Infectious Progression of Canine Distemper Virus from Circulating Cerebrospinal Fluid into the Central Nervous System

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ABSTRACT

In the current study, we generated recombinant chimeric canine distemper viruses (CDVs) by replacing the hemagglutinin (H) and/or phosphoprotein (P) gene in an avirulent strain expressing enhanced green fluorescent protein (EGFP) with those of a mouse-adapted neurovirulent strain. An *in vitro* experimental infection indicated that the chimeric CDVs possessing the H gene derived from the mouse-adapted CDV acquired infectivity for neural cells. These cells lack the CDV receptors that have been identified to date (SLAM and nectin-4), indicating that the H protein defines infectivity in various cell lines. The recombinant viruses were administered intracerebrally to 1-week-old mice. Fatal neurological signs of disease were observed only with a recombinant CDV that possessed both the H and P genes of the mouse-adapted strain, similar to the parental mouse-adapted strain, suggesting that both genes are important to drive virulence of CDV in mice. Using this recombinant CDV, we traced the intracerebral propagation of CDV by detecting EGFP. Widespread infection was observed in the cerebral hemispheres and brainstems of the infected mice. In addition, EGFP fluorescence in the brain slices demonstrated a sequential infectious progression in the central nervous system: CDV primarily infected the neuroependymal cells lining the ventricular wall and the neurons of the hippocampus and cortex adjacent to the ventricle, and it then progressed to an extensive infection of the brain surface, followed by the parenchyma and cortex. In the hippocampal formation, CDV spread in a unidirectional retrograde pattern along neuronal processes in the hippocampal formation from the CA1 region to the CA3 region and the dentate gyrus. Our mouse model demonstrated that the main target cells of CDV are neurons in the acute phase and that the virus spreads via neuronal transmission pathways in the hippocampal formation.

IMPORTANCE

CDV is the etiological agent of distemper in dogs and other carnivores, and in many respects, the pathogenesis of CDV infection in animals resembles that of measles virus infection in humans. We successfully generated a recombinant CDV containing the H and P genes from a mouse-adapted neurovirulent strain and expressing EGFP. The recombinant CDV exhibited severe neurovirulence with high mortality, comparable to the parental mouse-adapted strain. The mouse-infectious model could become a useful tool for analyzing CDV infection of the central nervous system subsequent to passing through the blood-cerebrospinal fluid barrier and infectious progression in the target cells in acute disease.

Canine distemper virus (CDV) is a member of the genus *Morbillivirus* in the family *Paramyxoviridae*, which also includes measles virus (MeV) and rinderpest virus (RPV); all of these viruses induce severe disease in their respective hosts. Since the late 1980s, novel CDV infections have occurred worldwide and have caused fatal disease in several species previously considered non-susceptible to CDV, such as felids, including hyenas and lions (1–3), and marine mammals (4). Furthermore, CDV outbreaks have recently emerged in nonhuman primates and have shown high mortality rates (5, 6).

CDV infection is characterized by pyrexia and nasal and ocular discharge; occasionally by diarrhea and pneumonia (7, 8); and, most critically, by neurologic deficit and leukopenia, resulting in death. Acute infection of animals with CDV is often accompanied by severe neurological manifestations.

Signaling lymphocyte activation molecule (SLAM), which is expressed on cells of the immune system, is a receptor for CDV (9) and other morbilliviruses (10). Using SLAM as a receptor, CDV primarily replicates in lymphocytes and macrophages in the respiratory tract, and it then propagates in lymph nodes before disseminating throughout the body (11). Nectin-4 has also been identified as a receptor for polarized epithelial cells (12–14). CDV also infects SLAM- and nectin-4-negative cell types in the central ner-

vous system (CNS) (15), suggesting the existence of an unidentified third receptor.

Possible routes of CDV transmission from the periphery to the CNS have been suggested, such as hematogenous spread of virus-infected lymphocytes across the blood-brain barrier (16, 17) and the blood-cerebrospinal fluid (CSF) barrier (18–20). Viral spread along olfactory nerves from the nasal concha is another possible route (17, 21). In contrast to the elucidated CDV entry mechanisms, the pattern of virus spread following invasion of the CNS is poorly understood.

Ferrets are often used as an animal model to analyze the neurovirulence of CDV (20, 22, 23). Recombinant neurotropic CDVs

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expressing fluorescent proteins, such as enhanced green fluorescent protein (EGFP) or red fluorescent protein (dTomato) (19), have been utilized to characterize CDV neuroinvasion and local immune activation in a ferret model (17, 20, 21). Mice are more tractable small animals, and a mouse model is commonly used for experimental infection, but mice are not a natural host and are not susceptible to CDV. Bernard et al. reported that the Onderstepoort strain of CDV, which is a vaccine strain passaged in Vero cells, was passaged 10 times in the brains of newborn Swiss mice (24). The mouse-adapted Onderstepoort strain showed typical lethal neuronal symptoms in weanling mice, indicating that the mouse is a suitable animal model for neurovirulence of CDV (24).

Morbilliviruses possess a single-stranded negative-sense RNA genome including six genes. Two surface glycoproteins, hemagglutinin (H) and fusion protein (F), play a key role in virus entry. The H protein is responsible for receptor binding, and the F protein mediates membrane fusion. The inner leaflet of the virion membrane is coated with matrix (M) protein. Inside the virion is the nucleocapsid core, consisting of approximately 16 kb of RNA genome encapsidated by nucleoprotein (N), which acts as a template for all viral RNA synthesis, and RNA-dependent RNA polymerase, composed of catalytic large (L) protein and its cofactor, phosphoprotein (P).

We previously established reverse genetics for the Yanaka strain of CDV, which enabled us to generate recombinant CDVs (25). The Yanaka strain is avirulent to dogs and confers protective immunity against virulent strains of CDV (26). Using this system, we generated recombinant CDV-Yanaka expressing enhanced green fluorescent protein (rCDV-EGFP). In the current study, we generated chimeric virus based on rCDV-EGFP in which certain genes were replaced with those from the mouse-adapted Onderstepoort strain and identified the genes that are necessary for infectivity *in vitro* and neurovirulence *in vivo*. Furthermore, using the recombinant virus, we traced the propagation of CDV in mouse brains with microscopic foci of fluorescence.

MATERIALS AND METHODS

Cells and viruses. B95a cells (Epstein-Barr virus-transformed marmoset B-lymphoblastoid cells) (27) were cultivated at 37°C in RPMI 1640 supplemented with 5% fetal calf serum (FCS). Vero cells (African green monkey kidney cells), HEK293 cells (human embryonic kidney cells), and 293SLAM cells (HEK293 cells expressing marmoset SLAM) (28) were cultivated at 37°C in Dulbecco's modified minimum essential medium (DMEM) supplemented with 5% FCS. IMR32 cells (human neuroblastoma cells) were cultivated at 37°C in DMEM supplemented with 10% FCS and 1% nonessential amino acid solution (Gibco). MGC cells (human glioma cells) were cultivated in DMEM-Hanks balanced salt solution supplemented with 10% FCS, and KG-1 cells (human oligodendroglioma cells) were cultivated at 37°C in DMEM supplemented with 10% FCS.

The mouse-adapted Onderstepoort strain of CDV established by Bernard et al. (24), provisionally named CDV-OndMB here, was propagated in Vero cells after isolation from infected mouse brain. rCDV-EGFP (25) was propagated in B95a cells.

Cloning of the H and P genes of CDV-OndMB and recovery of recombinant viruses. Total RNA was extracted from Vero cells infected with CDV-OndMB using Isogen (Nippongene) according to the manufacturer's instructions and was reverse transcribed using Superscript II (Invitrogen). The following primer pairs were used to amplify the genes for H and P, respectively: 5'-GCGATCGCGGTACCATATCTCGCTTGA TTGCCA-3' and 5'-GAGTCGACTTAATTAAGTGTGAGGGATTGTA CG-3'; 5'-AAGGCCGCGCTCGAGGTCTTGACATCAG-3' and 5'-TATC TAGAGTTTAAACTTAAGCATGAGTAATACT-3' (unique restriction

enzyme recognition sites are underlined). The amplified products were inserted into an EcoRV-digested pBluescript SK vector (Stratagene) for H or a pCR2.1-TOPO vector (Invitrogen) for P.

To generate recombinant viruses, subcloned H or P gene fragments from CDV-OndMB were digested with SgfI and PacI or FseI and PmeI, respectively, and each or both of the genes were replaced with the corresponding genes in the rCDV-EGFP full-length genomic cDNA (25). The resulting plasmids were designated pCDV-omH, pCDV-omP, and pCDV-omHP (omH for the H gene of CDV-OndMB; omP for the P gene of CDV-OndMB) (Fig. 1A). The recombinant viruses were recovered by reverse genetics as described previously (25). Briefly, HEK293 cells inoculated with the recombinant vaccinia virus encoding T7 RNA polymerase were transfected for 1 h with 1 µg of pCDV-omH, pCDV-omP, or pCDV-omHP; 1 µg of pKSN1; 1 µg of pKSP; and 0.3 µg of pGEM1, which expresses the N, P, and L proteins (29), per well in 6-well plates using Eugene 6 (Roche). After 3 days of incubation, the cells were cocultivated with B95a cells (for pCDV-omP) or Vero cells (for pCDV-omH and pCDV-omHP) and were further incubated in culture medium until extensive cytopathic effects (CPE) appeared.

Growth kinetics. A subconfluent B95a cell monolayer in a 12-well plate was infected with rCDV-EGFP or rCDV-omP at a multiplicity of infection (MOI) of 0.1, and a subconfluent Vero cell monolayer was infected with CDV-OndMB, rCDV-omH, or rCDV-omHP at an MOI of 0.01. In both experiments, after 1 h at 37°C, unbound virus was removed, the cells were washed with phosphate-buffered saline (PBS), and medium supplemented with 2% FCS was added. On different days and at different time points postinfection, infected cells and medium were harvested, frozen and thawed three times, and centrifuged at 3,000 × g for 5 min. The virus titers of the supernatants were determined as 50% tissue culture infective dose (TCID₅₀) values with B95a or Vero cells.

Fluorescence microscopy *in vitro*. The cells were cultured in 12-well plates and infected with CDV-OndMB or recombinant viruses at an MOI of 0.01 for 1 h at 37°C. The infection and propagation of the recombinant viruses were observed by the expression of EGFP using a confocal fluorescence microscope (Fluoview FV500; Olympus). CDV-OndMB was detected by an immunofluorescence assay. In brief, the infected cells were fixed with 3% formaldehyde in PBS for 20 min and were then permeabilized with 0.5% Triton X-100 in PBS for 5 min. The cells were incubated for 1 h with anti-N monoclonal antibody 3 (30), followed by incubation with Alexa 488-conjugated goat anti-mouse immunoglobulin G (Molecular Probes) for 1 h. The fluorescence images were acquired using a confocal fluorescence microscope.

Animal experiments. All animal experiments were approved by the Animal Experiment Committee at the University of Tokyo and were performed in accordance with the Regulations for Animal Care and Use of the University of Tokyo. One-week-old BALB/c mice were purchased from Clea Japan, Inc. Litters were infected intracerebrally with 10⁶ TCID₅₀ per 25-µl dose of rCDV-EGFP, rCDV-omH, rCDV-omP, rCDV-omHP, and CDV-OndMB, using craniopuncture under mild ether anesthesia. Injection was performed at the same site, at the vertex of a regular triangle joining the right eye and ipsilateral ear, beside the cranial suture (Fig. 2A). After infection, body weight and clinical signs, including neurological manifestations, such as myoclonus and convulsions, were recorded daily over 2 weeks. Animals that became moribund before the end of the observation period were sacrificed, and the brains were collected for fluorescence imaging. The Wilcoxon log-rank test was performed for the analysis of differences in survival curves, using JMP software (JMP Pro 10.0.2; SAS Institute Inc., Cary, NC, USA). The Macro-Illumination imaging system (VB-7010 microscope system; Keyence) was used to observe EGFP expression in fresh brains.

Tracing virus spread in the brains of mice inoculated with rCDV-omHP. The brains of mice infected with rCDV-omHP were collected at 2 days postinoculation (dpi) or at 4 or 5 dpi when they became moribund and were sectioned serially at 250-µm thickness along the axial plane in ice-cold cutting solution (120 mM choline chloride, 3 mM KCl, 8 mM

FIG 1 Construction of recombinant viruses. The white and hatched boxes represent the genes of the Yanaka and CDV-OndMB strains, respectively. The shaded boxes represent the EGFP gene. (B) Kinetics of recombinant viral growth. B95a or Vero cells were infected with recombinant viruses or CDV-OndMA at an MOI of 0.1 or 0.01, respectively, and then harvested on the indicated days. The titers of the viruses were determined using a TCID₅₀ assay. (C) Infectivities of recombinant CDVs in different cells. Shown is EGFP fluorescence in cells infected with rCDV-EGFP, rCDV-omP, rCDV-omH, and rCDV-omHP or immunohistochemical staining with CDV N antibody in CDV-OndMB-infected cells. The arrowheads indicate CPE. (D) Summary of the infectivity shown in panel B. + + +, efficient infection with syncytia; ±, limited infection without syncytia. omP, P gene of CDV-OndMB; omH, H gene of CDV-OndMB.

MgCl₂, 1.25 mM NaH₂PO₄, 20 mM glucose, and 26 mM NaHCO₃) using a vibratome (Leica). The sections were placed on a culturing membrane (pore size, 0.4 µm; Millicell-CM filter inserts; Millipore) and cultivated in DMEM-Hanks supplemented with 25% heat-inactivated horse serum, 0.65% sucrose, and 100 units of penicillin-streptomycin until required. Then, the sections were examined using a confocal fluorescence microscope, and several dozen images were acquired on a BZ-8000 microscope (Keyence), collected, and connected using BZ analyzer software (Keyence).

Immunohistochemistry of brain slices. Following the analysis of EGFP fluorescence, serial sections were fixed with 0.1 M phosphate buffer

containing 0.1% glutaraldehyde and 4% formaldehyde at pH 7.4 for 3 h, treated with 0.4% Triton X-100 in PBS (PBT), and kept in 10% normal horse serum in PBT. Anti-microtubule-associated protein (MAP) 2ab antibody (Chemicon), anti-myelin basic protein (MBP) antibody (Bioss), and anti-glial fibrillary acidic protein (GFAP) antibody (Sigma) were used as primary antibodies for neurons, astrocytes, and oligodendrocytes, respectively. The slices were incubated with the respective primary antibodies diluted 1:200 in PBS overnight at 4°C, followed by incubation with Alexa 568-conjugated goat anti-mouse antibodies (Molecular Probes) for MAP and MBP or anti-rabbit antibody (Invitrogen) for GFAP diluted 1:300 in PBS for 1 h.

FIG 2 Experimental infection of mice with recombinant CDVs and CDV-OndMB. Mice were intracerebrally infected with rCDV-EGFP, rCDV-omP, rCDV-omH, rCDV-omHP, or CDV-OndMB and monitored for 2 weeks. (A) Diagram showing the injection sites. (B) Survival curves of 1-week-old mice after infection with 10^6 TCID₅₀ of virus. *, $P < 0.001$ (Wilcoxon rank sum test). (C) Clinical signs in mice. The asterisks indicate signs observed in a surviving mouse. (D) Conjunctivitis in infected mice. (E) Macroscopic fluorescence imaging. Massive infection of rCDV-omHP was detected in extensive areas of the cerebral hemisphere (left) and brainstem (right). The arrowhead indicates the inoculation point. (F) Megascopic observation of brain mock infected or infected with rCDV-omHP at 5 dpi. The arrowhead indicates a hollow area of the cerebral hemisphere.

RESULTS

Generation of recombinant CDVs expressing EGFP. Previously, we examined genes that elicit cross-species virulence of RPV using reverse genetics. We generated chimeric viruses based on an avirulent vaccine strain of RPV in which genes were replaced with those of a virulent strain of RPV that had been lapinized. *In vitro* and animal experiments using these viruses clearly demonstrated that the H protein is required for infection of cells but that the P protein is necessary for severe pathogenicity in rabbits (31, 32). Based on this information, we attempted to generate a recombinant CDV-Yanaka strain that possessed the H and/or P gene of CDV-OndMB. The H and P genes of CDV-OndMB from RNA isolated from infected cells were subcloned and then used to replace the H and/or P gene in the full-length cDNA from rCDV-EGFP (Fig. 1A). These full-length genomic plasmids were subjected to reverse genetics. Three recombinant chimeric CDVs, rCDV-omH, rCDV-omP, and rCDV-omHP, were successfully rescued.

Characterization of infection with the recombinant CDVs *in vitro*. The growth curves of the rescued viruses were determined with cell lines utilized for the respective virus preparations. Compared to the parental viruses, rCDV-omHP showed slightly slower growth, but all the viruses reached comparable maximum titers (Fig. 1B). The recombinant viruses were tested for infectivity in various cell lines: lymphoid cells (B95a cells), nonpolarized epithelial cells (Vero and HEK293 cells), and neural cells (KG-1, MGC, and IMR32 cells). To confirm whether the presence of SLAM contributed to the virus infection, SLAM-expressing HEK293 cells (293SLAM cells) were also infected. The results are shown in Fig. 1C and summarized in Fig. 1D. All the viruses grew efficiently in the SLAM-positive cells, B95 cells, and 293SLAM cells. In the epithelial and neural cells, only rCDV-omH and rCDV-omHP showed infection with marked syncytia, as well as CDV-OndMB, whereas rCDV-EGFP and rCDV-omP generated a limited number of single EGFP-positive cells. Thus, CDV-OndMB acquired infectivity in SLAM-negative (and nectin-4-

negative) epithelial and neural cells, and the H gene of CDV-OndMB is essential for infection in these cells.

Neurovirulence of the recombinant CDVs in mice. To assess the pathogenesis of the recombinant CDVs in mice, 1-week-old mice were intracerebrally infected with each virus at the injection site indicated in Fig. 2A. As expected, CDV-OndMB showed high fatality, and rCDV-EGFP was avirulent to mice (Fig. 2B). Among the recombinant CDVs, all the mice infected with rCDV-omP or rCDV-omH survived, whereas rCDV-omHP caused high fatality (Fig. 2B). The clinical signs that developed in this experiment are summarized in Fig. 2C. rCDV-EGFP and rCDV-omP did not cause clinical signs of disease, except for one mouse infected with rCDV-omP, which showed transient depression at a later disease stage. Three of the eight mice inoculated with rCDV-omH exhibited a temporary decrease in body weight (data not shown), depression, and neurological signs, such as nervousness and transient mild convulsions that did not lead to death, and all three mice eventually recovered. In contrast, rCDV-omHP and CDV-OndMB caused depression and severe neurological symptoms, including circling, myoclonus and/or convulsions, and seizures, which led to the death of most of the infected mice. Conjunctivitis was observed only in the surviving mice infected with rCDV-omHP and CDV-OndMB (Fig. 2D). These results indicate that both the H and P genes of CDV-OndMB are important for neurovirulence in mice.

Macroscopic examination of the brains revealed widespread infection of the cerebral hemispheres (Fig. 2E, left) and brainstem (Fig. 2E, right) in rCDV-omHP-infected mice at the clinical stage. The fluorescent area of the cerebral hemispheres was diffused caudally, both dorsally and ventrally, but not near the dorsal rostral inoculation point (Fig. 2E, arrowhead). The dissemination proves that CDV injected via craniopuncture entered the subarachnoid space and was then transmitted by circulating CSF. In some rCDV-omHP-infected mice, a hollow area of the cerebral hemisphere, probably induced by encephalotrophy, was observed (Fig. 2F). Brains from animals that were infected with other viruses did not show any evidence of macroscopic fluorescence at necropsy (data not shown).

These results demonstrate that rCDV-omHP reproduced the CDV-OndMB disease phenotype in mice; therefore, rCDV-omHP was used for the subsequent analysis of viral progression in the CNS.

Tracing virus spread in the brains of mice infected with rCDV-omHP. The brains of rCDV-omHP-infected mice were examined for the expression of EGFP at the subclinical (2 dpi) and clinical (4 and 5 dpi) stages of infection, when severe neurological symptoms appeared. In the subclinical stage, the EGFP-expressing foci were restricted to areas around the ventricles, particularly the third ventricle, lateral ventricle, and dorsal third ventricle (Fig. 3A, B, and C). In the hippocampus, the fluorescence was observed in close proximity to the lateral ventricle and the pyramidal cell layer of the hippocampal CA1 region (Fig. 3C). Neurons expressing EGFP were detected in the cortex (Fig. 3D) and hippocampus (Fig. 3E) around the lateral ventricle.

In the clinical stage, the fluorescence extended from the lateral ventricle side to the parenchyma and cortex. In the brain collected at 4 dpi, one lateral ventricle was significantly enlarged compared to the contralateral in several mice (Fig. 3G, star). At 5 dpi, the fluorescence extended from the ependymal lining of the third ventricle to the parenchyma to a much greater extent than at 2 or 4 dpi

(Fig. 3A, F, and I) and extended throughout the cortex (Fig. 3J and K). EGFP-expressing neurons were also detected in the parenchyma and cortex (Fig. 3L and M). Infection in the hippocampus spread into not only the CA1 region, but also the CA3 region at 4 dpi (Fig. 3H), and the virus spread reached the dentate gyrus at 5 dpi (Fig. 3K). This observation indicates that in the hippocampus, CDV initially infects pyramidal cells of the CA1 region and is transmitted to the CA3 region and dentate gyrus by spreading.

To identify the types of infected cells, brain sections were stained with antibodies recognizing neurons (MAP), astrocytes (GFAP), and oligodendrocytes (MBP). EGFP-positive neurons were found surrounding the third ventricle and hippocampus, whereas EGFP-positive astrocytes and oligodendrocytes were rarely found in these areas (Fig. 4).

DISCUSSION

In this study, we successfully generated a mouse-neurotropic CDV expressing fluorescent protein (rCDV-omHP) by inserting the H and P genes of the mouse-adapted strain, CDV-OndMB, and thus visualized the spread of CDV into the CNS.

In vitro, recombinant viruses possessing the H gene of CDV-OndMB showed the same infectivity in various cell lines as CDV-OndMB (Fig. 1B and C), indicating that H protein defines the cell tropism. Pratakpiriya et al. detected nectin-4 in dog brain and postulated that CDV can infect the CNS through nectin-4 (14). Conversely, Alves et al. did not detect nectin-4 in primary dog brain cell cultures or in the white matter of dog brain tissues (15). In our study, Vero and HEK293 cells were nectin-4 negative, which suggests that the H protein of CDV-OndMB likely uses an unidentified receptor for entry into these cells. Our previous study demonstrated that rCDV-EGFP infects various SLAM/nectin-4-negative cell lines by binding to glycosaminoglycans on the cell surface (25). However, this binding was facilitated by the F protein of CDV instead of the H protein (25). Therefore, the infectivity of CDV-OndMB through the H protein results from a gain of function to bind to an alternative receptor on these cells.

In mice, rCDV-omHP showed high neurovirulence with marked neurological signs leading to high mortality, similar to CDV-OndMB, whereas infection with rCDV-omH showed only mild disease in 3/8 mice (Fig. 2B and C). These results clearly indicate that the P gene of CDV-OndMB is critical for neurovirulence following a successful H protein-mediated infection.

CDV has been postulated to enter the dog brain hematogenously via infected lymphocytes, penetrating not only the blood-brain barrier, but also, and possibly more importantly, the blood-CSF barrier through the epithelial cells of the choroid plexus (33). In the latter case, subsequent virus release into the CSF would enable viral fusion with the ependymal lining of the ventricle, resulting in periventricular and subpial lesions (34–37). In addition, previous reports demonstrated that viral spread into the subarachnoid space resulted in widespread infection of cells of the pia and arachnoid matter of the leptomeninges over a large area of the cerebral hemispheres (20). In our mouse model, rCDV-omHP injected into the subarachnoid space via craniopuncture demonstrated the sequential spread of CDV following invasion into the CSF and subsequent entry into the CNS tissue.

In the CNS tissues, the virus first infected neuroependymal cells lining the ventricular wall and neurons of the hippocampus and cortex adjacent to the ventricle (Fig. 3A to C). In the later clinical stages of the disease, multiple fluorescent foci were ob-

FIG 3 Distribution of CDV infection in brain sections at subclinical and clinical stages. Areas around the third ventricle (A, F, and I), lateral ventricle (B, G, and J), and hippocampus (C, H, and K) at 2 dpi (subclinical stage) and 4 and 5 dpi (clinical stage) are presented. The star in panel G marks an enlarged lateral ventricle. (D and E) Areas indicated by arrows 1 and 2 in panel C, respectively, at higher magnification. (L and M) Areas indicated by arrows in panels J and K, respectively, at higher magnification. (N) Approximate localization of the coronal brain sections. Panels A, B, F, G, I, and J are located in section a, and panels C, H, and K are located in section b. 3V, third ventricle; LV, lateral ventricle; D3V, dorsal third ventricle; Cx, cortex; DG, dentate gyrus. Fluorescent images merged with the light field are connected.

served over a large proportion of the brain surface by macroscopic and microscopic analyses (Fig. 2E and 3F to M). These data indicate that CDV was swiftly transferred into the ventricle through the circulating CSF and spread from there into the cerebral cortex, with extensive viral infection in the subarachnoid space into the meningeal cells of the pia and arachnoid cell layers.

The hippocampal formation consists of the hippocampus, dentate gyrus, and subicular cortex. The hippocampus is divided into four sectors (CA1, CA2, CA3, and CA4). The primary cell type within the hippocampus is the pyramidal cell, and the principal cell type of the dentate gyrus is the granule cell. The axons of the granule cells in the dentate gyrus, called mossy fibers, synapse with pyramidal cells in the CA3 region, and most of the axons from CA3 pyramidal cells form synapses onto CA1 pyramidal cells (38). In a previous study using MeV expressing EGFP, MeV-infected rat hippocampal-slice cultures exhibited a unidirectional retrograde spread of the virus along neuronal processes connect-

ing CA1, CA3, and the dentate gyrus (39). This study showed that MeV spreads much more efficiently through synapses from CA1 to CA3 than in the reverse direction. In our study, rCDV-omHP initially infected CA1 pyramidal cells adjacent to the lateral ventricle (Fig. 3C), and thereafter, the virus extended to CA3 pyramidal cells and the granule cells of the dentate gyrus (Fig. 3H and K). Therefore, CDV transmission from CA1 to CA3 and the dentate gyrus through synapses is likely similar to the retrograde spread of MeV.

In an acute-disease model in ferrets, neurons were preferentially targeted, undergoing morphological changes indicative of severe neuronal injury, including acute neuronal necrosis, atrophy, and ballooning reminiscent of karyolysis (22). We showed that CDV mainly targets neurons in the cerebral parenchyma of the brainstem, the cortex, and the hippocampus (Fig. 2 to 4). Ependymal and meningeal barriers are in intimate contact with an underlying astroglial cell layer (glia limitans). The progressive loss

FIG 4 Identification of EGFP-positive cells in brain. Brain slices from mice at 4 and 5 dpi, shown in Fig. 3, were stained with antibodies of neurons (MAP), astrocytes (MBP), and oligodendrocytes (GFAP). (Left) EGFP fluorescence. (Middle) Immunofluorescent staining of CNS cells. (Right) Colocalization of CNS cells (red) and EGFP fluorescence (green). The arrows indicate EGFP-positive neurons.

of infected neurons in these areas correlates with the development of neurological signs, especially seizures and circling. CDV invasion into the CSF during the acute phase may infect neurons predominantly and induce transsynaptic spread, and the neurological complications in subsequent demyelinating distemper encephalitis would be caused by viral cell-to-cell spread through astrocytes (40).

In the case of MeV, several rodent brain-passaged strains have been established (41). In particular, the neurovirulence of the CAM/RB strain has been well studied (42–44); MeV antigen was detected mainly in neurons in the hippocampus and cortex, as is observed with CDV-OndMB (Fig. 3 and 4). In contrast, MeV-CAM/RB was not found in the ependymal cells of the ventricular layer or meninges, where CDV-OndMB was observed. This difference may rely on the difference in neurotropic strength between CDV and MeV. Our reverse genetics and resultant recombinant CDVs will be useful for further detailed studies.

In summary, our system could be used as a mouse model to provide more precise information about the infectious progression of CDV from the CSF into the CNS tissue, facilitating the explanation of morbillivirus-induced neurological diseases in general.

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