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Pathogenesis of canine distemper virus in experimentally infected raccoon dogs, foxes, and minks

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ABSTRACT

Canine distemper virus (CDV) infects a broad range of carnivores and causes a highly contagious disease with severe immunosuppression. The disease severity markedly varies in different species. To investigate the pathogenesis of CDV in raccoon dog (*Nyctereutes procyonoides*), fox (*Vulpes vulpes*) and mink (*Neovison vison*) species, three groups of CDV sero-negative animals were infected with CDV strain LN(10)1. This CDV strain belongs to the Asia-1 genotype, which is epidemiologically predominant in carnivores in China. CDV infection provoked marked differences in virulence in the three species that were studied. Raccoon dogs developed fever, severe conjunctivitis, and pathological lesions, with 100% (5/5) mortality and with high viral RNA loads in organs within 15 days post infection (dpi). In infected foxes, the onset of the disease was delayed, with 40% (2/5) mortality by 21 dpi. Infected minks developed only mild clinical signs and pathological lesions, and mortality was not observed. Raccoon dogs and foxes showed more severe immune suppression (lymphopenia, decreased lymphocyte proliferation, viremia and low-level virus neutralizing antibodies) than minks. We also observed a distinct pattern of cytokine mRNA transcripts at different times after infection. Decreased IFN-γ and IL-4 mRNA responses were evident in the animals with fatal disease, while up-regulation of these cytokines was observed in the animals surviving the infection. Increased TNF-α response was detected in animals with mild or severe clinical signs. Based on the results, we could distinguish three different patterns of disease after experimental CDV infection, e.g. a mild form in minks, a moderate form in foxes and a severe disease in raccoon dogs. The observed differences in susceptibility to CDV could be related to distinct host cytokine profiles. Comparative evaluation of CDV pathogenesis in various animal species is pivotal to generate models suitable for the evaluation of CDV–host interactions and of vaccine response.

1. Introduction

Canine distemper virus (CDV) is an enveloped negative strand RNA virus, which, along with measles virus (MV), rinderpest virus (RPV), *pestedespetits ruminants* virus (PPRV), cetacean morbillivirus (CMV) and phocine distemper virus (PDV), belongs to the *Morbillivirus* genus, in the family *Paramyxoviridae* (King et al., 2011).

CDV is highly contagious and is transmitted by aerosol, causing a systemic and often fatal infection in carnivores (Krakowka et al., 1985). The virus enters the host by the nasal or oral route and initiates replication by using the signaling lymphocyte activation molecule (SLAM/CD150) receptor, which is expressed on the surfaces of immune cells, such as alveolar macrophages and/or dendritic cells of the respiratory tract. Infected circulating immune cells disseminate the virus throughout the lymphatic system between 3 and 6 days, followed by subsequent spread to epithelial tissues at about 10 days post-infection (von Messling et al., 2003, 2004; Beineke et al., 2009; Sawatsky et al., 2012). Nectin-4 (poliovirus-receptor-like-4, PVRL4), an epithelial cell receptor, plays a role in transmission during the late stages of CDV pathogenesis (Pratakpiriya et al., 2012; Noyce et al., 2013; Delpeut et al., 2014). The virus is amplified and secreted from the epithelial cells of the respiratory, gastrointestinal and urinary systems of the

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infected host (Ludlow et al., 2014). In addition to respiratory and gastrointestinal symptoms, the disease is characterized by the rapid onset of severe leukopenia/lymphopenia and inhibition of lymphocyte proliferation during the first weeks of infection (von Messling et al., 2004, 2006). Viral proteins are associated with modulation and inhibition of innate immunity within the host cell. In particular, the block of type I and II interferon (IFN) signal transduction are the main features of CDV immune modulatory actions (Röthlisberger et al., 2010; Svitek et al., 2014).

The mortality rates associated with CDV infections vary among susceptible species, and range from 0% in domestic cats (Ikeda et al., 2001), to 30% in lions and monkeys (Roelke-Parker et al., 1996; Qiu et al., 2011), 50% in domestic dogs (Ek-Kommonen et al., 1997), and 100% in ferrets (Williams et al., 1988). Raccoon dogs (*Nyctereutes procyonoides*), foxes (*Vulpes vulpes*) and minks (*Neovison vison*) are also susceptible to CDV infections with high mortality (Zhao et al., 2010a, 2014; Cha et al., 2012; Nikolin et al., 2012b; Martella et al., 2010). Previous field epidemiology and observational studies in China suggested that CDV infection was more common and more relevant clinically in raccoon dogs and foxes than in minks (Zhao et al., 2010a; Wang et al., 2009). Accordingly, it is possible to assume that raccoon dogs or foxes are more susceptible than minks to CDV, although virus-related factors should be also considered when assessing CDV pathogenesis (Nielsen et al., 2009; Jensen et al., 2015; de Vries et al., 2014). For this reason, the differences in susceptibility between these species required thorough investigation using experimental infections. Developing reliable models for CDV pathogenesis in raccoon dogs, foxes or minks would strongly contribute to better understanding of CDV host range, CDV pathogenesis and epidemiology in different hosts.

So far, only one serotype of CDV is recognized; however, several co-circulating CDV genotypes of different virulence and cell tropism have been found (Nielsen et al., 2009; Nikolin et al., 2012b; Takenaka et al., 2014). In this study, raccoon dogs (*N. procyonoides*), foxes (*V. vulpes*) and minks (*N. vison*) were infected experimentally with the CDV strain LN(10)1, representative of the CDV genotype (Asia-1) that is predominant in carnivores in China. Differences in virulence and immunosuppressive activities were observed among the three species. Variations in tissue tropism and cytokine responses in the experimental animals were also documented.

2. Materials and methods

2.1. Viruses and animals

The wild-type CDV strain LN(10)1 (Genbank accession no. KP765764) was isolated from an Arctic fox in 2010 in China. The virus was propagated on Vero cells stably expressing the SLAM receptor from raccoon dog (Vero-rSLAM) (Zhao et al., 2012).

Eight raccoon dogs, 8 Arctic foxes and 8 American minks, of 5–6 months of age, were purchased from a fur animal farm in Jilin province of China. The body weight was 3.5–4.0 kg for raccoon dogs and foxes and 1.5–2 kg for minks. The animals were clinically healthy and had no virological/serological evidence of parvovirus infection (Kang et al., 2013; Wang et al., 2014). All the animals were serologically negative for CDV. The animals were housed in separate cages at the animal house of the Central Laboratory for Animal Infectious Diseases of the Institute of Special Animal and Plant Sciences (ISAPS).

2.2. Animals infection and samples collection

All animal work and experimental procedures were conducted with the ethics approval from the Institutional Animal Care and Use Committee of the ISAPS. Three groups of fifteen animals [raccoon dogs (R1-R5), foxes (F1-F5) and minks (M1-M5)] were separately housed and infected intramuscularly with 10^4 $TCID_{50}$ of CDV strain LN(10)1. Additionally, three raccoon dogs, 3 foxes and 3 minks were mock-infected as controls.

Clinical signs, feces characteristics and rectal temperatures were monitored daily up to 21 days post-infection (dpi). The animals either died from infection or were humanely euthanized for ethical reasons using intramuscular premedication with ketamine (20 mg/kg) and xylazine (1 mg/kg) (Intervet, Netherlands). Nasal and rectal swabs and whole blood samples were collected at 0, 3, 7, 10, 14 and 21 dpi. Blood samples from raccoon dogs and foxes were collected via the femoral veins while blood from minks was obtained by clipping nails of the hind legs or, at the end of the experiments, by cardiac puncture. The sera were separated from blood cells and stored at −20 °C until use. Nasal and rectal swabs were suspended in 0.5 ml sterile phosphate buffered saline (PBS) and stored at −20 °C until used for RNA extraction.

2.3. Isolation and real-time RT-quantitative PCR (RT-qPCR) detection of CDV RNA

A total of 140 μl of suspensions from nasal and rectal swabs were subjected to RNA extraction using the QIAamp Viral RNA Kit (Qiagen, Germany). The RNA was extracted from 25 mg of tissues using the RNeasy Mini Kit (Qiagen, Germany). The QIAamp Blood RNA Mini Kit (Qiagen, Germany) was used to extract CDV RNA from 200 μl of EDTA-treated whole blood samples. RNA extraction was performed following the manufacturer's instructions. Viral RNA was reverse transcribed using random hexamer primers and the Prime Script II 1st Strand cDNA Kit (Takara, Japan). Primers and the TaqMan probe for CDV real-time RT-PCR (RT-qPCR) were used as previously described (Elia et al., 2006). Briefly, a plasmid containing the NP gene of CDV LN(10)1 strain was used as standard DNA template for the CDV RT-qPCR. Two microliter of cDNA or plasmid standard was added to the 20 μl reaction master mix. The master mix consisted of 10 μl HS Ex *Taq* Premix reaction mix (Takara, Japan), 0.6 μM of each primer and 0.4 μM probe. The RT-qPCR was performed in a LightCycler96 (Roche, Germany), using the following protocol: denaturation at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, 48°Cfor 1 min, and 60 °C for 1 min.

2.4. Histology and immunohistochemistry

Necropsy and histological examinations were performed on all the infected animals. The tissues were fixed in 10% formaldehyde in PBS and processed for histopathology. Paraffin-embedded tissue sections (lung, spleen, bladder, brain and mesenteric lymph nodes) were obtained and stained with hematoxylin and eosin (Sakai et al., 2013).

Detection of CDV antigen in tissues in immunohistochemistry (IHC) was carried out using a mouse CDV-NP monoclonal antibody (*VMRD*, USA). Immunohistochemical analysis s was performed on paraffin-embedded sections using the EnVison/HRP Systems kit (Dako, Denmark) as previously described (Sakai et al., 2013).

2.5. Measuring lymphocyte numbers, proliferation activity, and virus neutralizing antibody titers (VNT)

EDTA-treated whole blood samples (about 1.5 ml) from infected animals were collected on dpi 0, 3, 7, 10, 14 and 21 and used to assess the lymphocyte count and proliferation activity as follows: (1) 15 μl of EDTA-treated whole blood samples was used to estimate the number of lymphocytes per μl using an automated blood analyzer pocH-100i (*Sysmex*, Japan). (2) Peripheral blood