Canine distemper virus (CDV) causes acute systemic infection in dogs and other Canidae, with symptoms of fever, coughing, vomiting, diarrhea, ataxia, and paralysis. It has long been thought that only animals in the family Canidae are susceptible to CDV infection in nature. However, during the last 2 decades, animals of many other species, such as Ailuridae (1), Mustelidae (2), Viverridae (3, 4), Procyonidae (5), Phocidae (6), and Felidae (7, 8), have been infected with CDV in nature.

CDV belongs to the genus Morbillivirus within the family Paramyxoviridae (9). Signaling lymphocyte activation molecule (SLAM) is a principal receptor of CDV. Other members of the Morbillivirus genus, namely, measles virus (MV), rinderpest virus, and peste des petits ruminants virus, are also known to utilize human SLAM and nectin4, respectively, as a receptor. These viruses preferentially use the SLAM of their host animals but have the ability to use other SLAMs of nonhost animals with reduced efficiency (10). Recently, human nectin4 and dog nectin4 have been identified as epithelial cell receptors for MV (11, 12) and CDV (13), respectively.

Importantly, CDV outbreaks have recently emerged with a high mortality rate in nonhuman primates. The first outbreak occurred in 1989 in Japan (14). Twenty-two Japanese monkeys (Macaca fuscata) in the wild were captured and later shown to have CDV infections. Two of them developed neurological symptoms, and one died of encephalitis (14). Recently, large CDV outbreaks have occurred in rhesus monkeys (Macaca mulatta) at a breeding farm in Guangxi province, China, with a mortality rate of 5 to 30% (15). In 2008, an animal center in Beijing, China, experienced another CDV outbreak in rhesus monkeys (16). This outbreak was likely associated with the Guangxi outbreaks. Following these outbreaks in China, a CDV outbreak occurred in cynomolgus monkeys (Macaca fascicularis) in Japan in 2008. These monkeys were imported from China, and some 46 cynomolgus monkeys out of 432 imported were euthanized or died from severe pneumonia, diarrhea, and anorexia during a quarantine period. A CDV strain was isolated from a moribund monkey, and phylogenetic analysis of its genome sequence showed that the CDV strain was closely related to the CDV strains associated with recent outbreaks in rhesus monkeys in China, suggesting continuing chains of CDV infection in monkeys. In vitro, CYN07-dV uses macaca SLAM and macaca nectin4 as receptors as efficiently as dog SLAM and dog nectin4, respectively. CYN07-dV showed high virulence in experimentally infected cynomolgus monkeys and excreted progeny viruses in oral fluid and feces. These data revealed that some of the CDV strains, like CYN07-dV, have the potential to cause acute systemic infection in monkeys.

MATERIALS AND METHODS

Cells. Vero cells constitutively expressing dog SLAM (Vero.DogSLAMtag) and dog nectin4 (Vero/dNectin4) were used (13, 17). Vero cells expressing human SLAM (Vero/hSLAM) (10) were also used. Vero cells constitutively expressing macaca SLAM (Vero/macSLAM) and macaca nectin4 (Vero/macNectin4) were generated in the present study. Total RNAs obtained from peripheral blood mononuclear cells (PBMCs) and a kidney from a cynomolgus monkey were used to synthesize cDNAs of macaca SLAM and macaca nectin4, respectively. The nucleotide sequences of cytoplasmic domains of macaca SLAM and macaca nectin4 were deposited in GenBank with accession numbers AB742520 and AB742522, respectively. The macaca SLAM and macaca nectin4 cDNA fragments were inserted into the pCXN2 vector (18), generating pCXN2-macSLAM and pCXN2-macNectin4, respectively. Vero/macSLAM and Vero/macNectin4 cells were generated by transfecting Vero cells with pCXN2-macSLAM and pCXN2-macNectin4, respectively, and were selected in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% fetal bovine serum (FBS) and 0.5 mg/ml Geneticin (G418; Invitrogen). Expression of macaca SLAM and nectin4 in Vero cells was confirmed by immunofluorescence.
staining using a goat anti-human SLAM and nectin4 polyclonal antibody, respectively.

**Virus isolation.** Tissue samples obtained from the spleens of moribund or dead monkeys were suspended in phosphate-buffered saline (PBS) supplemented with antibiotics and were homogenized. The homogenates were centrifuged at 10,000 × g for 5 min, and supernatants were inoculated to monolayers of Vero.DogSLAMtag cells.

**RNA extraction and RT-PCR.** Viral and total RNAs were extracted from culture media and cells, respectively, using ISOGEN-LS (Nippon Gene). Reverse transcription (RT) was carried out with Superscript III (Invitrogen) using primers of random nucleotide hexamers (TaKaRa Bio Inc.). Then, PCR was performed to amplify CDV-specific cDNA fragments.

**Sequential and phylogenetic analysis of the CDV isolate.** PCR amplions were used as templates for sequencing on an Applied Biosystems 3130 automated DNA sequencer using a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems Japan). The entire genome nucleotide sequence of each extremity was determined by the rapid amplification of cDNA ends (RACE) method. The sequence was further confirmed by using a Roche GS Junior sequencer. Nucleotide and amino acid sequence identities were calculated using the pairwise distance algorithm (p distance) with MEGA 4 software (19). Phylogenograms were reconstructed using a neighbor-joining algorithm with MEGA 4 software. The robustness of the resulting branching patterns was tested using the bootstrap method with 1,000 replicates. Sequence relatedness is shown as percentage identity.

**Histopathological examination of monkeys infected with CDV during the 2008 outbreak.** Three cynomolgus monkeys (11, 12, and 13) infected with CDV during the 2008 outbreak were euthanized by exsanguination under excess ketamine anesthesia and autopsied for histopathological examination. Tissue samples were immersed in 10% phosphate-buffered formalin. Fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Immunohistochemical analysis for the detection of the CDV antigens was performed on paraffin-embedded sections using EnVision/HRP Systems (Dako). After deparaffinization with xylene, the sections were dehydrated in ethanol and immersed in PBS. Antigens were retrieved by hydrolytic autoclaving for 15 min at 121°C in a sodium citrate-sodium chloride buffer (10 mM, pH 6.0). Endogenous peroxidase was blocked by incubation in 1% hydrogen peroxide in methanol for 30 min. The sections were incubated with a monoclonal antibody against CDV nucleoprotein (NP) (VMDR Inc.) and then with biotin-conjugated anti-mouse IgG. Peroxidase activity was detected by development with diaminobenzidine containing hydrogen peroxide, and then the nuclei were counterstained with hematoxylin.

Double immunofluorescence stainings were also performed for the various tissues of the CDV-infected cynomolgus monkey 11. Rabbit anti-wide-spectrum cytokeratin antibody (ab9377; Abcam), rabbit anti-neuron-specific β III tubulin antibody (ab56110; Abcam), rabbit anti-CD3 antibody [SP7] (ab21703; Abcam), goat anti-nectin4 polyclonal antibody (R&D Systems), and the monoclonal antibody against CDV NP were used as primary antibodies. Normal rabbit, goat, and mouse sera were used as negative-control antibodies (Dako). The sections were deparaffinized, rehydrated, and immersed in PBS. Antigens were retrieved by hydrolytic autoclaving in the retrieval solution (pH 9.0; Nichirei) for 15 min at 121°C. After the sections were cooled, to block background staining, normal goat or donkey sera were used. The sections were incubated with the monoclonal antibody against CDV NP overnight at 4°C. The sections were washed and incubated with antibodies to the cell markers for 60 min at 37°C. The sections were washed and incubated with goat anti-rabbit IgG-Alexa Fluor 568 (Invitrogen) and goat anti-mouse IgG-Alexa Fluor 488 (Invitrogen) antibodies or donkey anti-goat IgG-Alexa Fluor 568 (Invitrogen) and donkey anti-mouse IgG-Alexa Fluor 488 (Invitrogen) antibodies for 60 min at 37°C. After being washed, the sections were mounted with SlowFade Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). The images were captured by a fluorescence microscope (IX71; Olympus) equipped with a Hamamatsu high-resolution digital black and white charge-coupled-device (CCD) camera (ORCA2; Hamamatsu Photonics).

**Experimental infection of cynomolgus monkeys.** Five cynomolgus monkeys of 5 to 11 years of age were obtained from the Tsukuba Primate Research Center (National Institute of Biomedical Innovation, Ibaraki, Japan). They were free from simian retrovirus type 4 (SRV) and were confirmed to be free from MV and CDV antibodies. Four of them (no. 4450, 4571, 4965, 4969) were male, while one (no. 4970) was female. Their cages were placed in negatively pressurized glove boxes. They were anesthetized with ketamine (0.1 ml/kg) and inoculated intranasally with 5 × 10^5 PFU of CDV in 0.5 ml of DMEM using a spray (0.25 ml in each nostril; Keytron). On the day of inoculation, and at 3, 7, 10, and 15 days after inoculation, body weight and body temperature were measured, and throat and rectal swabs and peripheral blood were obtained. PBMCs were isolated using Percoll gradients (GE Healthcare), adjusted to a concentration of 10^7/ml, and divided into 2-fold serial dilutions. Then, a 500-µl aliquot of each diluted PBMC sample was inoculated into Vero, DogSLAMtag cells. On the assumption that one CDV-infected PBMC was contained in the maximum diluted PBMC sample that induced syncytium, the number of CDV-infected PBMC per 10^5 PBMCs was calculated. All monkeys were euthanized 15 days after inoculation by exsanguination under excess ketamine anesthesia, and tissue samples were collected for histopathological examination and virus isolation. For virus isolation, tissue homogenates were prepared in PBS containing antibiotics and clarified by centrifugation. These samples were inoculated to Vero, DogSLAMtag cells. When no cytopathic effect (CPE) was observed, RT-PCR was performed for the detection of CDV-specific RNAs. When no CDV-specific cDNA was amplified, samples were determined as being negative for CDV. Total numbers of blood cells were measured using an autoanalyzer (Cell Tuck; Nihon Koden). Numbers of neutrophils, lymphocytes, monocytes, eosinophils, and basophils were determined by microscopic analysis. A virus-neutralization test for CDV was performed using a plaque reduction method with a constant amount of virus and various serum dilution. Sample sera were serially diluted 4-fold and mixed with equal volumes of 100 PFU of CDV units. The neutralizing antibody titer was calculated at the 50% plaque reduction point by the Behrens-Kaerber method.

**Multiplex analysis of cytokines and chemokines in monkey sera.** Monkey sera were subjected to multiplex cytokine analysis using the human cytokine 25-plex antibody bead kit (Invitrogen) with Luminex 100 (Luminex Co.) according to the manufacturer’s instructions. Enzyme-linked immunosorbent assays (ELISAs) were performed in duplicate, and the averages of each assay are shown.

**Replication kinetics of the CDV isolate in Vero cells expressing SLAM and nectin4 of various animal species.** Vero, Vero.DogSLAMtag, Vero/macSLAM, Vero/hSLAM, Vero/dNectin4, and Vero/macNectin4 cells (2 × 10^5 cells/well) were cultured in 24-well plates and infected with the CDV isolate at a multiplicity of infection (MOI) of 0.01. The cells were adsorbed with the virus for 1 h at 37°C, and then the virus inoculum was removed and the cells were rinsed twice with DMEM. The cells were cultured in DMEM supplemented with 1% fetal calf serum (FCS) at 37°C. The cells and culture supernatants were harvested every 12 h until 3 days postinfection (p.i.). The harvested samples were stored at −80°C until use. The samples were centrifuged at 1,000 x g and titrated with plaque assay.

**Cell-to-cell fusion assay.** DNA fragments encoding H and F protein of the CDV isolate were amplified by RT-PCR and cloned into the pCAGGS vector (18). Vero, Vero.DogSLAMtag, Vero/macSLAM, Vero/hSLAM, Vero/dNectin4, Vero/macNectin4, and Vero cells in 24-well plates were transfected with the F protein-expressing plasmid together with or without the H protein-expressing plasmid. To detect syncytia clearly, a fluorescent protein-expressing plasmid, pEGFP-C1 (Clontech Laboratories),

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was cotransfected. The cell monolayers were observed using an Axio Observer.D1 microscope at 16 and 24 h posttransfection.

**Entry assay using pseudotyped viruses.** To analyze the efficiency of virus entry using SLAM and nectin4 in more detail, a vesicular stomatitis virus (VSV) pseudotype system (17) was employed (VSVΔG* was kindly provided by M. A. Whitt, The University of Tennessee Health Science Center). A VSV pseudotype bearing the H protein and the F protein of the CDV strain on the surface of the virion (referred to as VSVΔG*-F-dVH) was constructed. VSV pseudotype bearing only the F protein (referred to as VSVΔG*-F) was also constructed. Vero, Vero.DogSLAMtag, Vero/macSLAM, Vero/dNectin4, and Vero/macNectin4 cells were infected with the VSV pseudotypes. Infectivity titers of the VSVΔG*-F-dVH and VSVΔG*-F were determined at 24 h p.i.

**Ethics statement.** The experiments with animals were performed at animal biological safety level 2 in strict accordance with the Animal Experimentation Guidelines of the National Institute of Infectious Diseases. The protocol was approved by the Institutional Animal Care and Use Committee of the institute (permit number 611001). Collection of the specimens from the monkeys was performed under ketamine hydrochloride anesthesia, and all efforts were made to minimize suffering.

**RESULTS**

**Clinical and pathological features of cynomolgus monkeys naturally infected with CDV in the outbreak in Japan.** Forty-six monkeys out of 432 cynomolgus monkeys died or were euthanized during a quarantine period after import from China in Japan in 2008, resulting in a fatality rate of 10.6% (46/432) if euthanized animals were considered to be fatal. Clinical signs of sick monkeys were characterized by eye mucus, nasal mucus, rhinitis, coughing, anorexia, diarrhea, fever, and generalized rash, which are similar to those observed in acute measles in humans and in monkeys in recent CDV outbreaks in China. Swelling of the footpads was also observed in sick monkeys. Three moribund monkeys were autopsied for histopathological examination. Histopathologically, two monkeys (animals 11 and 12) were in the acute phase of systemic CDV infection. One monkey (animal 13) was considered to be in a convalescent phase. Various stages of giant cell pneumonia were found in all three monkeys (Fig. 1A). CDV antigen-positive syncytial pneumocytes were seen in the lungs of two monkeys (animals 11 and 12) (Fig. 1B). In the thymus, spleen, tonsils, and lymph nodes of all monkeys, almost all lymphocytes were depleted, suggesting severe immune suppression. The remaining mononuclear cells were positive for CDV antigen in the lymph tissues of two monkeys (animals 11 and 12) (Fig. 1C and D). Focal gliosis and demyelination were found in the cerebrum and/or cerebellum of all monkeys. In these lesions, some neurons and glia cells were positive for CDV antigen in all monkeys (Fig. 1E and F). In other organs, including the skin, small and large intestines, kidneys, salivary glands, and testes, giant cells and/or CDV antigen-positive cells were observed. Interestingly, CDV antigen-positive cells were not observed in the tissues except for the brain and testis of monkey 13. The types of cells with CDV antigens in the tissues were identified by dual staining with antibodies to CDV NP and various cell markers (Fig. 2). CDV antigens were detected in some of the cytokeratin-positive giant cells in the bronchi and bronchiole (Fig. 2). The bronchiolar epithelial cells with CDV antigens were also nectin4 positive (Fig. 2). In addition, the virus antigens were also detected in the CD3+ lymphocytes in the lymph node and the IIII tubulin-positive neurons in the brain (Fig. 2). Sera of the three monkeys (no. 11, 12, and 13) were subjected to multiplex cytokine analysis. Compared with eight normal monkeys from Tsukuba Primate Research Center, naturally CDV-infected monkeys in the outbreak showed upregulated levels of proinflammatory cytokines and chemokines, such as interleukin 1β (IL-1β), IL-6, macrophage inflammatory protein 1α (MIP-1α), MIP-1β, monocyte chemoattractant protein 1

![FIG 1](https://jvi.asm.org/article-pdf/87/2/1107/1107-0531608935327524643.pdf)

Histopathological analyses of cynomolgus monkeys naturally infected with CDV in the 2008 outbreak. Tissue sections obtained from cynomolgus monkey 11 were examined by hematoxylin and eosin staining (HE) and immunohistochemistry (IHC) using anti-CDV-NP monoclonal antibody. Giant cell pneumonia (A) and CDV antigen in the syncytial pneumocytes (B) were seen in the lung. Lymphocyte depletion (C) and CDV antigen in the mononuclear cells (D) were observed in the lymph node. Focal and slight microglial cell infiltration (E) and CDV antigen in neurons and glia cells (F) were observed in the cerebrum. HE, original magnification ×20; IHC, ×40.
(MCP-1), and eotaxin. Proinflammatory cytokines associated with T cell activation, gamma interferon (IFN-γ) and IL-15, were also found. Anti-inflammatory responses of IL-1 receptor antagonist (IL-1ra) were also upregulated in the monkeys (Table 1).

**Isolation of CDV from monkeys using Vero.DogSLAMtag cells.** Typical syncytia developed in monolayers of Vero. DogSLAMtag cells at as early as 2 days p.i. of spleen homogenates obtained from dead or moribund monkeys. However, syncytia were not observed in Vero cells inoculated with the spleen homogenates (data not shown). One of the CDV isolates was named CYN07-dV.

**Relationship between CYN07-dV and Chinese CDV strains associated with monkey outbreaks.** The entire genome nucleo-

![Image of double immunofluorescence staining](image)

**FIG 2** Double immunofluorescence staining of tissues of cynomolgus monkey 11 naturally infected with CDV in the 2008 outbreak. Tissue sections obtained from cynomolgus monkey 11 were examined by double immunofluorescence staining with anti-CDV-NP antibody and rabbit anti-cytokeratin, rabbit anti-neuron-specific β III tubulin, rabbit anti-CD3, or goat anti-nectin4 antibody. CDV-NP antigens were detected in the bronchi, bronchiole, lymph node, and brain. Some CDV-NP antigen-positive cells were positive for cytokeratin, nectin4, or CD3. A few CDV-NP antigen-positive neurons were positive for β III tubulin.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Monkeys naturally infected with CDV</th>
<th>Normal monkeys</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (pg/ml) SD No. positive/ no. tested</td>
<td>Median (pg/ml) SD No. positive/ no. tested</td>
</tr>
<tr>
<td>IL-1β</td>
<td>120  66  2/3</td>
<td>&lt;17  0  0/8</td>
</tr>
<tr>
<td>IL-6</td>
<td>94   72  3/3</td>
<td>&lt;9   0  0/8</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>228  78  3/3</td>
<td>84   48  8/8</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>157  61  3/3</td>
<td>40   36  7/8</td>
</tr>
<tr>
<td>MCP-1</td>
<td>3,917 2,286 3/3</td>
<td>461  245 8/8</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>2,121 1,096 3/3</td>
<td>413  203 8/8</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>509  150 3/3</td>
<td>261  183 3/8</td>
</tr>
<tr>
<td>IL-15</td>
<td>89   65  2/3</td>
<td>64   23  1/8</td>
</tr>
</tbody>
</table>

* Asterisks indicate statistically significant differences between monkeys naturally infected with CDV and normal monkeys (*, P < 0.05; **, P < 0.001).
The tide sequence of the CDV strain CYN07-dV was determined (DDBJ/GenBank accession number AB687720). A phylogenetic analysis of H protein indicated that CYN07-dV belongs to the Asia-1 clade and is closely related to monkey-BJ-01 and monkey-KM-01 strains (GenBank accession numbers FJ405223 and FJ405224, respectively) isolated from rhesus monkeys in China in 2008 (15) (Fig. 3). Comparative analyses throughout the genomes of CDV strains revealed that CYN07-dV showed the highest homology to the monkey-KM08 strain isolated from a rhesus monkey in China in 2008 (GenBank accession number HM852904) (15) (99.6%; 15,632/15,690 nucleotides). A phylogenetic analysis indicated that CYN07-dV showed high homology with CDV isolates from different animal species in China (NM strain isolated from foxes in China and 17 strains isolated from dogs in China), suggesting a Chinese source of the CYN07-dV strain.

Experimental infection of cynomolgus monkeys with CYN07-dV. Five cynomolgus monkeys were infected intranasally with CYN07-dV. In these animals, no lethal infection was observed during an experimental period of 15 days, and clinical symptoms were less severe than those observed in the moribund and dead monkeys during the outbreak. However, all five monkeys had appetite loss at 7 to 12 days p.i. Body weight was decreased in three of the five monkeys (Fig. 4A). The rectal temperature was transiently increased at 3 to 7 days p.i. in some monkeys (Fig. 4B). In all five monkeys, the numbers of white blood cells and lymphocytes were decreased (Fig. 4C and D), as the numbers of CDV-infected PBMCs were increased (Fig. 4E). Neutralizing antibodies against CDV were detected in the monkey sera at 7 days p.i., and then the titers of antibodies were raised at 10 days p.i. (Fig. 4F). Infectious CDV was isolated from various tissues of autopsied specimens at 15 days p.i., including local lymph nodes, lungs, liver, kidneys, intestinal tracts, and central nervous tissues of monkeys (Table 2, 3). In monkey 4965, giant cells were observed in the alveolar area of the lungs where the syncytial pneumocytes were positive for CDV antigen (Fig. 5A and B), and lymphocytes were depleted in the lymph nodes where the follicular cells and mononuclear cells were positive for CDV antigen (Fig. 5C and D). These histopathological findings were also observed in...
other monkeys but were less severe than those in monkey 4965 (data not shown). The experimentally CDV-infected monkeys showed upregulated levels of IFN-α and IL-1ra at 7 to 10 days p.i. Some monkeys showed upregulated levels of MIP-1β, MCP-1, eotaxin, IFN-γ, and IL-15 (data not shown).

The nucleotide and amino acid sequences of SLAM and nectin4 of cynomolgus monkeys. The nucleotide and deduced amino acid sequences of SLAM and nectin4 of cynomolgus monkeys were determined (DDBJ/GenBank accession numbers AB742520 and AB742522, respectively). Cynomolgus monkey

![FIG 4](https://example.com/figure4.jpg) Changes in body weight, rectal temperature, and the numbers of white blood cells, lymphocytes, and CDV-infected PBMCs and neutralization antibody level in the experimentally CDV-infected monkeys. (A) Body weight; (B) rectal temperature; (C) white blood cell count; (D) lymphocytes count; (E) CDV-infected PBMC count; (F) neutralizing antibodies against CDV.

### TABLE 2 CDV isolation from various organs in the experimentally infected cynomolgus monkeys at 15 days p.i.

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Skin</th>
<th>Respiratory</th>
<th>Liver</th>
<th>Kidney</th>
<th>Intestine</th>
<th>Spleen</th>
<th>Lymph node&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Thymus</th>
<th>Tonsil</th>
<th>Central nervous system</th>
</tr>
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<tbody>
<tr>
<td>4450</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
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<td>4571</td>
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<td>−</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>4969</td>
<td>−</td>
<td>−</td>
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<td>−</td>
<td>−</td>
<td>−</td>
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<td>+</td>
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<td>−</td>
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</tbody>
</table>

<sup>a</sup> −, CDV negative; +, CDV positive.

<sup>b</sup> Cervical and intestinal lymph node.
SLAM showed high levels of identity to rhesus monkey SLAM (DDBJ/EMBL/GenBank accession no. XM_001117605) and human SLAM (DDBJ/EMBL/GenBank accession no. U33017), with 99.9% (100%) and 97.6% (96.7%) nucleotide (amino acid) identity, respectively. Amino acid sequences of the SLAM were completely conserved among three macaques: cynomolgus monkey, rhesus monkey, and pig-tailed monkey (AB742521) (data not shown). On the other hand, cynomolgus monkey SLAM showed a lower level of identity to dog SLAM (DDBJ/EMBL/GenBank accession no. AF390108), with 76.5% (65.0%) nucleotide (amino acid) identity.

Cynomolgus monkey nectin4 showed identity to dog nectin4 (DDBJ/EMBL/GenBank accession no. AB755429), rhesus monkey nectin4 (DDBJ/EMBL/GenBank accession no. XM_001117709), and human nectin4 (DDBJ/EMBL/GenBank accession no. NM_030916), with 89.0% (94.1%), 100% (100%), and 98.3% (99.4%) nucleotide (amino acid) identity, respectively.

**CDV strain CYN07-dV utilizes macaca SLAM and macaca nectin4 as receptors.** The replication kinetics of CYN07-dV was analyzed in Vero, Vero.DogSLAMtag, Vero/macSLAM, Vero/hSLAM, Vero/dNectin4, and Vero/macNectin4 cells. In Vero and Vero/hSLAM cells, CYN07-dV replicated inefficiently (Fig. 6A). On the other hand, it replicated efficiently in Vero.DogSLAMtag, Vero/macSLAM, Vero/dNectin4, and Vero/macNectin4 cells. The peak titer and replication kinetics of the virus in Vero/macSLAM cells were comparable to those in Vero.DogSLAMtag cells (Fig. 6A). The peak titer of the virus in Vero/macNectin4 cells was also comparable to that in Vero/dNectin4, although virus production was slightly delayed in Vero/macNectin4 cells (Fig. 6A). The wild-type CDV strain Ac96I (13), isolated from

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Throat swabs</th>
<th>Rectal swabs</th>
<th>Feces</th>
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<tr>
<td></td>
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<tr>
<td>4970</td>
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*a* – CDV negative; +, CDV positive.

**FIG 5** Histopathological analyses of cynomolgus monkeys experimentally infected with CDV. Tissue sections obtained from cynomolgus monkey 4965 were examined by hematoxylin and eosin staining and immunohistochemistry using anti-CDV-NP monoclonal antibody. Giant cells (A) with CDV antigen (B) were observed in the alveolar area in the lung. Lymphocyte depletion (C) and CDV antigen-positive cells were observed in the follicular area (D) in the lymph node of CYN07-dV-infected monkey 4965. HE, ×20; IHC, ×40.
a sick dog, also replicated in the Vero/macSLAM cells; however, the appearance of synsytia was delayed in the Vero/macSLAM cells compared with that in the Vero.DogSLAMtag cells, and the peak titer of the virus in the Vero/macSLAM cells was significantly lower than that in the Vero.DogSLAMtag cells (data not shown).

To clarify whether the H and the F proteins of the virus induce syncytia in the cells expressing either SLAM or nectin4, cell-to-cell

FIG 6 CYN07-dV utilizes macaca SLAM and macaca nectin4 as receptors. (A) Growth kinetics of CYN07-dV in Vero.DogSLAMtag, Vero/hSLAM, Vero/macSLAM, Vero/dNectin4, Vero/macNectin4, and Vero cells. The cells were infected with the virus at an MOI of 0.01, and titers at the indicated points were shown. (B) Induction of syncytium upon transfection with a mixture of plasmids expressing enhanced green fluorescent protein (EGFP) and CDV F with or without a plasmid expressing CDV H. (C) Infectivity of VSV pseudotype bearing H and F proteins of CYN07-dV (VSVΔG*-F-dVH) or that bearing the F protein (VSVΔG*-F) in Vero.DogSLAMtag, Vero/hSLAM, Vero/macSLAM, Vero/dNectin4, Vero/macNectin4, and Vero cells.
fusion assay was performed upon transfection of the plasmids expressing the F and the H proteins of CYN07-dV. No syncytium formation was detected when the F protein of CYN07-dV alone was expressed in the cells (data not shown). On the other hand, many syncytia were observed in Vero/DogSLAMtag, Vero/macSLAM, Vero/dNectin4, and Vero/macNectin4 cells, but not in Vero and Vero/hSLAM cells, when the F and the H protein of CYN07-dV were expressed together (Fig. 6B). The syncytium formation was most remarkable in Vero/macSLAM cells (Fig. 6B). Although syncytia in nectin4-expressing Vero cells (Vero/dNectin4 and Vero/macNectin4) were smaller than those in SLAM-expressing cells (Vero/DogSLAMtag and Vero/macSLAM), syncytia in Vero/macNectin4 and Vero/dNectin4 cells were comparable (Fig. 6B).

To clarify the function of the H and the F proteins on entry of the virus via the SLAM and the nectin4, the infectivities of the VSV pseudotype bearing the H and the F protein of CYN07-dV, VSVΔG*-F-dVH, in various cells were compared. The VSV pseudotype bearing the F protein alone, VSVΔG*-F, did not infect any cells tested, whereas the VSVΔG*-F-dVH efficiently infected Vero/macSLAM cells and Vero/DogSLAMtag cells (Fig. 6C) but did not infect Vero/hSLAM cells. The VSVΔG*-F-dVH also efficiently infected Vero/macNectin4 cells and Vero/dNectin4 cells (Fig. 6C). These data showed that the CYN07-dV H protein efficiently utilizes macaca SLAM and macaca nectin4 as receptors.

**DISCUSSION**

Viruses in the genus *Morbillivirus* often cause severe diseases in animals and humans. Generally, symptomatic infection with each morbillivirus occurs in specific animal species or humans. Among the morbillviruses, MV is the one that causes an acute febrile and systemic infection in humans. Although CDV also shows host specificity and causes acute infections primarily in dogs, it often affects animals in different species, including nonhuman primates, showing a high mortality rate. In the CDV outbreak that occurred among Japanese monkeys in Japan in 1989, only one monkey out of 34 died (14). However, in recent outbreaks in China and Japan, higher mortality rates were recorded: 4,250 monkeys out of ~10,000 died in Guangxi Province in 2006, 12 out of 20 died in Beijing in 2008, and 46 out of 432 died in Japan in 2008. Most authorized animal suppliers in China receive monkeys from a Guangxi farm and distribute monkeys to researchers throughout the mainland of China. Laboratory investigations of clinical specimens from moribund and/or dead monkeys in the present study and in earlier studies (15, 16) fulfilled the two criteria of Koch’s postulates: (i) detection of CDV in sick animals and (ii) isolation of CDV in cultured cells. The experimental infection in the study further fulfilled the remaining postulates: (iii) induction of a comparable disease in the original host and (iv) reisolation of CDV from experimentally infected animals. These findings proved that CDV is the primary cause of the outbreak in monkeys. Moreover, the numbers of monkeys infected with CDV in the Guangxi farm decreased greatly after the introduction of attenuated CDV vaccination in early 2009, even though a few cases have still been reported every year (15).

In the present study, mortality was not observed in experimentally CDV-infected monkeys. However, many monkeys, especially those that eventually became CDV antibody-positive at the outbreak, showed mild or no symptoms. Severe symptoms were observed only in some moribund and dead monkeys in the outbreak. Thus, mortality might have been observed if more monkeys were experimentally infected, even though we could not rule out the possibility of enhanced pathogenicity of CDV by coinfection of some other agent during the outbreak. To date, no other particular agents were detected in the monkeys. However, we could not rule out the possibility that isolation and passage of the virus in Vero/dog.SLAM could have caused a partial attenuation of the virus. This may be clarified in future to analyze the quasispecies of genome sequences of the virus in the original clinical samples of the monkey and to compare them to the sequence of the isolated virus.

In the present study, three moribund monkeys in the 2008 CDV outbreak showed upregulated levels of proinflammatory cytokines and chemokines, such as IL-1β, IL-6, MIP-1α, MIP-1β, MCP-1, eotaxin, IFN-γ, and IL-15. Anti-inflammatory responses of IL-1ra were also upregulated. In the rhesus monkeys infected with measles virus, suppression of IL-12 in the sera was reported (20). The induction of IFN-γ, IL-2, and MCP-1 in the sera of the measles virus-infected cynomolgus monkeys was also reported (21). However, comparable levels of upregulation in proinflammatory cytokines, chemokines, and anti-inflammatory responses of IL-1 receptor antagonist observed in the CDV-infected monkeys were not reported for the measles virus-infected monkeys. These observations are rather similar to rhesus monkeys infected with a lethal dose of Zaire ebolavirus (22). The mixed anti-inflammatory response syndrome in Zaire ebolavirus-infected monkeys is characterized by highly elevated levels of IL-13 and IL-1ra, which are similar to the CDV-infected monkeys in the outbreak. Thus, unbalanced responses of cytokines and chemokines may have contributed to the pathogenesis of fatal cases of CDV infection in monkeys in the outbreak.

The CYN07-dV efficiently infected Vero cells expressing dog and macaca SLAM but not the cells expressing human SLAM. This was confirmed by syncytium induction upon transient expression of the H and the F proteins of the virus and also by infectivity of the VSV pseudotype bearing the H and the F proteins of the virus. On the other hand, the CYN07-dV efficiently utilized nectin4 of dog and macaca. Thus, the CYN07-dV is capable of utilizing macaca SLAM and macaca nectin4 as receptors, as efficiently as dog SLAM and dog nectin4, respectively. These findings were consistent with the experimental infection of the CYN07-dV to cynomolgus monkeys, in which the virus infected PBMCs and epithelial cells expressing macaca SLAM and nectin4, respectively. Interestingly, the CYN07-dV did not efficiently utilize human SLAM as a receptor even though the SLAMs of human and macaca are highly conserved. Thus, at the moment, CDVs like CYN07-dV may not be a direct threat to humans. However, the expansion of host animal species of CDV to include primates might be a global threat in the future. Wild-type CDV strains isolated from dogs with distemper were recently shown to efficiently utilize both dog SLAM and dog nectin4 as receptors (13, 17). However, the wild-type CDV strain Ac96I also replicated in the Vero/macSLAM cells even though less efficiently than in the Vero.DogSLAMtag cells. This suggested that some wild-type CDV strain is capable of utilizing macaca SLAM as a receptor per se, even though the CYN07-dV utilizes macaca SLAM more efficiently. Thus, the CYN07-dV is considered to be adapted to spread among monkeys using macaca SLAM and macaca nectin4.

**Nucleotide and amino acid sequence accession numbers.** The complete nucleotide sequence of the CYN07-dV has been deposited in GenBank under the following accession numbers: JN412454 (H) and JN412453 (F).
deposited in DDBJ/GenBank under accession number AB687720, while the amino acid sequence of hemagglutinin protein has been deposited in DDBJ/GenBank under accession number BAM15593. The complete nucleotide sequences of mRNAs of cynomolgus monkey SLAM, pig-tailed monkey SLAM, and cynomolgus monkey nectin4 have been deposited in DDBJ/GenBank under accession numbers AB742520, AB742521, and AB742522, respectively.

ACKNOWLEDGMENTS

We thank Yusuke Yanagi for providing Vero.DogSLAMtag and Vero/hSLAM cells.

This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan (grants H22-shinkou-ippan-006 and H24-kokui-shitei-003) and KAKENHI [Grant-in-Aid for Young Scientists (B), 22700459] from the Japan Society for the Promotion of Science (http://www.jsps.go.jp/english/).

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