Neuropathogenesis of a mouse-adapted porcine epidemic diarrhea virus infection in suckling mice

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A mouse-adapted porcine epidemic diarrhea virus, MK-p10, showed higher neurovirulence in suckling mice than a non-adapted MK strain. There was no difference in virus growth, whereas clear differences between these two virus infections existed in the type of target cells infected, the spread of virus and the cytokine levels produced in the brain. In the early phase of infection, neurons, astrocytes and neural progenitor cells were infected by MK-p10, whereas neural progenitor cells were the only target cells infected by MK. On days 4–5 post-inoculation, MK-p10 antigens were distributed in a number of neurons in a wide area of the brain; however, antigens were restricted in MK infection. In moribund mice in both infection groups, viral antigens were found in a wide area of the brain. The wide spectrum of initial target cells following MK-p10 infection, as well as its faster spread in the brain, may be evidence of enhanced virulence in suckling mice.

Porcine epidemic diarrhea virus (PEDV) is an enveloped alphacoronavirus with a single-stranded, positive-sense RNA genome of about 30 kb and is a causative agent for diarrhoea in pigs. It induces mild intestinal disease in adult pigs, whereas it causes lethal diarrhoea in piglets (Pensaert & de Bouck, 1978). However, the pathogenesis of diseases caused by PEDV is not well understood, since there are no animal models available for the study of infection.

We have recently obtained a PEDV strain adapted to suckling mice (Shirato et al., 2010). Although the neurovirulence of PEDV has not been described thus far in pigs, we reported the establishment of a highly neurovirulent strain of PEDV via adaptation of a cell-adapted MK strain (Kusanagi et al., 1992) to the mouse brain (Shirato et al., 2010). MK showed weak neurovirulence when injected into a suckling mouse brain, whereas MK-p10 showed increased neurovirulence. When inoculated with MK-p10 via the oral route, mice showed slight weight loss with no other clinical symptoms, but no viral antigen or histological changes were observed in the intestine. In this study, we investigated the enhanced neuropathological features of the MK-p10 strain compared with those of the MK strain in suckling mice by histopathological and immunochemical analysis. Our study suggests that the alteration of the cell tropism in the suckling mouse brain, as well as the faster spread of the virus to a variety of cells, mainly neurons, leads to higher neurovirulence in the adapted MK-p10 strain.

MK and MK-p10 were prepared as previously reported (Shirato et al., 2010). Pregnant BALB/c mice were obtained from Japan SLC and Sankyo Labo Service Corporation, and newborn mice were inoculated intracerebrally with 10 μl containing ca 10^4 p.f.u. virus solution. Those mice were clinically observed and their brains were histologically or immunohistochemically examined. Kaplan–Meier analysis was used for statistics, and P<0.05 was considered to be statistically significant. Animal experiments were approved by the Committees on Experimental Animals, National Institute of Infectious Diseases, Japan and Nippon Veterinary and Life Science University, Japan.

A supplementary table is available with the online version of this paper.
We first examined the mortality kinetics of mice to confirm the previous observation (Shirato et al., 2010). MK-inoculated mice showed the clinical signs of Straub tail and tremor at days 12–14 post-inoculation (p.i.), whereas all of the MK-p10-inoculated mice exhibited listlessness and unconsciousness at day 5 p.i. Approximately 20% of the MK-inoculated mice died, whereas MK-p10-inoculated mice had a 100% mortality rate at 10 days p.i. (Fig. 1a). However, there were no differences in virus titres in the brains of MK- and MK-p10-inoculated mice at days 2, 4 and 5 p.i. (Fig. 1b), results which are similar to our previous observations (Shirato et al., 2010).

To elucidate the immune responses of the mice after infection, the levels of 20 different cytokines and chemokines [interleukins (ILs)-1α, 1β, 2, 4, 5, 6, 10, 12p40/p70, 13 and 17, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein-1α, tumour necrosis factor (TNF)-α, interferon (IFN)-γ, IFN-inducible protein (IP)-10, fibroblast growth factor basic, granulocyte–macrophage colony-stimulating factor, keratinocyte-derived chemokine, monokine induced by IFN-γ and vascular endothelial growth factor] in their brain homogenates were measured by using the Mouse Cytokine 20-Plex Panel (Invitrogen) on a Luminex 100 (Luminex). The brains of MK-p10-inoculated mice had significantly higher levels of macrophage-associated cytokines and chemokines (IL-1α, IL-1β, IL-12 p40/p70 and IP-10) than those of MK-inoculated mice on day 4 p.i. (Fig. 1c). In contrast, the levels of lymphocyte-associated cytokines (IL-4, IL-5, IL-6, IL-10 and IFN-γ) were undetectable in the mice brains of both groups. Since IP-10 is a microglia- and astrocyte-related, proinflammatory chemokine (Babcock et al., 2003), the above observations indicate that the proinflammatory cytokine responses related to the microglia and astrocyte were induced earlier in MK-p10-inoculated mice than they were in MK-inoculated mice. The levels for all of

\[\text{Fig. 1. Comparison of the pathogenicity of MK and MK-p10 in suckling mice. (a) Survival rate of mice after infection: 1-day-old mice were intracerebrally inoculated with 10^4 p.f.u. virus and were observed for any clinical symptoms. Mortality rates were noted (n=16 for each group; □, MK; ■, MK-p10). (b) Virus titres in the brains of mice: mice were inoculated, as described above, and sacrificed at intervals, and virus titres in the whole brains were measured (n=3 for each group; white bars, MK; black bars, MK-p10). (c) Chemokine/cytokine levels in the brains of mice: brain homogenates prepared from MK- and MK-p10-infected mice were used to analyse the levels of chemokines/cytokines (n=3 for each group; white bars, MK; black bars, MK-p10). The dotted lines indicate the chemokine/cytokine levels for medium-inoculated mice. Asterisks indicate significant differences between MK- and MK-p10-inoculated mice (P<0.05). Error bars represent SD.}\]
the above mediators were either trace or under the detection limits in the medium-inoculated mice (Fig. 1c).

Then, the viral antigens were detected in the brains of infected mice by immunohistochemistry with a labelled streptavidin–biotin method by using an anti-PEDV-rabbit antibody. Both MK- and MK-p10-inoculated mice showed a few viral antigen-positive cells in the hypothalamus on day 2 p.i. Some of them were revealed to have an astrocytic morphology. On day 4 p.i., viral antigen-positive cells were detected in the cerebral cortex, hippocampus, thalamus, hypothalamus, midbrain and cerebellum of MK-p10-inoculated mice, whereas a few positive cells were observed in the cerebral cortex and thalamus of MK-inoculated mice. On days 5–6 p.i., the MK-p10-inoculated mice showed more serious neuronal degeneration and loss, with oedema in the cerebral cortex, than the MK-inoculated mice (Fig. 2a, two left-most panels). Immunohistochemistry revealed that both MK- and MK-p10-inoculated mice had virus antigen-positive cells in the temporal cortex, hippocampus, thalamus and midbrain on day 5 p.i. However, the antigen-positive cells were detected in the parietal cortex, optic nerve and retina of the MK-p10-inoculated mice, but not in those areas of the MK-inoculated mice (Fig. 2a, two right-most panels, not all data shown). To quantitatively compare the numbers of viral antigen-positive cells in the brains of MK- and MK-p10-inoculated mice, antigen-positive cells were counted in the parietal and temporal cortices. The graph in Fig. 2(a) shows that there were significant differences in the numbers of viral antigen-positive cells between the two infection groups on day 5 p.i. On day 8 p.i., severe liquefactive necrosis was observed in the cerebral cortex of both MK- and MK-p10-inoculated mice (Fig 2b, c). Degenerated cells, including those displaying pyknosis and karyorrhexis, were also found in the necrotic area.

Huge numbers of activated and phagocytic microglia were present in the brains in which early infection was observed. Activated astrocytes were present, along with necrotic areas, in the peripheral cortex of the temporal lobes of MK-p10-infected mice on day 8 p.i. (Fig. 2c). Those cells were stained with glial fibrillary acidic protein (GFAP; Dako) antibody and Iba1 (Wako) antibody (Fig. 2b, right-most panel). There was very tiny cuffing and weak meningitis with mononuclear cells in the sucking mice after either the MK or the MK-p10 inoculation. When examined in combination with a report that the cerebral cortex, thalamus and striatum are connected by thalamocortical and thalamostriatal projections (Alexander & Crutcher, 1990), the above observations suggested that MK-p10 infection spreads to a variety of areas in the brain faster than MK infection, as summarized in Fig. 2(c).

Activated microglia were morphologically observed as cells without projections, similar to macrophages, while resting microglia have a number of projections (Kreutzberg, 1996). Some *Griffonia simplicifolia* (GS)-lectin-positive activated microglia, i.e. not resting microglia, were co-localized with viral antigen on day 8 p.i. in both infection groups (data not shown). Only a small number of T lymphocytes identified by a CD3 antibody (Abcam) had invaded mice brains on days 2, 4 and 5 p.i. of the two viruses (data not shown), although the T-cell response was reported to significantly contribute to the neurovirulence of the MHV-JHM. JHM induces a weak T-cell response, while the less virulent MHV-A59 induces strong responses (Iacono et al., 2006; Rempel et al., 2004). Conclusively, microglia infiltration was predominant in the brains of sucking mice infected with MK and MK-p10 strains.

To identify the cell types infected by MK and MK-p10 strains in mice, double-immunofluorescent staining was performed in paraffin-embedded sections. To define neuron and glia cells, primary and secondary antibodies, listed in Table S1 (available in JGV Online), were used. For staining of mouse tissue with mouse mAb, a mouse stain kit was used (NICHIREI BIOSCIENCES). SlowFade Gold antifade reagent with DAPI (Invitrogen) was used for counterstaining.

On day 2 p.i. a small number of double-positive cells with viral antigen and the glutamate-aspartate transporter (GLAST) were detected in MK infection, whereas in MK-p10 infection, antigen-positive cells were revealed to be microtubule-associated protein 2 (MAP2)-positive neurons, GLAST–GFAP-positive astrocytes or GLAST-positive neural progenitor cells in the thalamus (Fig. 3a). In both mice, myelin CNPase-positive oligodendrocytes, GS-lectin-positive resting microglia and Musashi-1-positive neural stem cells were negative for viral antigen. These results clearly revealed that the neuronal progenitor is the only cell type initially infected with MK; however, neurons, neural progenitor cells and astrocytes were infected with MK-p10 on day 2 p.i., which may mean that the target cells initially infected are different for the two viruses. However, on day 4 p.i., most PEDV antigen-positive cells were neuronal marker-positive pyramidal cells, and some were GLAST and/or GFAP-positive astrocytes or neural progenitor cells in the cerebral cortex and thalamus in both infections (Fig. 3b). These results suggest that astrocytes or neural progenitor cells might have a key role in the spread of the virus to neurons. Most viral antigen-positive nerve cells were pyramidal and seemed to connect to GLAST-positive cells (Fig. 3b, arrowheads).

The present study indicated that the MK-p10 strain obtained a higher affinity to neurons and astrocytes after in vivo passage. This could be caused by an increase in the cell-to-cell spread of the virus in the brain. It was also indicated that affected microglia and astrocytes secreted acute inflammatory chemokines (IP-10, MCP-1) and cytokines (IL-1, TNF-α, IL-12) in the brains of the MK-p10-inoculated mice, although there was no difference in virus growth in the brain. The expression of type I IFN mRNA (IFN-α and IFN-β), as examined by quantitative RT-PCR, was also significantly higher (from 4.8 to 7.9-fold) in MK-p10-inoculated mice than in MK-inoculated
mice on day 5 p.i. (data not shown). These results suggest that the chemokine/cytokine levels are more significantly related to neurovirulence in the mouse rather than to virus growth in the brain. After intracerebral inoculation, strong and widespread neuronal damage was seen earlier in MK-p10-infected mice than in MK-infected mice. Consequently, the MK-p10 strain exhibited higher neurovirulence than the original MK strain in the suckling mice.

Viral growth in the brain failed to explain the difference in virulence between MK-p10 and MK. Both viruses grew equally in the brain; however, the former showed higher virulence and stronger cell damage, as well as a wider/faster distribution of viral antigen. In cultured cells, the spread of PEDV from infected cells to neighbouring cells was influenced by host proteases that enhanced the spread of the virus, although the intracellular virus titre was not affected by the proteases (Shirato et al., 2011b). This finding in cultured cells is somehow suggestive of the phenomenon observed in PEDV infection in the brain shown in this study. Namely, that the titre of the two viruses in the total brain was not different, but the

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**Fig. 2.** Histopathological findings in the brains of mice after intracerebral inoculation. (a) Histopathological (two left panels, HE) and immunohistochemical (two right panels, IHC) analyses, and a graph comparing viral antigen-positive cells (right). Mice inoculated with either MK or MK-p10 were sacrificed on day 5 p.i. Brain specimens were stained with HE, and the viral antigens in the brain were stained with anti-PEDV serum. The number of antigen-positive cells in MK-infected mice was compared with that in MK-p10-infected mice; the number shows the antigen-positive cells out of 1000 neural cells counted in the cerebral area (graph) (n=3 for each group). Bars, 1 mm (first column), 200 μm (second column) and 50 μm (third and fourth columns). P<0.05. Error bars represent so. (b) Histopathological (two right-most panels) and immunohistochemical analyses (two left-most panels) in terminal infectious brain disease: mice inoculated with MK-p10 were sacrificed on day 8 p.i. and samples were stained with HE or observed via IHC analysis using an Iba1 antibody. The two left-most panels are observed under low magnification and the two right-most under high magnification (n=3 for each group). Asterisks indicate liquefactive necrosis in the cerebral cortex. HE, haematoxylin and eosin; IHC, immunohistochemistry. Bars, 1 mm and 100 μm for low and high magnification, respectively. (c) Comparison of virus antigen distribution and neural lesions: mice inoculated with MK or MK-p10 were sacrificed on days 4, 5 and 8, and on days 4, 6 and 8 p.i., respectively (n=3 for each group). We examined the viral antigens present in the different areas of mice brains under the microscope and showed the whole results in schematics.
extracellular spread was higher for MK-p10 than for MK. Some cellular factor, perhaps equivalent to the proteases that enhanced the virus release in cultured cells, could be involved in MK-p10, but not MK infection, in the mouse brain. To analyse such factors, studies are in progress to establish PEDV infection in primary, mixed neural cell cultures.

Some coronaviruses (CoVs) exhibit neurovirulence in animals. Porcine haemagglutinating encephalomyelitis virus (HEV) infects the neurons in piglets and causes encephalomyelitis (Greig et al., 1962). Experimentally, it infects the peripheral neurons of rats and spreads to the central nervous system (Hirano et al., 1995). In contrast, infectious peritonitis virus was found to infect the tissue macrophages in a variety of organs in kittens, and the infected macrophages invaded the brains, possibly causing encephalitis (Weiss & Scott, 1981). It was reported that human CoV (HCoV)-OC43 infected neural cells, suggesting that this virus is a possible candidate agent in the causation of multiple sclerosis (Riski & Hovi, 1980; Arbour et al., 2000). The present study showed that the neurovirulence of MK-p10 is attributable to a higher affinity for neurons and a more efficient spread to neurons, findings that are similar to those of HEV or MHV-JHMV infection.

Some CoVs cross species barriers and gain virulence in a new host, as reported in studies of HCoV-OC43 and severe acute respiratory syndrome coronavirus (SARS-CoV) (Jacomy & Talbot, 2003; Roberts et al., 2007). The increase in virulence after passage in a new host is, at least partly, explained by the usage of new, otherwise low-functioning receptors. Thus far, mutations in the spike proteins of MHV-JHMV, HCoV-OC43 and SARS-CoV were reported to cause adaptation to a new animal species (Phillips et al., 2002; Butler et al., 2006; Kan et al., 2005). However, recent studies have shown that other viral proteins such as haemagglutinin-esterase, nucleocapsid and integral membrane proteins are involved in efficient replication in animals (Iacono et al., 2006; Roberts et al., 2007). Thus, these proteins may participate in the adaptation to a new animal species, though the factor involved in the neurovirulence of PEDV was not clarified. We believe reverse genetics will provide an excellent means of exploring the viral factors related to the higher neurovirulence of MK-p10.

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