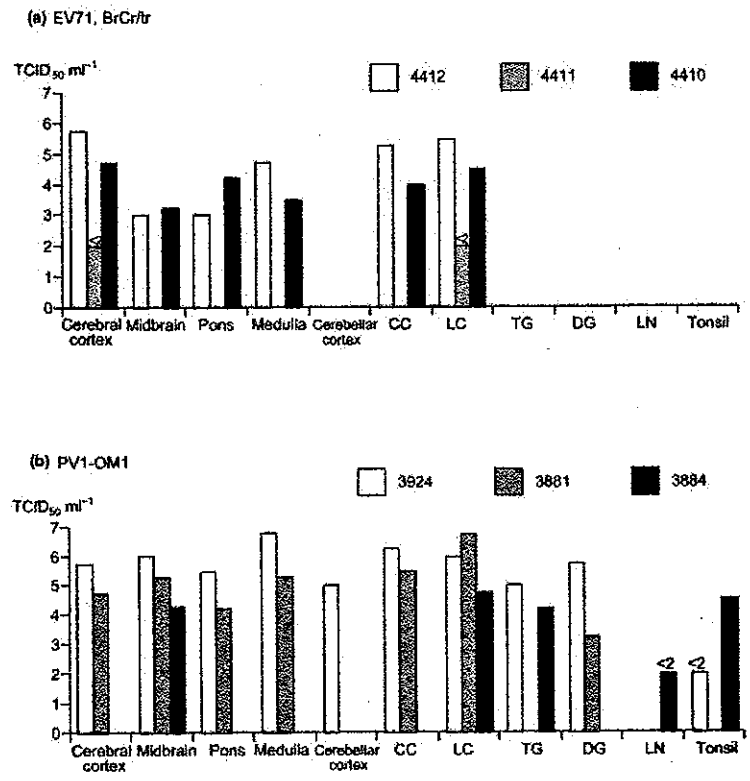


Fig. 2. Virus infectivity titres in tissues of monkeys inoculated intravenously with EV71 BrCr/tr (a) or PV1-OM1 (b). CC, Cervical cord; DG, dorsal root ganglion; LC, lumbar cord; LN, lymph node; TG, trigeminal ganglion.

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

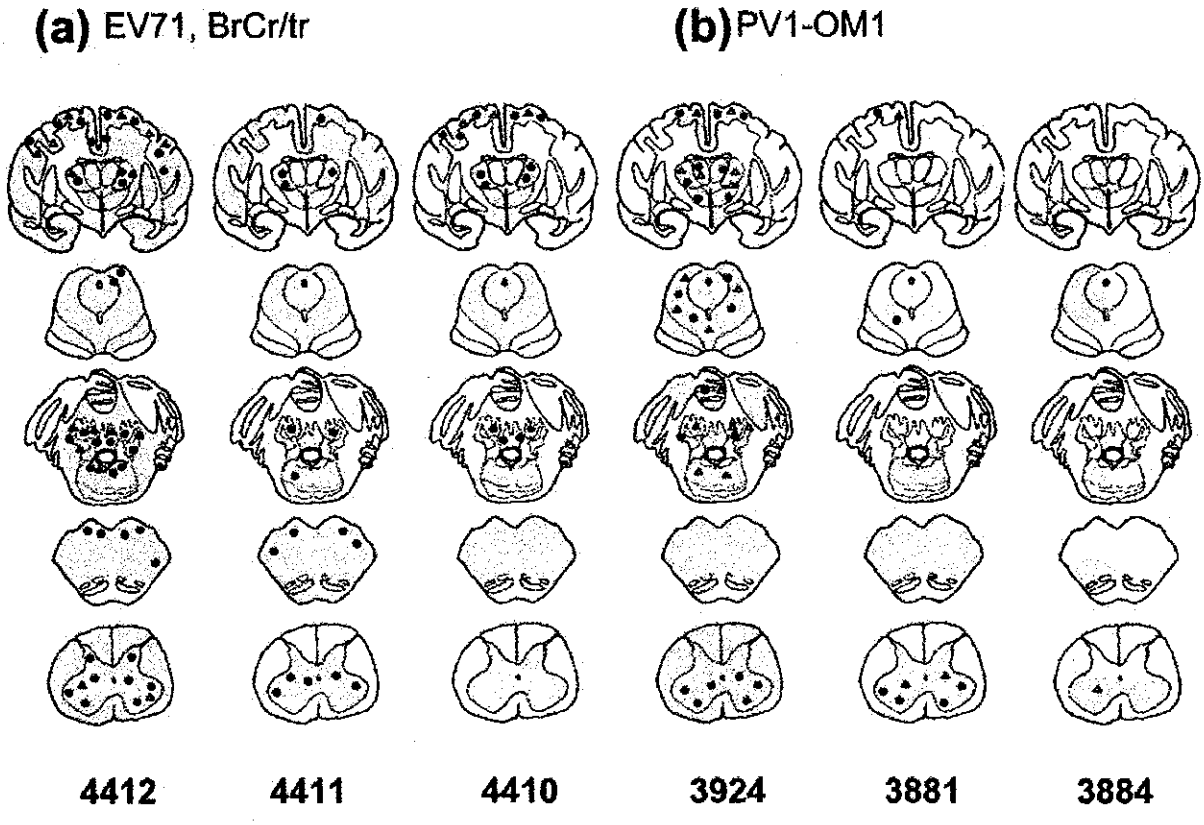


Fig. 3. Distribution of virus-induced lesions in infected monkeys. Rows from top to bottom show the cerebrum and diencephalon, midbrain, pons and cerebellum, medulla oblongata and spinal cord, respectively. Results are shown for six monkeys, each inoculated intravenously with either EV71BrCr/tr (a) or PV1-OM1 (b). △, Virus antigen; ●, neuronal damage; grey areas, inflammation.

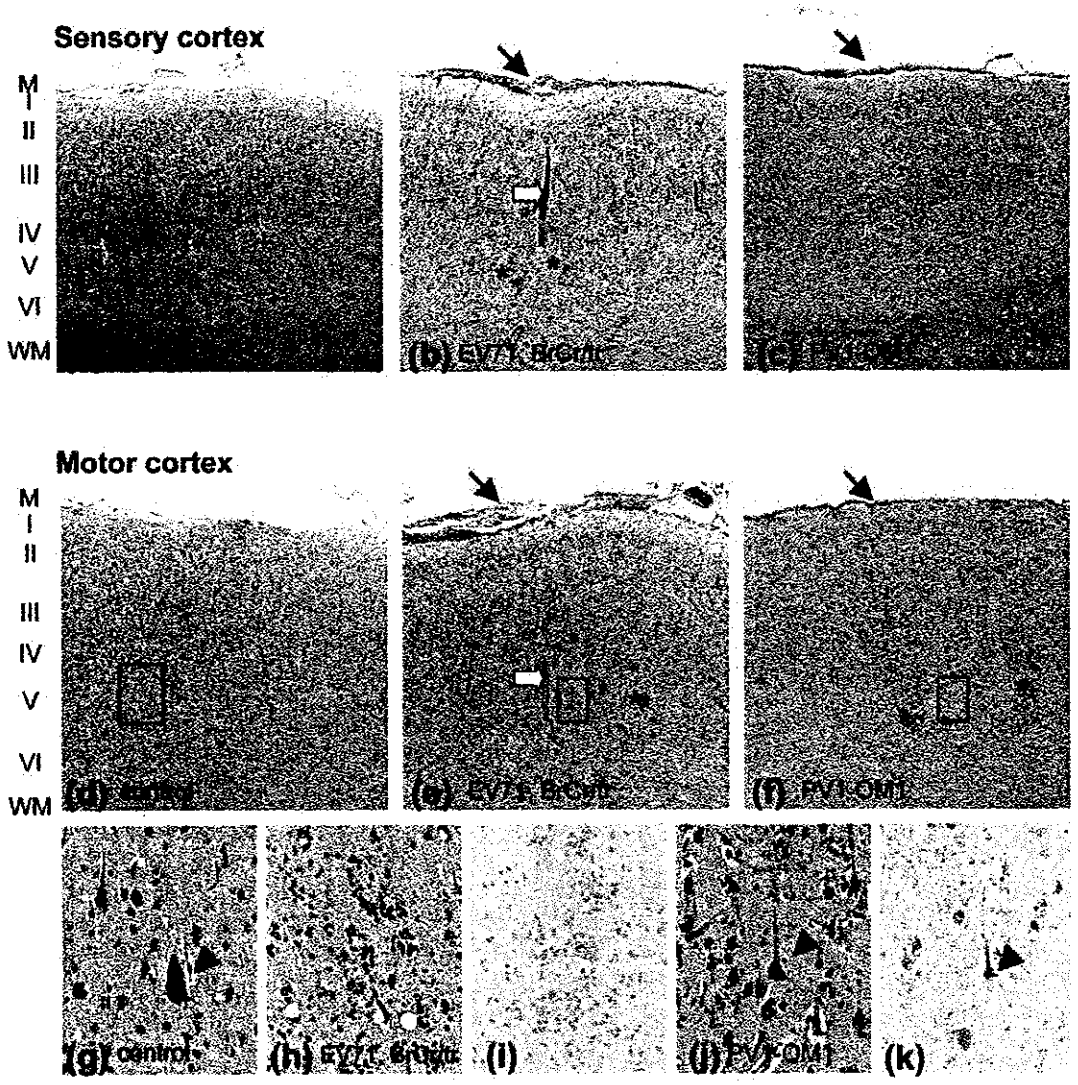


Fig. 4. Histology and immunohistochemistry of the sensory (a–c) and motor (d–f) cortices of control (uninoculated) monkeys (a, d, g) and monkeys inoculated intravenously with (b, e, h, i) EV71 BrCr/tr (no. 4412) or (c, f, j, k) PV1-OM1 (no. 3924). The boxes in parts (d–f) are shown at higher magnification in (g), (h and i) and (j and k), respectively. Perivascular cuffing (open arrows), neuronophagia (asterisks) and oedematous changes with subarachnoid infiltrates (arrows) were observed to be diffuse in the sensory and motor cortices (b and e). Neuronal degeneration and neuronophagia (asterisk) by neutrophils and mononuclear cells were seen at higher magnification (h) of the box in (e). EV71 antigen was detected in some neurons (i). Focal inflammatory changes were observed only in the motor cortex (f), but there was no change in the sensory cortex (c). Subarachnoid infiltration (arrows) was mild. Pyramidal cells (arrowheads) in the motor cortex became degenerative and neuronophagia (asterisks) was noted (j). Poliovirus antigen was detected in these neurons (k) (arrowheads). Parts (a–h) and (j) were stained with H&E; parts (i) and (k) were stained by immunohistochemistry. Magnification, ×40 (a–f); ×400 (g–k).

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

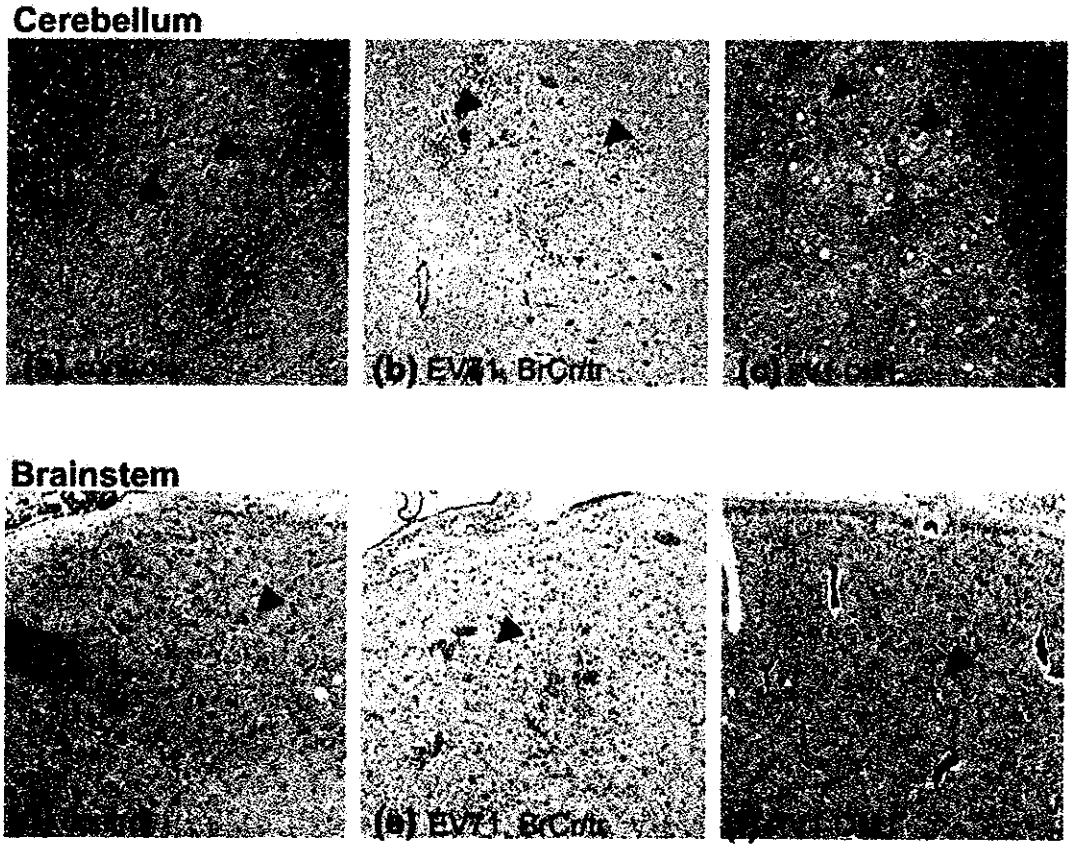
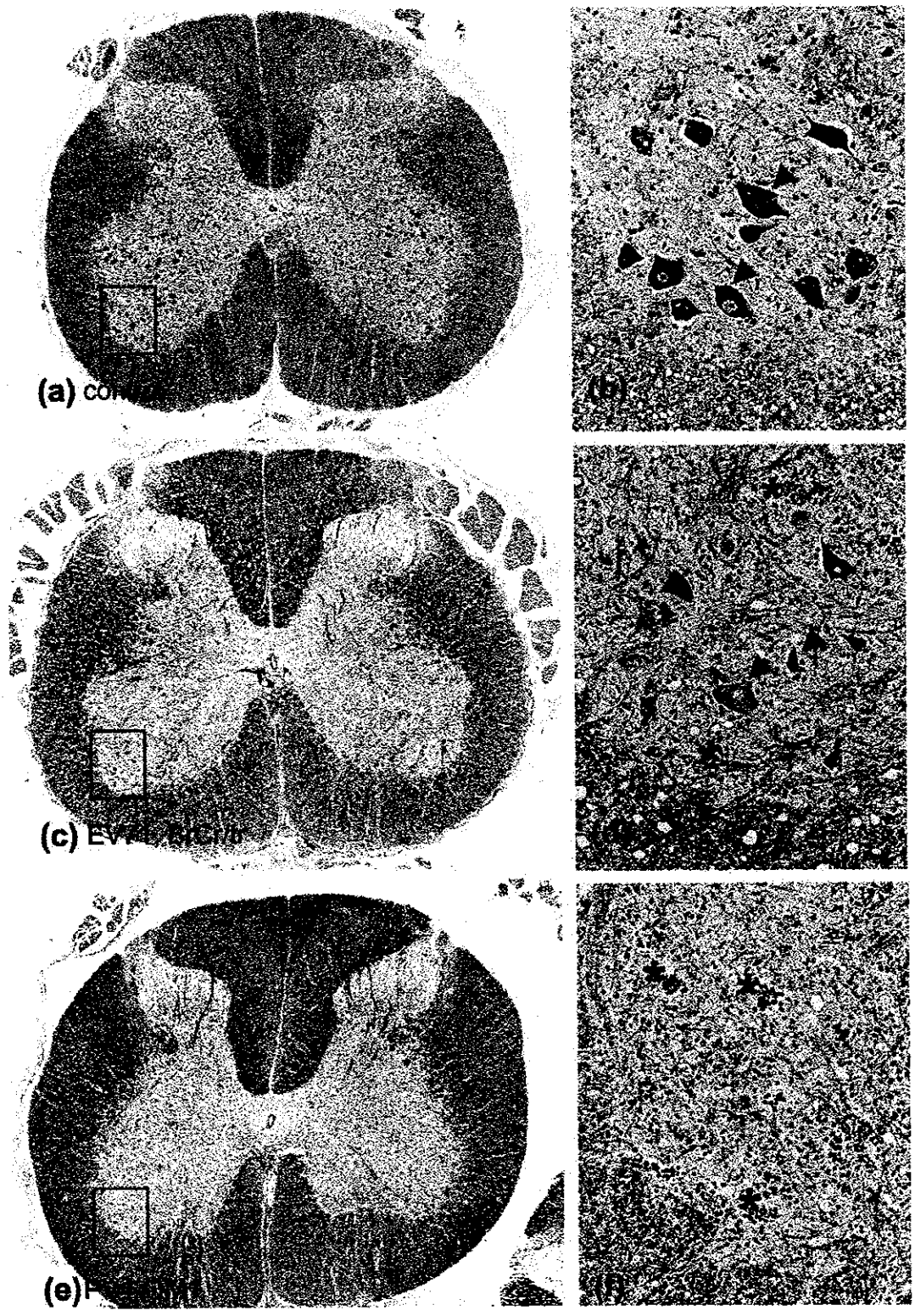


Fig. 5. Cerebellar and brainstem changes in control (uninoculated) monkeys (a, d) and in monkeys inoculated intravenously with EV71 BrCr/tr (b, e) or PV1-OM1 (c, f). Prominent neuronal degeneration and necrosis (arrowheads) were observed in the dentate nuclei of monkey no. 4410, which was inoculated 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.

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.

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 PV1 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).



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 lumbar 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 Lα, 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 Lα 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 bulbar poliomyelitis (Baker, 1949). In human cases with bulbar poliomyelitis, poliovirus involvement in the medial 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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Theme Papers

Circulating vaccine-derived polioviruses: current state of knowledge

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Abstract Within the past 4 years, poliomyelitis outbreaks associated with circulating vaccine-derived polioviruses (cVDPVs) have occurred in Hispaniola (2000–01), the Philippines (2001), and Madagascar (2001–02). Retrospective studies have also detected the circulation of endemic cVDPV in Egypt (1988–93) and the likely localized spread of oral poliovirus vaccine (OPV)-derived virus in Belarus (1965–66). Gaps in OPV coverage and the previous eradication of the corresponding serotype of indigenous wild poliovirus were the critical risk factors for all cVDPV outbreaks. The cVDPV outbreaks were stopped by mass immunization campaigns using OPV. To increase sensitivity for detecting vaccine-derived polioviruses (VDPVs), in 2001 the Global Polio Laboratory Network implemented additional testing requirements for all poliovirus isolates under investigation. This approach quickly led to the recognition of the Philippines and Madagascar cVDPV outbreaks, but of no other current outbreaks. The potential risk of cVDPV emergence has increased dramatically in recent years as wild poliovirus circulation has ceased in most of the world. The risk appears highest for the type 2 OPV strain because of its greater tendency to spread to contacts. The emergence of cVDPVs underscores the critical importance of eliminating the last pockets of wild poliovirus circulation, maintaining universally high levels of polio vaccine coverage, stopping OPV use as soon as it is safely possible to do so, and continuing sensitive poliovirus surveillance into the foreseeable future. Particular attention must be given to areas where the risks for wild poliovirus circulation have been highest, and where the highest rates of polio vaccine coverage must be maintained to suppress cVDPV emergence.

Keywords Poliovirus/genetics/isolation and purification; Poliovirus vaccine, Oral/adverse effects; Poliomyelitis/etiology/chemically induced/prevention and control; Immunization programs; Disease outbreaks/Review literature (*source: MeSH, NLM*).

Mots clés Poliovirus humain/génétique /croissance et développement; Vaccin antipoliomyélitique Sabin/effets indésirables; Poliomyélite antérieure aiguë/étiologie/induite chimiquement/prévention et contrôle; Programmes de vaccination; Revue de la littérature (*source: MeSH, INSERM*).

Palabras clave Poliovirus/genética/crecimiento y desarrollo; Vacuna antipolio oral/efectos adversos; Poliomielitis/etiología/inducida químicamente/prevención y *control*; Programas de inmunización; Literatura de revisión (*fuente: DeCS, BIREME*).

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Voir page 21 le résumé en français. En la página 21 figura un resumen en español.

Introduction

The oral poliovirus vaccine (OPV) of Albert Sabin is nearly ideal for use in polio eradication (*1–3*). OPV is easily administered by mouth, facilitating its widespread use; it induces intestinal immunity, making recent OPV recipients resistant to infection by wild polioviruses and effectively blocking wild poliovirus transmission when used in mass campaigns; and it provides long-term protection against polio through durable humoral immunity. OPV virus can spread to and immunize unvaccinated contacts of vaccine recipients, increasing the impact of OPV beyond those actually immunized. Through effective use of this excellent vaccine, the WHO Global Polio Eradication Initiative has nearly achieved its goal of eradicating wild polioviruses (*4, 5*).

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Despite its many advantages, OPV use carries certain liabilities (*3, 6*). Genetic stability was a prime concern during OPV development, and a delicate balance was struck between attenuation of neurovirulence, immunogenicity in humans, and genetic stability (*7, 8*). The first evidence of the clinical consequences of the genetic lability of OPV was the appearance of cases of vaccine-associated paralytic poliomyelitis (VAPP) soon after licensure and widespread use of OPV (*1*). The much higher incidence of polio from wild poliovirus infections at the time, however, mitigated concern over the rare occurrence of VAPP (*1*), and it has only been in recent years that VAPP has become an increasingly significant proportion of the global polio burden (*1*). Occasionally, immunodeficient persons exposed to OPV become chronically infected (*9–11*), excreting derivatives of the OPV strains for many months or years (*1, 12–14*). Chronic OPV excretors, however, seem to be very rare, and have so far been found only in upper- and middle-income countries where appropriate clinical management of immunodeficiency is available (*15*).

In retrospect, it is remarkable that OPV has attained such an outstanding record of safety and efficacy over the four decades of worldwide use (*3, 6*). It is now known that most RNA viruses have highly mutable genomes that are potentially capable of very rapid evolution, many orders of magnitude faster than the genomes of DNA viruses or cellular organisms (*16, 17*), and polioviruses are among the most rapidly evolving of all RNA viruses (*1, 12, 14, 18, 19*). Moreover, the attenuating mutations of the OPV strains are strongly selected against when the vaccine replicates in the intestinal tract of OPV recipients (*20–22*). To counter the daunting challenges of delivering a live, attenuated RNA virus vaccine via its natural route of infection, immunization strategies were developed to minimize adverse events (*1, 23*). In developed countries, OPV was first delivered in mass campaigns to achieve high rates of coverage, and this was followed by a strategy of comprehensive routine immunization. Similar strategies were adopted in developing countries, with mass OPV campaigns often playing a more prominent role than routine immunization. In most instances, OPV was delivered in the context of pre-existing high population immunity to poliovirus, because of recent exposure to circulating wild polioviruses or, as with developed countries in the early 1960s, from the combination of immunity acquired from natural infection and immunity acquired from several years of immunization with the inactivated poliovirus vaccine (IPV). These strategies probably minimized the epidemiological consequences of the frequent phenotypic reversion of the OPV strains.

Recent years have seen a rapidly changing risk profile from OPV exposure. In most of the world, population immunity to poliovirus is maintained only by immunization. Where polio vaccine coverage rates decline but OPV use continues, conditions may arise that increase the likelihood of person-to-person spread of vaccine-derived polioviruses (VDPVs). The duration and extent of spread are dependent on the magnitude of the immunity gap and the intensity of other risk factors favouring poliovirus circulation. This long-discussed hypothetical concern (*6*) has been realized by the recent occurrence of outbreaks of paralytic polio associated with circulating VDPVs (cVDPVs). Although several important themes are common to all of the outbreaks, each outbreak has taught its own important lesson about the parameters for the safe administration of OPV in a world free of circulating wild polioviruses.

Recent cVDPV outbreaks

Hispaniola, 2000–01

The immediate public health importance of cVDPVs was underscored by the occurrence of a polio outbreak associated with type 1 cVDPVs on the Caribbean island of Hispaniola in 2000–01 (Fig. 1) (*24*). The first indication of an outbreak was the isolation of poliovirus type 1 in the summer of 2000 from two patients with acute flaccid paralysis (AFP) in the Dominican Republic and Haiti. Because the indigenous wild poliovirus type 1 had been eradicated by the late 1980s, imported wild poliovirus was suspected. However, molecular characterization of the two case isolates, comparing sequences encoding the major capsid surface protein VP1 (~900 nucleotides), showed that they were unrelated to wild type 1 polioviruses previously endemic to Hispaniola or to any wild poliovirus currently found in other parts of the world (*24*). Instead, the Haitian and Dominican isolates were closely related (~ 97.7% VP1 sequence identity) to the Sabin type 1 OPV strain, and to each other (98.0% VP1 sequence identity). The degree of VP1 sequence similarity to the OPV strain was substantially lower than is normally observed (>99.5%) in isolates from cases of AFP or VAPP.

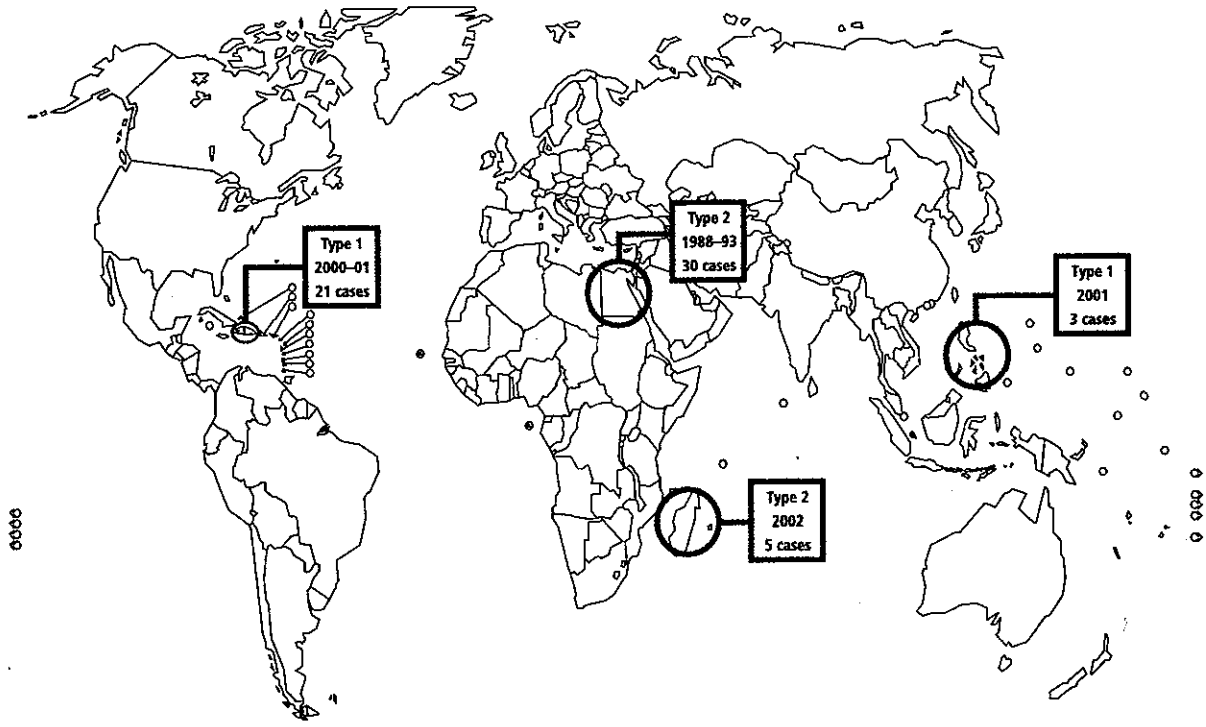
Active search for AFP cases detected a total of 21 confirmed polio cases (13 in the Dominican Republic in 2000, and eight in Haiti in 2000–01). It was possible to reconstruct the patterns of cVDPV transmission from the sequence properties of the isolates because of the rapid, stepwise evolution of poliovirus genomes (about 1% nucleotide substitutions per site per year). Relationships among VP1 sequences of the 31 type 1 VDPV outbreak isolates suggested that they were derived from an OPV dose given in late 1998 or early 1999 (*24*). The close sequence relationships among isolates from the Dominican Republic indicated that the outbreak there began with the importation of cVDPV from Haiti in the spring of 2000. By contrast, the Haitian isolates were more diverse, and appeared to have diverged into at least four separate lineages in 1999.

Circulation of VDPV occurred in an environment of low OPV coverage throughout Haiti (<30% nationwide, and as low as 7% in some areas) and in the affected communities of the Dominican Republic (20–30%). All but one of the patients were either unvaccinated children or incompletely vaccinated children. No mass OPV immunization campaigns in the form of national immunization days (NIDs) had been conducted in either country within the past 5 years. The outbreak stopped in both countries after mass administration of OPV in NIDs (*24*).

Philippines, 2001

The Hispaniola outbreak showed conclusively that low OPV coverage carried a risk of cVDPV emergence and prompted a reassessment by WHO of the strategies both for polio immunization and poliovirus surveillance. In 2001, the Global Polio Laboratory Network implemented additional testing requirements for all polioviruses under investigation, in order to increase sensitivity for detecting VDPVs (*25*). Soon thereafter, cVDPV was detected in the Philippines (*26, 27*). Specimens from three cases of AFP, reported during March to July 2001, tested positive for type 1 cVDPV. The isolates were closely related to the Sabin 1 OPV strain (~97% VP1 sequence identity), but even more so to each other (>99% VP1 sequence identity). The VP1 sequence relationships among the isolates suggested that the VDPV circulation began with an OPV dose given in 1998. Although the three cases occurred in separate communities (two

Fig. 1. **Location of the four polio outbreaks associated with circulating vaccine-derived polioviruses (cVDPVs).** Shown are the serotypes of the cVDPV isolates, the years of cVDPV virus isolation, and the number of reported polio cases associated with cVDPVs



Note: In each of these areas, the spread of cVDPVs followed the elimination of the corresponding serotype of indigenous wild poliovirus, but with the continued introduction of oral poliovirus vaccine into communities with growing immunity gaps.
All of the cVDPV outbreaks were detected first by the laboratory, using sequence data and evolutionary analyses.

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in Luzon, one in Mindanao), the close sequence similarities among the cVDPV isolates suggested that the virus had spread via a single, minimally branched chain, in contrast to the pattern of multichain transmission found in Hispaniola.

Wild poliovirus was last reported in the Philippines in 1993 (*26*), and no national immuniztion days (NIDs) had been conducted since 1997, although subnational campaigns had been conducted in 1998 and 1999 outside the affected areas. Nationwide routine OPV coverage had been approximately 80% during much of the 1990s; however, shortages of OPV in the 2 years before the appearance of cases probably led to gaps in coverage, particularly in slum areas (*26*). An important new lesson from the Philippines cases was that transient immunity gaps in very densely populated areas with poor hygiene/sanitation and tropical climates may permit cVDPV emergence.

Madagascar, 2001–02

Five cases of AFP associated with type 2 cVDPV were reported from two different communities in the southern province of Madagascar (*28*). The first case (onset in October 2001) was from the urban district of Toliara, whereas the remaining four cases (onsets in March to April 2002) were clustered in a rural village ~400 km from Toliara. None of the patients had been fully immunized against polio. The type 2 polioviruses isolated from patients and contacts from the two areas represented two geographically separate, genetically distinct, independent cVDPV lineages (*28*). The urban isolates differed from the Sabin 2 OPV strain at ~1% of VP1 nucleotides, whereas the rural isolates differed from Sabin 2 at ~2.5% of VP1 nucleotides.

OPV coverage was <50% nationwide in Madagascar, and wild poliovirus was last reported in 1997 (*28*). The detection of two distinct VDPV lineages in Madagascar underscored the point that cVDPVs can emerge independently in localities where gaps in polio immunity arise.

Evidence of past circulation of VDPVs

Egypt, 1983–93

From 1988 to 1993, 30 cases of polio associated with type 2 cVDPV were found in seven governorates of Egypt. The cases occurred at a time of low OPV coverage and after the apparent eradication of the type 2 wild poliovirus indigenous to Egypt (last known isolate, 1979) (*29*). The type 2 isolates were initially thought to be wild polioviruses, but recent molecular studies have shown them to be cVDPVs (*29*). The sequence properties of the isolates suggested that VDPV circulation in Egypt started with an OPV dose given in 1983, and that progeny from the initiating infection circulated for approximately a decade within Egypt. Like wild polioviruses, the type 2 cVDPVs established independent reservoirs of endemicity within the country. VDPV circulation ceased with rising OPV coverage, and VDPVs were last detected in Egypt in 1993.

The important lesson learned from the situation in Egypt was that cVDPVs can circulate indefinitely in countries with persistently low rates of polio vaccine coverage.

Poland, 1968

In 1968, a large polio outbreak in Poland immediately followed a field trial of an experimental type 3 OPV strain, USOL-D-bac (*30*). The environment in which the outbreak occurred was one of low population immunity to poliovirus type 3, because the Sabin type 3 component was never included in OPV and the replacement type 3 IPV failed to induce high levels of immunity (*30*). Although the epidemiological findings implicated a breach in quarantine of USOL-D-bac recipients during the field trial (*30*), conclusive evidence for the vaccine origin of the outbreak came from retrospective oligonucleotide fingerprinting (*31*) and nucleotide sequencing (*32, 33*) studies of outbreak isolates.

The outbreak in Poland is of current importance because it shows that vaccine virus progeny can circulate widely in developed countries with temperate climates and moderate population densities if immunity to at least one poliovirus serotype is low. Although the implicated vaccine virus was genetically distinct from Sabin 3 (*32, 33*), the outbreak in Poland is the only one known to be associated with any type 3 vaccine strain.

Other possible examples of VDPV circulation

A recent retrospective study found evidence for the circulation of type 2 vaccine-related virus in Belarus following local cessation of OPV use from 1963 to 1966 (*34*). In the months after the limited reintroduction of OPV in 1965, type 2 vaccine-related poliovirus was isolated from nine healthy unvaccinated children. The sequence properties of three of the four available isolates showed evidence of prolonged vaccine virus replication (6–9 months) (*34*). In Romania in 1980, a type 1 VDPV was isolated from a patient with "community acquired" VAPP (*35*). The patient was immunocompetent, but lived in a community with low rates of OPV coverage and poor hygiene/sanitation. In the Russian Federation in 1999, an immunocompetent 7-month-old orphanage child contracted VAPP associated with a type 1 VDPV (*36*). These findings reinforce the point that uniformly high rates of polio vaccine coverage are necessary to prevent the emergence of cVDPVs in industrialized countries that continue to use OPV.

Other current (*25, 37*) and retrospective (unpublished data) studies have found single VDPV isolates (all type 2) with genetic properties similar to those of the well-documented cVDPV outbreak isolates in other tropical developing countries in communities where the rates of OPV coverage were low.

Risk factors for cVDPV emergence and spread

The most significant risk factor for cVDPV outbreaks, like wild poliovirus outbreaks, is insufficient population immunity. The risk is also a function of other factors favouring poliovirus circulation: the number and density of non-immune susceptible persons, the birth rate, deficiencies in hygiene/sanitation, and the seasonal duration of tropical conditions (*38*). The previous elimination of indigenous wild poliovirus circulation increases the risk because non-immune susceptibles will accumulate rapidly in the absence of high rates of polio vaccine coverage and naturally acquired immunity. The cVDPV outbreaks are similar to outbreaks from imported wild poliovirus, except that the outbreak agents emerge endogenously. Virus excreted by OPV recipients may frequently recover the capacity for spread beyond immediate contacts, but spread is normally limited by population immunity. Outbreaks occur when the density of non-immune susceptibles rises to the point where the chains of cVDPV transmission can propagate (*6, 38*). The threshold point (per cent susceptible) for sustained person-to-person transmission of cVDPVs is probably lowest for type 2, and perhaps highest for type 3. The size of a cVDPV outbreak is a function of the size of the non-immune population and the potential for transport of outbreak virus to susceptible communities elsewhere. Countries that were (or are) major reservoirs for wild poliovirus circulation, and where the potential for person-to-person poliovirus transmission is greatest, are at particularly high risk for cVDPV emergence, and maintenance of high rates of polio vaccine coverage in these settings is essential.

Insensitive surveillance is an additional risk factor for the spread of cVDPV. Apart from the outbreaks in the Philippines and Poland, VDPV circulation occurred in areas with very low rates of AFP case reporting. VDPV circulation in Hispaniola, Madagascar, and the Philippines apparently began 2–3 years before the first cases were detected. The genetic diversity of the cVDPV isolates from Egypt and Haiti suggested that the large majority of polio cases went unreported (*24, 29*), and the case counts probably seriously underestimate the impact of VDPV circulation in the two countries.

Properties of known cVDPV isolates

The most important biological properties of cVDPV isolates are their increased capacity to cause paralytic disease in people and their capacity for sustained person-to-person transmission. When tested experimentally, cVDPV isolates have been found to be as neurovirulent as wild polioviruses for transgenic mice expressing the human receptor for poliovirus (*24, 27, 29*). Like wild polioviruses, cVDPV isolates have been shown to replicate to high titres in cell culture at supraoptimal temperatures (*24, 29*). All cVDPV isolates characterized so far have antigenic properties that more closely resemble wild polioviruses than the original Sabin strains (*24, 29*). The antigenic differences from the Sabin strains are less pronounced for type 2 than for type 1 cVDPVs, possibly because selection against the Sabin 2 antigenic sites is less intense (*39*). Although these experimentally determined properties may correlate with clinically relevant properties, they are not unique to cVDPV isolates, as less highly evolved (OPV-like) vaccine-related polioviruses isolated from healthy individuals and VAPP patients may share some or all of these traits (*20, 22, 39*).

The extensive sequence divergence from the respective OPV strain is a distinguishing feature of VDPVs (*12–14, 27–29*). A vaccine-related isolate is considered a VDPV if it has diverged by ≥1% of VP1 nucleotides from the reference OPV strain (*25, 37*). The demarcation of 1% VP1 divergence implies that replication of vaccine virus had occurred for ~1 year. It does not imply that isolates having <1% divergence would lack the capacity for person-to-person transmission in poorly immunized populations, as it is likely that the critical attenuating mutations of the Sabin strains generally revert well before nucleotide substitutions accumulate to the level of 1% (*20–22*). By this definition, nearly all minimally diverged "OPV-like" isolates would be excluded, and VDPVs that had replicated for at least 1 year would be included (*25, 37*).

All cVDPVs, but none of the iVDPVs (VDPVs isolated from immunodeficient chronic poliovirus excretors) described thus far appear to be recombinants with enteroviruses closely related to polioviruses (*24, 27–29*). The possible role of

recombination in the phenotypic reversion of OPV is unclear. Recombination with other enteroviruses appears to be an indicator of circulation, as the cVDPVs in Hispaniola and Egypt had participated in successive rounds of recombination during the outbreaks (*24, 29*), as frequently occurs during the circulation of wild polioviruses (*40, 41*).

Laboratory surveillance for VDPVs

The occurrence of cVDPV cases also highlights the need for countries to maintain sensitive poliovirus surveillance into the foreseeable future. Laboratory-based surveillance for VDPVs began in 1997 in the Americas, with the sequencing of the VP1 genes of poliovirus isolates from AFP cases in the region (*42*). Unfortunately, this approach did not provide an early warning of the Hispaniola cVDPV outbreak because no polioviruses were isolated in the 5 years preceding the outbreak (*24*). Following that outbreak, intensive screening of recent poliovirus isolates for cVDPVs was initiated by laboratories within the entire WHO Global Polio Laboratory Network (*25, 37*). Since 2001, all vaccine-related poliovirus isolates from AFP cases have been screened for evidence of prolonged replication or circulation (*25, 37*). Poliovirus isolates are identified according to their genetic properties by probe hybridization (*43*), diagnostic polymerase chain reaction (PCR) assays (*44, 45*), or PCR-restriction fragment polymorphism analysis (*46*). All isolates are also tested for antigenic change by using specific cross-absorbed sera in an enzyme-linked immunosorbent assay (ELISA) format (*47*) or panels of monoclonal antibodies in neutralization tests (*47*). Alternatively, isolates have been screened for recombinant sequences using PCR primers targeting non-capsid region sequences characteristic for each Sabin strain (D.R. Kilpatrick, unpublished observations). Any isolate having "non-vaccine-like", "double-reactive", or "non-reactive" antigenic properties or having a recombinant genome is further characterized by VP1 sequencing (*25, 37*). WHO is notified of any current isolates having ≥1% VP1 divergence, and both the case and the associated isolate are investigated further.

To date, over 7300 vaccine-related isolates from 1999–2003 AFP cases from all WHO regions have been screened for VDPVs (*25, 37*). The large majority (>95%) of isolates had "vaccine-like" antigenic properties and were usually not investigated further. Of the remainder, 44 were cVDPVs (all from the three recent outbreaks), 3 were iVDPVs isolated from immunodeficient chronic poliovirus excretors, 11 were uncategorized VDPVs, and 125 were antigenic variants of OPV-like virus (*25, 37*). A subset (1980) of the vaccine-related isolates was screened for the presence of recombinant non-capsid sequences of non-Sabin origin. A small proportion (<1%) of the isolates had non-Sabin sequences, and only one of these was an uncategorized VDPV. None of the other VDPVs were associated with more than one patient.

The potentially higher risk of type 2 cVDPV

Several observations indicate that the risk for emergence cVDPVs may be highest for poliovirus type 2 (*6*). The type 2 OPV strain appears to spread most readily to unimmunized people, as shown by its more frequent association with contact cases of VAPP (*1*) and by the much higher seroprevalence to poliovirus type 2 (relative to types 1 and 3) found among unvaccinated individuals in the United States and Europe (*1, 6*). Moreover, VP1 sequence comparisons (described above) found that vaccine-related isolates with the more divergent genomes (>0.5%) were most frequently type 2 (*42*). Limited, localized spread of type 2 vaccine-related virus may occur in some areas. Because paralytic attack rates for type 2 poliovirus infections are low (*38*), circulation of type 2 VDPVs is the most difficult to detect by AFP surveillance. Consequently, early detection of any future type 2 cVDPV outbreaks will require maintenance, and possible augmentation in some countries, of the current very high global standard for AFP and poliovirus surveillance (*4, 25, 37, 48*).

A changing risk profile for cVDPVs

Currently, the major risk for polio worldwide is from wild poliovirus infection (*4*). Most of this risk is localized to a few reservoir areas in Africa and Asia. In the rest of the world, the chief risk derives from continued use of OPV. It has been estimated that the global VAPP burden is 250–500 cases annually (*1, 49*), and most VAPP cases occur outside of the remaining reservoir areas. About half of all VAPP cases are associated with the type 2 OPV strain (*1*), whose wild counterparts were eradicated in 1999 (*4, 25, 37, 48*). It is likely that all polio cases will soon be associated with OPV use, causing the risk-benefit ratio for continued OPV use to shift dramatically.

Considerations for transition away from OPV use have focused largely on VAPP, for which the risk is quantifiable and the cases dispersed (*49*). More difficult to assess is the risk of outbreaks from cVDPVs. Here the potential risk may be very high. Fortunately, the recent cVDPV outbreaks were restricted to islands. However, if a cVDPV outbreak were to occur in a populous mainland country, the case burden could far outstrip that from VAPP, and could be of the scale of the past wild poliovirus outbreaks. Experience has shown that it becomes increasingly difficult to maintain high levels of polio vaccine coverage in countries and regions that have been certified as polio free. Thus, it is unlikely that the high rates of OPV coverage necessary to suppress cVDPV emergence in areas at greatest risk can be sustained much past global certification. Because OPV is the vaccine of choice for eradicating wild poliovirus, especially in tropical developing countries, its continued use in those settings is recommended until wild poliovirus circulation ceases (*48, 49*). Thereafter, all OPV use should be discontinued as soon as is safely possible (*50*).

Implications of cVDPVs to the "endgame strategy" for global polio eradication

Currently, the most urgent priority is to eliminate the remaining reservoirs of wild poliovirus endemicity (*4, 5, 48, 50*). In the remaining polio-endemic countries, the mass immunization campaigns currently under way to eliminate the last pockets of poliovirus circulation will also effectively prevent dissemination of cVDPVs. In polio-free areas with inadequate rates of routine OPV coverage, it is crucial to close the immunity gap. To achieve this, WHO has recommended maintenance or reinstatement of mass immunization campaigns in such areas (*48*). The appropriate frequency of the mass campaigns follows from the rate of accumulation of non-immune susceptibles in the highest risk populations of each area (*6*).

Recognition of the risks posed by cVDPVs and other VDPVs has prompted a reassessment of global strategies for maintaining polio-free status after wild poliovirus circulation has ceased (*2, 3, 48, 50*). The number of viable options for the "endgame strategy" using the existing polio vaccines now appear to be quite limited (*2, 3, 48*). WHO must develop a comprehensive strategy for the prompt cessation of OPV use as soon as

possible after global certification (*50*). Cessation of OPV use should be closely coordinated by WHO, as uncoordinated discontinuation by countries is likely to create unacceptable risks for emergence of cVDPVs. Synchronous cessation of OPV use immediately after coordinated mass OPV campaigns (in countries that had continued to use OPV) would maximize global immunity to polio at the time of OPV cessation (*3, 48*). Transition to IPV should be encouraged at the present time in developed countries in temperate zones where IPV efficacy is known to be high and where high rates of IPV coverage can be maintained through routine immunization (*48, 49*). The use of IPV in tropical developing countries presents special challenges because the rates of routine immunization are often inadequate, IPV efficacy is uncertain, and logistical and financial challenges persist (*49*). This option requires further, careful analysis (*50*). Stockpiles of polio vaccine must be established at strategic sites to enable a rapid response to the detection of any poliovirus infection in the post-OPV era (*3, 48*). Finally, sensitive field and laboratory surveillance must be maintained until there is compelling evidence that the risk of any poliovirus re-emergence is negligible (*3, 48, 50*). ■

Conflicts of interest: none declared.

Résumé

Poliovirus circulants dérivés de souches vaccinales : état des connaissances

Au cours des quatre dernières années, on a observé des flambées de poliomyélite associées aux poliovirus circulants dérivés de souches vaccinales (PcDSV) sur l'île d'Haïti (2000-2001), aux Philippines (2001) et à Madagascar (2001-2002). Des études rétrospectives ont également décelé la circulation d'un PcDSV endémique en Egypte (1988-993) et une propagation localisée probable d'un virus dérivé du vaccin antipoliomyélitique buccal (VPO) en Biélorussie (1965-1966). Pour toutes les flambées dues à des PcDSV, la couverture insuffisante par le VPO et l'éradication préalable du poliovirus sauvage autochtone étaient les principaux facteurs de risque. Des campagnes de vaccination de masse par le VPO ont interrompu ces flambées. Pour améliorer la sensibilité de la détection des PcDSV, le réseau mondial de laboratoires pour la poliomyélite requiert désormais des tests supplémentaires pour tous les poliovirus isolés. Cette démarche a permis de reconnaître rapidement les flambées des Philippines et de Madagascar, mais il n'y en a pas d'autres actuellement. L'interruption de la circulation du poliovirus sauvage dans la plupart des régions du monde ces dernières années a entraîné une augmentation spectaculaire du risque potentiel d'émergence de PcDSV. Ce risque semble le plus élevé avec la souche de type 2 du VPO, en raison de sa plus forte propension à se propager aux sujets contacts. L'émergence de PcDSV souligne bien l'importance cruciale d'éliminer le poliovirus sauvage dans les dernières poches où il circule, de maintenir la couverture universelle de la vaccination, d'arrêter d'administrer le VPO dès que tout risque est écarté et d'assurer une surveillance suffisamment sensible des poliovirus dans un proche avenir. Les régions où le risque de circulation du poliovirus sauvage a été le plus élevé et où les couvertures vaccinales doivent être les plus étendues pour supprimer l'émergence de PcDSV doivent tout spécialement retenir l'attention.

Resumen

Poliovirus circulantes de origen vacunal: estado actual de los conocimientos

En los últimos cuatro años se han declarado brotes de poliomielitis asociados a poliovirus de origen vacunal circulantes (PVOVc) en La Española (2000-2001), Filipinas (2001) y Madagascar (2001-2002). Estudios retrospectivos han detectado también la circulación de PVOVc endémicos en Egipto (1988-1993) y la probable propagación localizada del virus derivado de la vacuna antipoliomielítica oral (OPV) en Bielorrusia (1965-1966). Las lagunas existentes en la cobertura de OPV y la previa erradicación del serotipo correspondiente al poliovirus salvaje autóctono fueron los factores de riesgo críticos de todos los brotes de PVOVc. Éstos fueron atajados mediante campañas de inmunización masiva con OPV. A fin de aumentar la sensibilidad de detección de los PVOVc, en 2001 la Red Mundial de Laboratorios para la Poliomielitis implantó nuevos requisitos de análisis para todos los aislados de poliovirus sometidos a investigación. Este enfoque permitió reconocer rápidamente los brotes de PVOVc de Filipinas y de Madagascar, pero no así otros brotes recientes. El riesgo potencial de aparición de PVOVc ha aumentado extraordinariamente en los últimos años, debido al cese de la circulación del poliovirus salvaje en la mayor parte del mundo. La cepa OPV de tipo 2 parece ser la que más riesgo plantea, debido a su mayor tendencia a propagarse a los contactos. La aparición de PVOVc subraya la importancia que revisten la eliminación de las últimas bolsas de circulación del poliovirus salvaje, el mantenimiento de unos niveles universalmente altos de cobertura con vacuna antipoliomielítica, la interrupción del uso de OPV tan pronto como la seguridad alcanzada lo permita, y el mantenimiento de sistemas sensibles de vigilancia del poliovirus en un futuro próximo. Hay que prestar especial atención a las áreas que presenten el máximo riesgo de circulación del poliovirus salvaje, y a aquellas donde deban mantenerse las mayores tasas de cobertura con vacuna antipoliomielítica para evitar la aparición de PVOVc.

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The Alpha/Beta Interferon Response Controls Tissue Tropism and Pathogenicity of Poliovirus

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Poliovirus selectively replicates in neurons in the spinal cord and brainstem, although poliovirus receptor (PVR) expression is observed in both the target and nontarget tissues in humans and transgenic mice expressing human PVR (PVR-transgenic mice). We assessed the role of alpha/beta interferon (IFN) in determining tissue tropism by comparing the pathogenesis of the virulent Mahoney strain in PVR-transgenic mice and PVR-transgenic mice deficient in the alpha/beta IFN receptor gene (PVR-transgenic/*Ifnar* knockout mice). PVR-transgenic/*Ifnar* knockout mice showed increased susceptibility to poliovirus. After intravenous inoculation, severe lesions positive for the poliovirus antigen were detected in the liver, spleen, and pancreas in addition to the central nervous system. These results suggest that the alpha/beta IFN system plays an important role in determining tissue tropism by protecting nontarget tissues that are potentially susceptible to infection. We subsequently examined the expression of IFN and IFN-stimulated genes (ISGs) in the PVR-transgenic mice. In the nontarget tissues, ISGs were expressed even in the noninfected state, and the expression level increased soon after poliovirus infection. On the contrary, in the target tissues, ISG expression was low in the noninfected state and sufficient response after poliovirus infection was not observed. The results suggest that the unequal IFN response is one of the important determinants for the differential susceptibility of tissues to poliovirus. We consider that poliovirus replication was observed in the nontarget tissues of PVR-transgenic/*Ifnar* knockout mice because the IFN response was null in all tissues.

The replication of many viruses is restricted to certain cells and tissues in the host. This tissue tropism results in a distinct disease pattern unique for each virus. Since virus infection initiates after binding of the virion to a receptor on the cell surface, cellular receptors for viruses have been considered the primary determinants of tissue tropism. However, following the identification of receptors for a number of viruses, it became apparent that receptor distribution in the host is wider than the virus replication sites (38). This indicates that virus tropism may be determined by another factor(s) in addition to the virus receptor.

Poliovirus, belonging to the genus *Picornaviridae*, is the causative agent of an acute human central nervous system disease, poliomyelitis (33). After poliovirus infection, the virus first multiplies in the oropharyngeal and intestinal mucosa and then in the lymphatic tissues, such as the tonsils and Peyer's patches. The virus drains into the blood and circulates within the body. Because visceral tissues, except for adipose tissues, seem to be nonpermissive for poliovirus infection, apparent pathological lesions are not observed in the nonneural tissues. Therefore, the site of virus multiplication during the viremic phase has not been identified. Finally, poliovirus reaches the central nervous system, which leads to the development of a paralytic disease in less than 1% of persons naturally infected with wild-type poliovirus (4, 23, 35). Even in the central nervous system, the poliovirus antigen, nerve cell changes, and inflammatory reactions are localized mainly in motor neurons in the anterior horn of the spinal cord and neurons in the brainstem. The brainstem as far as the hypothalamus and thalamus bears most of the cerebral pathological changes in poliomyelitis. The cerebral cortex (except for the motor cortex), basal ganglia (except occasionally for the globus pallidus), and the cerebellar cortex (except for the vermis) are rarely affected (2, 6). In terms of modern molecular biology, tissue tropism may be determined by interactions between host and viral factors. It is therefore important to elucidate the molecular mechanism responsible for tropism.

The poliovirus receptor (PVR) has been considered a major determinant of poliovirus tissue tropism (13). The molecular cloning of the human *PVR* gene was reported more than a decade ago (18, 24). With cultured cells, susceptibility of poliovirus infection completely correlates with the presence of functional PVR. However, in vivo, this rule is not always true. Analyses of PVR expression in humans revealed that there are many tissues, such as the liver and kidneys, that express PVR but are not involved in the infection (18, 24). Transgenic mice expressing the human *PVR* gene with its natural promoter (PVR-transgenic mice) were produced as a new animal model

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for the study of poliovirus pathogenicity (21, 32). PVR-transgenic mice exhibited a paralytic disease that resembled human poliomyelitis after poliovirus infection. PVR mRNA was detected in all tissues of PVR-transgenic mice by Northern blot hybridization, although expression was restricted to certain cells, such as neurons in the central nervous system (20, 32) and Bowman's capsule and tubules in the kidney (32). PVR was also detected in the glomerulus in the kidney by immunofluorescent staining (15). These data suggest that PVR is necessary for poliovirus infection but may not be the sole determinant of tissue tropism (reviewed in reference 29). The results also suggest the existence of other factors that determine tissue tropism concomitantly with PVR expression. It is possible that host factors required for poliovirus replication are abundant only in target tissues or that host factors inhibiting virus replication are present in nontarget tissues.

To identify possible host factors, we produced another transgenic mouse strain in which there is ubiquitous PVR expression under the control of the CAG promoter (14). If we hypothesize that host factors required for poliovirus replication are present only in susceptible tissues, the distribution of poliovirus replication sites in the new transgenic mice would be the same as or wider than that in the transgenic mice previously produced, since the distribution of the PVR has broadened. However, after intracerebral infection, poliovirus propagated to a slight degree in neurons and glial and ependymal cells near the inoculation sites on day 1 postinfection (p.i.) but the virus titer decreased from day 2 p.i. without development of fatal encephalitis and poliomyelitis. After intraperitoneal and intravenous infection, no apparent signs of illness or virus replication were observed. It seemed to us that an unknown factor(s) that prevented virus replication and spreading was induced. This led us to hypothesize that an innate immune response, such as the production of interferon (IFN), may influence the pathogenesis of poliovirus.

Picornaviruses are sensitive to IFNs (7, 8, 25, 27, 49). IFN plays a central role in the innate immune antiviral response. Infected cells produce alpha/beta IFNs, which induce a number of genes, called IFN-stimulated genes (ISGs), that confer an antiviral state (36, 39, 41). In some viruses, including coxsackievirus and Theiler's virus, alpha/beta IFN plays an important role in the pathogenicity and tissue tropism of the virus (9, 10, 28, 34, 44). However, little is known about the role of alpha/beta IFNs in poliovirus pathogenesis.

Here we report the results of experiments that we conducted to assess the contribution of alpha/beta IFN in the pathogenesis of poliovirus infection with a PVR-transgenic mouse model (20, 21). PVR-transgenic mice were crossed with IFN-α/β receptor (IFNAR) knockout mice in which alpha/beta IFN signaling is disrupted (26). Poliovirus infection of the resulting PVR-transgenic/*Ifnar* knockout mice revealed that the IFN system is an important determinant of poliovirus tissue tropism and poliovirus pathogenesis.

MATERIALS AND METHODS

Cells, viruses, and mice. African green monkey kidney (AGMK) cell line JVK-03 (19) was maintained in Eagle's minimal essential medium supplemented with 5% fetal bovine serum. The poliovirus type 1 Mahoney strain was obtained by transfection of in vitro-synthesized RNA from infectious cDNA clone pOM (40) into JVK-03 cells. Virus titer was determined by a plaque assay on JVK-03 cells. A transgenic mouse strain, ICR-PVRtg21 (20, 21), was backcrossed for 10 generations with C57BL/6 mice, and then N10 mice were mated to obtain homozygotes (PVR-transgenic mice). The mouse strain deficient in the *Ifnar* gene, A129 (26), was purchased from B&K Universal Limited (United Kingdom) with the permission of M. Aguet. A129 was backcrossed for five generations with C57BL/6 mice and then with PVR-transgenic mice for two generations. $PVR^{+/+}$ $Ifnar^{-/-}$ mice were obtained by intercrossing these PVR-transgenic/*Ifnar* knockout mice.

Until the infection experiments, mice were maintained in an animal facility free of the following pathogens: *Citrobacter rodentium*, *Corynebacterium kutscheri*, *Mycoplasma pulmonis*, *Pasteurella pneumotropica*, *Salmonella* spp., cilia-associated respiratory bacillus, *Helicobacter hepaticus*, *Pseudomonas aeruginosa*, *Clostridium piliforme*, *Mycoplasma pulmonis*, ectromelia virus, lymphocytic choriomeningitis virus, mouse hepatitis virus, Sendai virus (HVJ), EDIM virus (rotavirus), minute virus of mice, mouse encephalomyelitis virus, pneumonia virus of mice, mouse adenovirus, reovirus type 3, ectoparasites, intestinal protozoa, and pinworm. Six-week-old mice were used for the experiments. All experiments with mice were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Tokyo Metropolitan Institute for Neuroscience.

Poliovirus infection in mice. Poliovirus at the indicated doses was inoculated intracerebrally, intravenously, or intraperitoneally as described previously (21). The mice were observed daily for 3 weeks or sacrificed at the indicated time p.i. The 50% lethal dose (LD_{50}) was calculated by the method of Kärber (45). In poly(I):poly(C) [poly(I:C)] protection experiments, 200 μg of poly(I:C) (Calbiochem) in phosphate-buffered saline was administered intracerebrally, and poliovirus challenge (10^4 PFU) was performed with the same inoculation routes on the next day.

Determination of virus titer in mouse tissues. Six mice inoculated with poliovirus were sacrificed on day 3 p.i. They were anesthetized and blood was collected from the heart. After perfusion with 20 ml of phosphate-buffered saline, the tissues were removed and frozen at −80°C. The tissues were thawed and homogenized in 5 to 10 ml of minimal essential medium. After centrifugation for 20 min at 3,000 × g, the virus titer of the supernatant was determined.

Histological and immunological examinations. Three to six mice were sacrificed on day 1, 2, or 3 p.i. or when the mice showed paralysis and were then used for histological examination. The mice were anesthetized and perfused with 10 ml of phosphate-buffered saline followed by 10 ml of 4% paraformaldehyde in phosphate-buffered saline. The fixed tissues were embedded in paraffin, from which 3-μm-thick sections were prepared. The poliovirus antigen was detected with an immunoperoxidase method as described previously (21).

Alanine aminotransferase and amylase activity. To evaluate the extent of liver injury in the mice, the serum alanine aminotransferase (ALT) level was measured with the Transnase Nissui kit (Nissui Pharmaceutical Co. Ltd.). The serum amylase level in the mice was measured by the method of Henkel et al. (12) with the Cica Auto amylase kit (Kanto Chemical Co. Inc.).

Quantitative real-time PCR. Mice tissues were separated immediately after sacrifice and stored at −80°C. Total RNA was isolated from the tissue with the RNeasy mini RNA isolation kit (Qiagen, Valencia, Calif.) according to the supplier's instructions. After DNase I treatment, cDNA primed with a random hexamer was synthesized with the Taqman reverse transcription reagent (Applied Biosystems, Foster City, Calif.). The quantification of RNAs was performed with ABI Prism 7900HT. 18S rRNA was quantified by the SYBR Green method with 18S-rRNA-F (5′-GTA ACC CGT TGA ACC CCA TT-3′) and 18S-rRNA-R (5′-CCA TCC AAT CGG TAG TAG CG-3′) as primers. Poliovirus RNA was quantified by the Taqman method with PV c-2493F (5′-TGG TTG GTG ACA GTT CTT ACA CAT T-3′) and PV c-2629R (5′-CCA CTG TGG CAC ACA GTG ATG-3′) as primers and PV c-2579T (5′-FAM-CCA TGT CGA ACG CAA AGC GCC-TAMRA-3′) as the probe. The detection of IFN-β, 2′-5′ oligoadenylate synthetase (OAS)1a, OAS1g, OAS2, OAS3, OASL2, protein kinase R, RIG-I, and helicard mRNAs was performed with Assay-on-Demand PCR probes (Applied Biosystems). The amount of mRNA was determined by comparison with standard templates of cloned cDNAs of known copy number. The expression levels were then normalized to the level of 18S rRNA.

RESULTS

Increased susceptibility of PVR-transgenic/*Ifnar* knockout mice to poliovirus. We previously produced transgenic mice expressing the human *PVR* gene (20, 21). *PVR* mRNA was detected in the brain, spinal cord, thymus, lungs, heart, stom-