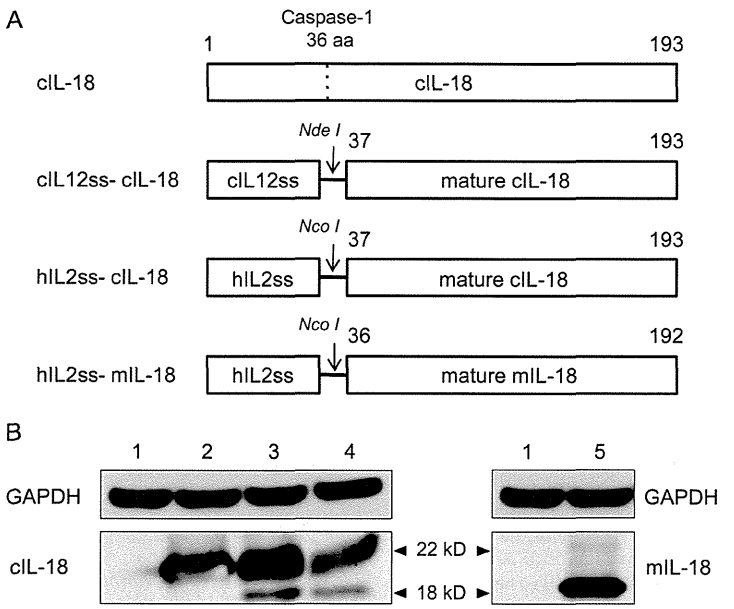

Fig. 1. Mammalian expression plasmids for canine and mouse IL-18. (A) Schematic representation of IL-18 constructs. From top to bottom: full-length sequence of canine IL-18, mature canine IL-18 fused to the canine IL-12 signal sequence, mature canine IL-18 fused to the human IL-2 signal sequence and mature mouse IL-18 fused to the human IL-2 signal sequence were inserted into the pCAGGS and pFuse–hIgG2–Fc2 vectors. (B) The expression of IL-18 by transfected 293T cells was analyzed with immunoblotting using anti-GAPDH, anti-cIL-18 and anti-mIL-18 antibodies. 1. Mock; 2. pCAG–cIL18; 3. pCAG–cIL12ss–cIL18; 4. pFuse–hIL2ss–cIL18; 5. pFuse–hIL2ss–mIL18.

with pET42-cIL18 was incubated at 37°C. When the optical density at 600 nm (OD_{600}) reached 0.4, expression of the recombinant protein was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside. After a 4-hr incubation, the *E. coli* was washed with PBS, suspended with sonication buffer (0.5% Triton X-100, 50 mM Tris-HCl [pH 8.0], 1 mM EDTA and 10 mM dithiothreitol) and sonicated with Sonifier450 (Branson, North Olmsted, OH, U.S.A.) for 5 min on ice. The lysate was incubated with 0.2 mg/ml lysozyme (Wako, Osaka, Japan), 10 μg/ml DNase I (Roche, Mannheim, Germany) and 1 mM $MgCl_2$ for 45 min at room temperature. The lysate was added with 7 mM EDTA, incubated for 30 min at 37°C and then centrifugation at 12,000 × *g* for 20 min. The pellet containing purified inclusion bodies of GST-cIL18 fusion protein (2.5 mg) was mixed with complete Freund's adjuvant (Difco, Sparks, MD, U.S.A.) and immunized into rabbit. After 2 weeks, the rabbit was immunized with 2.5 mg of GST-cIL18 mixed with incomplete Freund's adjuvant (Difco). After 2 weeks, the serum was collected. The specificity of the antiserum against cIL-18 was confirmed by immunoblotting (data not shown).

Immunoblotting: 293T cells were transfected with the mammalian expression plasmids using FuGENE®6 Transfection Reagent (Invitrogen), according to the manufacturer's instructions, and each cell lysate was subjected to immunoblotting analysis. Briefly, the lysates were separated with 12% SDS-PAGE, and the separated proteins were transferred onto an Immobilon-P membrane (Millipore, Billerica, MA, U.S.A.). The membrane was blocked with Block ACE reagent (DS Pharma Biomedical, Osaka, Japan) and then incubated with rabbit anti-cIL-18 antibody, rabbit anti-mIL-18 antibody (Invitrogen) or mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (GE Healthcare) overnight at 4°C. The membrane was washed 3 times with 0.05% Tween-PBS and incubated with a 1:2,000 dilution of horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin antibody or rabbit anti-mouse immunoglobulin antibody (Dako Cytomation, Glostrup, Denmark) for 1 hr at 37°C. The immunoreactive bands were detected with ECL Prime Western Blotting Detection Reagent (GE Healthcare). Chemiluminescence was scanned with a luminescent image analyzer (LAS-1000UV Minisystem; Fujifilm, Tokyo, Japan).

Recovery of recombinant viruses: To generate *r*CDV, we used reverse genetics to recover the recombinant virus previously established in *Paramyxovirus* [3, 23]. Plasmid pCDV containing the full-length cDNA of the RNA genome of the CDV Yanaka strain was constructed previously [10]. The entire coding region of cIL-18, hIL-2ss–cIL-18 and hIL-2ss–mIL-18 was amplified with primer pairs containing

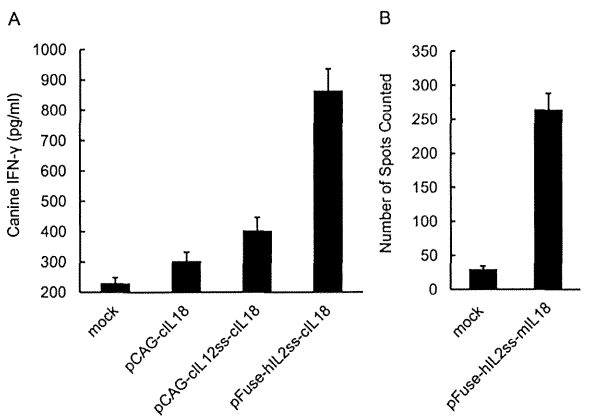

Fig. 2. Induction of IFN-γ by incubating canine or mouse immune cells with the supernatant from transfected cells. (A) The supernatant from transfected 293T cells or mock-transfected 293T cells was cocultured with canine PBMCs. After 48 hr, the supernatant was examined for IFN-γ production with an ELISA. Three independent experiments were performed (n=3). (B) The supernatant from transfected 293T cells or mock-transfected 293T cells was cocultured with mouse spleen cells in a polyvinylidene difluoride (PVDF)-backed microplate coated with monoclonal antibody specific for mouse IFN-γ in a humidified 37°C incubator for 48 hr. The supernatant was examined for IFN-γ production by counting the individual blue–black spots under a stereomicroscope. Three independent experiments were performed (n=3).

the CDV transcription signal unit and the *Fse*I restriction site: for entire cIL-18, *Fse*I-cIL18-CDV F and *Fse*I-cIL18-CDV R; for hIL-2ss–cIL-18, *Fse*I-hIL2ss-CDV F and *Fse*I-hIL2ss-cIL18-CDV R; and for hIL2ss-mIL18, *Fse*I-hIL2ss-CDV F and *Fse*I-hIL2ss-mIL18-CDV R (Table 1). The PCR products were inserted into the *Fse*I site in pCDV. The resulting full-genome plasmids were used to generate *r*CDVs with reverse genetics, as previously described [10]. In brief, HEK293 cells infected with vaccinia virus encoding T7 RNA polymerase were transfected with the full-genome plasmid, together with expression plasmids encoding rinderpest virus nucleoprotein (N), phosphoprotein (P) and large protein (L) (pKS–N, pKS–P and pGEM–L, respectively), using FuGENE®6 Transfection Reagent (Invitrogen). Two days later, transfected HEK293 cells were overlain with B95a cells. Syncytia were observed approximately 10 days later. The viruses were harvested, and their titers were determined with the limiting dilution method and expressed as the 50% tissue culture infective dose ($TCID_{50}$). To confirm IL-18 mRNA expression, the viral RNAs were extracted with Isogen reagent and reverse transcribed. PCR amplification was performed using the N gene primers (CDV-N F and CDV-N R, Table 1) or the cIL-18 or mIL-18 primers described above. Protein expression was confirmed with an immunoblotting analysis, as described above, using rabbit anti-N antibody [13].

Growth kinetics analysis: Monolayers of B95a cells in a 24-well plate were infected with virus at a multiplicity of infection of 0.01. The infected cells and their supernatants were harvested daily, and the titers of the released viruses and cell-associated viruses were determined as $TCID_{50}$ using standard methods.

In vitro bioassay for IL-18: Supernatants (100 μl) prepared from 293T cells transfected with the mammalian expression plasmids or B95a cells infected with CDVs were added to 10^5 canine PBMCs in 100 μl of culture medium in a 96-well plate. After 48 hr, the supernatant from each well was examined for canine IFN-γ with an ELISA (Quantikine Canine IFN-γ Immunoassay; R&D Systems, Minneapolis, MN, U.S.A.), according to the manufacturer's instructions. Alternatively, 50 μl of the supernatant was added to 10^6 mouse spleen cells in 50 μl of culture medium. After 48 hr, IFN-γ secretion was detected with the Mouse IFN-gamma ELISpot Kit (R&D Systems), and the frequency of mouse IFN-γ-secreting cells was quantified according to the manufacturer's instructions.

RESULTS

Bioactivity of recombinant IL-18 with different signal sequences in mammalian cells: First, we compared the secretion efficiency of the signal sequence with that of mature cIL-18. 293T cells were transfected with recombinant plasmids encoding entire cIL-18 (pCAG–cIL18) or mature cIL-18 fused to the cIL-12 signal sequence (pCAG–cIL12ss–cIL18) or to the human IL-2 signal sequence (pFuse–hIL2ss–cIL18) (Fig. 1A). The expression of cIL-18 protein in the cells was detected with an immunoblotting analysis. The proIL-18 (22 kDa) protein was detected in all the transfected cells, whereas mature cIL-18 (18 kDa) was detected in the cells transfected with pCAG–cIL12ss–cIL18 or pFuse–hIL2ss–cIL18, but not in cells transfected with pCAG–cIL18 (Fig. 1B).

To examine the bioactivity of the cIL-18 secreted from the transfected cells, the culture supernatant was cocultured with canine PBMCs, and the amount of canine IFN-γ produced by the PBMCs was measured. The supernatants of the cells expressing entire cIL-18 induced little IFN-γ production compared with the control, whereas the supernatants of cells expressing cIL-12ss–cIL-18 slightly enhanced the induction efficiency, as in the previous report [37]. Interestingly, the supernatant of cells transfected with pFuse–hIL2ss–cIL18 induced significant IFN-γ production (Fig. 2A). This result suggests that the human IL-2 signal sequence is more effective for expressing mature IL-18 *in vitro* than the canine IL12-p40 signal sequence. We then used hIL-2ss to express bioactive mouse IL-18 (Fig. 1A). We only detected mature mIL-18 in the pFuse–hIL2ss–mIL18-transfected cells (Fig. 1B). The supernatant from cells transfected with this plasmid also effectively stimulated mouse spleen cells to secrete IFN-γ (Fig. 2B). These results indicate that the human IL-2 signal sequence is suitable for the secretion of bioactive IL-18 protein from cells.

Generation of r*CDVs expressing canine or mouse IL-18*: To generate *r*CDVs expressing IL-18, we constructed CDV full-genome plasmids containing cDNA encoding the entire cIL-18, hIL-2ss–cIL-18 or hIL-2ss–mIL-18 sequence (Fig. 3A). The *r*CDVs were rescued from the plasmids as de-

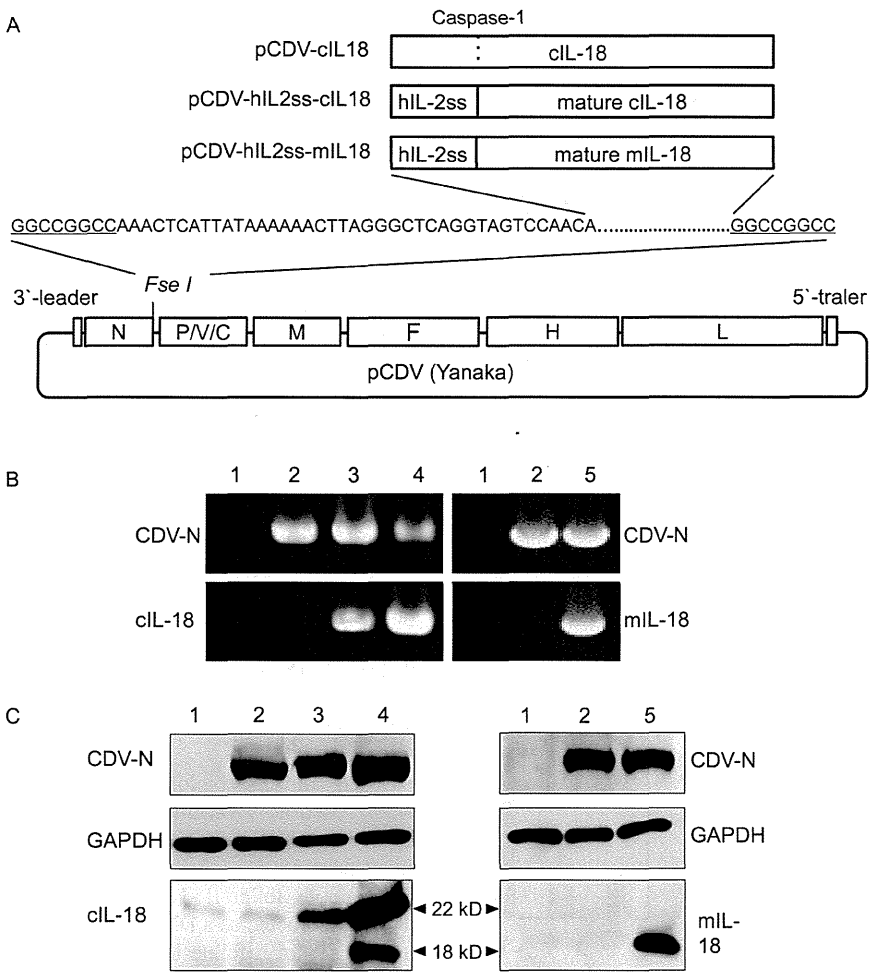

Fig. 3. Generation and *in vitro* characterization of *r*CDVs expressing mature canine or mouse IL-18 fused to the human IL-2 signal sequence. (A) Schematic model of the *r*CDV genome with the *Fse*I site introduced between the N and P genes and hIL2ss–IL18 inserted at the *Fse*I site. (B) The resulting viruses were harvested and identified by RT-PCR using primers complementary to the CDV-N gene and the canine IL-18 or mouse IL-18 gene. (C) Recombinant viruses were identified by immunoblotting analysis. Cell lysates were examined with anti-CDVN, anti-GAPDH, anti-cIL-18 or anti-mIL-18 antibody. 1: B95a; 2: parental CDV (Yanaka strain); 3: *r*CDV–cIL18; 4: *r*CDV–hIL2ss–cIL18; 5: *r*CDV–hIL2ss–mIL18.

scribed in the Methods section, and we successfully generated the *r*CDVs. The expression of mRNA from the inserted gene in each viral genome was confirmed by RT-PCR (Fig. 3B). To identify the IL-18 proteins expressed by the viruses, infected B95a cells were subjected to an immunoblotting analysis. Two bands corresponding to proIL-18 and mature IL-18 were detected (Fig. 3C), and each sample showed a similar band pattern, corresponding to that of the mammalian expression plasmids (Fig. 1B).

We then examined the growth kinetics of the *r*CDVs. As shown in Fig. 4, all the recombinant viruses showed slightly higher peaks for cell-associated virus than for released virus, and the maximum titers were similar to that of the parental Yanaka strain (Fig. 4). These results demonstrate that in B95a cells, *IL18* inserted into the CDV genome and expressed from recombinant viruses had no obvious effect on viral replication or growth kinetics.

Bioactivity of IL-18 produced by r*CDV*: To investigate whether the mature IL-18 protein expressed by *r*CDV was bioactive, we harvested the supernatants of the virus-infected cells and cocultured them with canine PBMCs. The supernatant contained infectious CDV released from the cells, but the parental CDV induced little IFN-γ production in the PBMCs (Fig. 5A) because CDV infection of canine PBMCs is inefficient [10]. The supernatant from the *r*CDV–cIL18-infected cells induced slight IFN-γ production, and

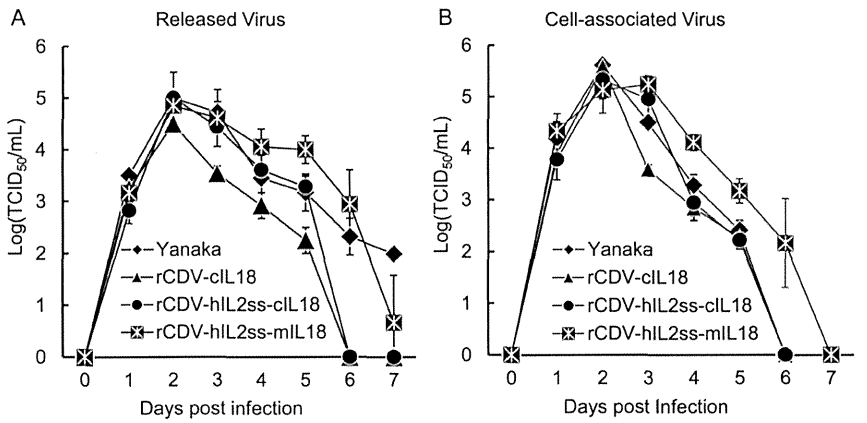

Fig. 4. Kinetics of recombinant viruses in B95a cells. The infected cells and supernatants were harvested separately every 24 hr after infection for 7 days. The titers of the viruses released into the supernatant (A) and the cell-associated viruses (B) were determined with a $TCID_{50}$ assay.

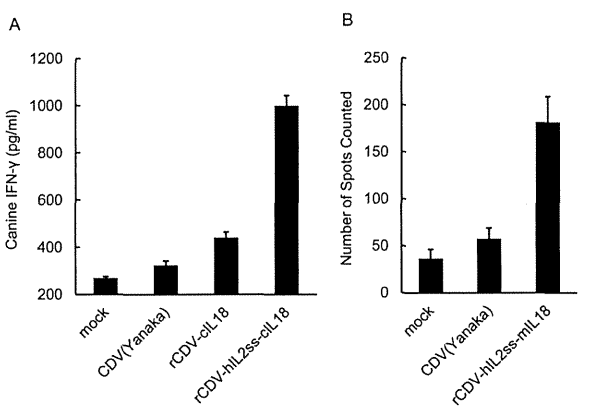

Fig. 5. Induction of IFN-γ production in canine or mouse immune cells after coculture with supernatant harvested from different recombinant-virus-infected B95a cells. (A) B95a cells were infected with parental CDV (Yanaka strain), *r*CDV–cIL18 or *r*CDV–hIL2ss–cIL18 for 48 hr. The supernatants were then harvested and cocultured with canine PBMCs for 48 hr. The supernatants were examined for IFN-γ production with an ELISA. Three independent experiments were performed (n=3). (B) B95a cells were infected with parental CDV (Yanaka strain) or *r*CDV–hIL2ss–mIL18 for 48 hr. The supernatants from the infected B95a cells or mock-infected B95a cells were then cocultured with mouse spleen cells in a PVDF-backed microplate coated with monoclonal antibody specific for mouse IFN-γ in a humidified 37°C incubator for 48 hr. The supernatants were then examined for IFN-γ production by counting the individual blue–black spots under a stereomicroscope. Three independent experiments were performed (n=3).

as expected, *r*CDV–hIL2ss–cIL18 induced significant IFN-γ production (Fig. 5A). Similarly, the supernatant from *r*CDV–hIL2ss–mIL18-infected cells also stimulated mouse spleen cells and induced IFN-γ production (Fig. 5B). The increased IFN-γ expression in PBMCs and splenocytes confirmed the biological activity of IL-18 secreted by *r*CDV–hIL2ss–cIL18- and *r*CDV–hIL2ss–mIL18-infected cells.

DISCUSSION

Based on its biological activity, IL-18 has been widely used as a vaccine adjuvant for infectious diseases [5, 6, 12, 18]. Various expression systems and cytokine signal peptides have been used to express bioactive IL-18. For example, feline IL-18 fused to human immunoglobulin κ was constructed as a DNA vaccine adjuvant and enhanced the efficacy of a feline leukemia virus DNA vaccine [14]. The signal sequence from the human IL-1β receptor antagonist protein fused to mature feline or equine IL-18 induced IFN-γ production in a KG-1 assay [24]. Porcine IL-18 fused to the baculovirus gp67-encoded signal sequence displayed bioactivity in a baculovirus system [22]. In the present study, we evaluated the utility of the human IL-2 signal sequence and demonstrated that it is more efficient than the previously reported canine IL12-p40 signal sequence [37] and that it can be used to express both canine and mouse IL-18.

The human IL-2 signal sequence contains 21 amino acids and shares the common characteristics of the signal peptides of other secretory proteins. Intracellular cleavage of the IL-2 signal peptide occurs after Ser20 and leads to the secretion of the antigenic protein. The signal sequences of canine and mouse IL-2 also consist of 20 amino acids, whereas the homology of the canine IL-2 signal sequence with that of the human sequence is 80% and that of the mouse IL-2 signal sequence is only 60%. Meanwhile, the human IL-2 signal sequence derived from the plasmid we used, pFuse–hIgG2–Fc2, can function in a variety of commonly used mammalian cells, from human cells to Chinese hamster cells. Therefore, *r*CDV–hIL2ss–cIL18 and *r*CDV–hIL2ss–mIL18 can secrete functional IL-18 after undergoing the proper processing in an appropriate host. The difference in the intracellular pro-

cessing efficiency of cIL-18 and mIL-18 (Figs. 1B and 3C) may be attributable to their respective primary sequences immediately downstream from the cleavage site (cIL-18: 37-YFGKLEPKLS, mIL-18: 36-NFGRLHCTTA). We will also examine the secretion efficiencies of cIL-18 and mIL-18 at protein level using ELISA in the future.

To date, divalent vaccines based on several recombinant viruses that encode CDV glycoproteins (hemagglutinin [H] and fusion [F]) have been investigated to protect against CDV and other pathogens. For example, vaccines based on recombinant vaccinia viruses or canarypox vectors engineered to express CDV glycoproteins have been tested in dogs and ferrets [30, 35]. These vaccines elicited protective immune responses, but it has yet to be determined whether the duration of these immune responses is equivalent to that of the responses induced with conventional live CDV vaccines [2]. It has been reported that 2 replication-competent canine adenovirus type 2 (CAV2)-based vaccines, expressing the CDV H and F antigens, protected dogs against CDV challenge [9]. It is well known that CAV2 vaccines are easily recovered from the respiratory tracts of sentinel dogs [7].

In the case of CDV, live attenuated vaccines are known to confer long-lasting immunity, and recombination of the CDV genome is impossible during the viral replication cycle. Therefore, the development of CDV-based multivalent vaccines is the most appropriate approach to the development of vaccines against CDV and other pathogens. A recent study demonstrated that an attenuated *r*CDV vaccine strain expressing RV-G conferred protective immunity against challenge with RABV in mice and dogs, providing particularly effective protection for more than a year in dogs [40]. This result confirms that CDV is a suitable vaccine vector.

Many recent studies have indicated that IL-18 plays an important role not only in host defenses against pathogens but also in immunotherapies for cancer [reviewed in 34]. Oncolytic virotherapy has been used as a novel strategy for cancer treatment. Interestingly, previous reports have shown that CDV and measles virus (MV), a human *Morbillivirus*, have oncolytic activity. CDV was shown to infect lymphoblasts isolated from canine lymphoma patients, leading to cell death by apoptosis [38]. In our previous study, we also demonstrated that recombinant MV, which was mutated so that it could not bind to the lymphoid-cell receptor SLAM, showed oncolytic activity against breast cancer *in vivo* [36]. Extrapolating from these data, we surmise that application of *r*CDV–hIL2ss–cIL18 plays a positive role in the cancer therapy using oncolytic activity of CDV.

In conclusion, we constructed an efficient secretion system of cIL-18 and succeeded in generating *r*CDV expressing bioactive IL-18. The recombinant CDV, *r*CDV–hIL2ss–cIL18, could offer a new approach to investigating the role of IL-18 in the host defense mechanism, the pathogenesis of infectious diseases and the treatment of cancer in the host.

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Characterization of two recent Japanese field isolates of canine distemper virus and examination of the avirulent strain utility as an attenuated vaccine

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ABSTRACT

Recently, several new strains of canine distemper virus (CDV) have been isolated in Japan. To investigate their pathogenesis in dogs, the Yanaka and Bunkyo-K strains were investigated by infecting dogs and determining clinical signs, amount of virus, and antibody responses. The Yanaka strain is avirulent and induced an antibody response. The Bunkyo-K strain induced typical CDV clinical signs in infected dogs and virulence was enhanced by brain passage. Molecular and phylogenetic analyses of H genes demonstrated the Bunkyo-K strains were of a different lineage from Asia-1 group including the Yanaka strain and Asia-2 group that contain recent Japanese isolates, which were recently identified as major prevalent strains worldwide but distinct from old vaccine strains. Based on these data, we tested the ability of the Yanaka strain for vaccination. Inoculation with the Yanaka strain efficiently induced CDV neutralizing antibodies with no clinical signs, and the protection effects against challenge with either old virulent strain or Bunkyo-K strain were equal or greater when compared with vaccination by an original vaccine strain. Thus, the Yanaka strain is a potential vaccine candidate against recent prevalent CDV strains.

1. Introduction

Canine distemper virus (CDV) is an enveloped, single-stranded, negative sense RNA virus in the genus *Morbillivirus* and family *Paramyxoviridae*, which includes measles virus (MeV) and rinderpest virus. CDV is highly infectious and causes an often-fatal systemic disease in dogs and other carnivores. CDV in dogs is generally transmitted as an aerosol infection to the upper respiration tract. Primary virus replication occurs in the lymphoid tissues leading to severe immunosuppression, and the incubation period may range from 1 to 4 weeks or more (Appel, 1969; Krakowka, 1982; Krakowka et al., 1980). Transient fever reaches a peak 3–6 days after infection and is associated with an initial virus spread throughout the body. At about 10 days post-infection, CDV spreads by cell-associated viremia from sites of primary replication to various epithelial tissues and the central nervous system (CNS) (Appel et al., 1982; Winters et al., 1983). Subsequent to

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epithelial infection, respiratory, intestinal and dermatological signs occur. The most serious complication is infection of the CNS, leading to a variety of neurological syndromes, frequently with a bad prognosis (Tipold et al., 1996).

CDV is a monotypic virus and a single exposure normally confers long-lasting immunity, similar to MeV. In general, the extensive use of live attenuated CDV vaccines introduced in the 1950s has drastically reduced the incidence of canine distemper in dogs. However, cases of CDV in vaccinated dogs have been reported since the 1990s, and the prevalence of sporadic cases and large outbreaks of CDV are increasing worldwide (Blixenkrone-Moller et al., 1993; Calderon et al., 2007; Ek-Kommonen et al., 1997; Haas et al., 1997; Jozwik and Frymus, 2002; Simon-Martinez et al., 2008). Sequencing analyses have demonstrated that most cases are considered to be caused by infection with newly prevalent strains of CDV, but not by reversion to virulence of vaccine viruses.

Recently, the number of CDV outbreaks has also increased in Japan in both dogs and wild animals (Iwatsuki et al., 2000; Kai et al., 1993; Lan et al., 2006; Shin et al., 1995; Uema et al., 2005). In particular, our previous epidemiological investigations revealed that 44 of 62 dogs (71%) clinically diagnosed with canine distemper from 1985 to 1994 in the Tokyo area were previously vaccinated (Gemma et al., 1996). Furthermore, among six new Japanese CDV strains isolated between 1992 and 1997, an antigenic region in the H protein responsible for neutralization was altered compared with current vaccine strains (Iwatsuki et al., 2000). To examine this phenomenon further, we investigated the virulence of two recent Japanese field isolates, the Yanaka and Bunkyo-K strains. We also examined the potential use of the Yanaka strain as a novel live vaccine against recent prevalent CDV strains.

2. Materials and methods

2.1. Ethical statement

All animal experiments followed the Regulations for Animal Care and Use of the University of Tokyo and were approved by the Animal Experiment Committee at The University of Tokyo. All surgery was performed under anesthesia with a Dormicum and Domitor, and all efforts were made to minimize animal suffering. At the end of the experimental period, the dogs were euthanized by anesthesia with a ketamine-xylazine combination followed by exsanguination.

2.2. Experimental animals

Female beagle puppies with ages varying between 4 and 17 weeks old (details described below), and confirmed to be free from CDV infection by anti-CDV antibody enzyme-linked immunosorbent assay (ELISA), were purchased from the Narc Co. (Chiba, Japan). Dogs were group-housed in cages with ample space provided for exercise. Groups of dogs were kept in strict isolation to prevent viral cross-contamination during the course of all experiments.

2.3. Cells and virus

Vero cells and B95a cells were cultivated at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) or RPMI 1640 containing 5% FCS, respectively (Kobune et al., 1990).

The Yanaka and Bunkyo-K strains were propagated in B95a cells as previously described (Iwatsuki et al., 1997). For challenge, the Snyder Hill strain, provided by Nippon Zenyaku Kogyo Co., Ltd (Fukushima, Japan), was intracerebrally inoculated into a 4-week old dog. After 7 days, homogenates of infected brain in phosphate-buffered saline (PBS) were used for testing as described below. The Onderstepoort strain, which was passaged in our laboratory (Yamanouchi et al., 1977), was grown in Vero cells and prepared as previously described (Gemma et al., 1995).

2.4. Clinical signs

Dogs were considered febrile if their body temperature increased more than 1 °C and leukopenic if white blood cells (WBC) decreased by more than 40% after challenge. Clinical signs specific for canine distemper (described below) were observed during virulence tests, counted daily and scored for diminished activity, depression, anorexia, lethargy, gastrointestinal signs (diarrhea, hematochezia), neurologic signs (convulsion, tremors, sialorrhea), and respiratory signs (sneezing, rhinitis, dyspnea).

2.5. Test for Yanaka strain virulence

To examine the virulence of the Yanaka strain, 500 μl of the virus solution diluted to 10^4 $TCID_{50}$/ml with maintenance medium were intracerebrally inoculated into six dogs with ages varying between 8 and 17 weeks old. Inoculated dogs were examined daily and rectal temperatures recorded.

The Yanaka strain (500 μl) was also inoculated intracerebrally into a weanling dog. After 7 days, a 20% (w/v) homogenate was produced from the brain and spleen in cold PBS. One dog was inoculated intracerebrally with 500 μl of brain homogenate and another intranasally with 500 μl and intravenously with 1 ml of spleen homogenate. Rectal temperature and clinical signs were monitored daily.

2.6. Test for Bunkyo-K strain virulence

Four weanling dogs (Nos.1-1 to 1-4) were inoculated intracerebrally with 500 μl of Bunkyo-K strain prepared in B95a cells at a titer of 10^4 $TCID_{50}$/ml, and examined daily for rectal temperature, leukocyte counts with a commercial kit (Unopette Test 58.56; Becton Dickinson), and clinical signs. Dogs were euthanized at 6 days post-infection (dpi) (1-2) or 7 dpi (1-4). At necropsy, the cerebrum, spleen, lung, and mesenteric lymph nodes were collected. Viral titers in 10% (w/v) homogenates of these tissues were determined in B95a cells and expressed as $TCID_{50}$/ml. Brain homogenates from infected dogs were inoculated intracerebrally into two additional dogs (Nos.

2-1 [7 weeks old] and 2-2 [6 weeks old]), and clinical sings were recorded. One dog (2-2) was euthanized at 8 dpi, and the brain was collected for a 10% (w/v) homogenate for subsequent challenge experiments.

2.7. Sequencing and phylogenetic analysis of the H gene of the Bunkyo-K strain

Total RNA was extracted from B95a cells infected with the Bunkyo-K strain using the Isogen reagent (Nippongene, Tokyo, Japan) following the manufacturer's instructions. A random 9-mer oligonucleotide was used for the reverse transcriptase (RT) reaction and three primer pairs, 1F (5′-AGGGCTCAGGTAGTCCAACA-3′) and 1R (5′-TTATAATGATACGGATAGGGGGA-3′), 2F (5′-GATGTCTTGACACCGCTCTT-3′) and 2R (5′-GTCTCCTCTACTTGCTTTGT-3′), and 3F (5′-CCTGCATTGGTCTCTGAGAA-3′) and 3R (5′-AACCATAGAAGGAAAGG-3′), were used for PCR. The H gene of the resulting RT-PCR products was sequenced using Big Dye terminator Cycle Sequencing System (Life Technologies, Carlsbad, California, USA). The sequence of 1824 nucleotides in the H gene open reading frame (ORF) was analyzed using Genetyx-Mac v10.0 (Software Development L.C., Tokyo, Japan). A phylogenetic tree based on the deduced amino acid sequences of the H proteins from Bunkyo-K, Convac (Z35493), Onderstepoort (AAG30920), Snyder Hill (AAG15490), black leopard (CAA87692), A92-6 (Q66000), raccoon (CAA87694), American dog (Z47762), Javelina (CAA87693), 111-03B (DQ494319), 2544 (CAB01252), Danish dog (CAA87690), 5804 (AY386315), CDTaichung (AY378091), NTU1 (DQ887547), Hamamatsu (BAA19585), Ueno (BAA19584), Yanaka (BAA19586), HM-3 (BAB39167), 26D (AB040766), 007Lm (AB212730), and 98-002 (BAA84208)) were used to construct a phylogenetic tree using ClustalX v2.1 and PHYLIP v3.6. The robustness of the grouping in the neighbor-joining analysis was assessed with 1000 bootstrap resampling.

2.8. Immunization and challenge testing

Animal experiments were conducted using 2 dogs per group for a total of 18 (Nos. 3-1 to 3-18) with ages varying between 3 and 5 weeks old (Tables 1 and 2). Dogs were subcutaneously inoculated with either the Yanaka or Onderstepoort strains, either on days 0 and 42 with 500 μl (titer of 10^4 $TCID_{50}$) or once with 500 μl (titer = 10^3 $TCID_{50}$). Unimmunized control dogs were inoculated with 500 μl of PBS.

Dogs were intracerebrally challenged with 10% brain homogenate infected with either the Snyder Hill or Bunkyo-K strain 14 days after the second immunization or 21 days after the single immunization. After challenge, the rectal temperature, leukocyte counts, and clinical signs were recorded daily as described above.

2.9. Measurement of neutralizing antibodies

CDV neutralizing titers in serum were determined using B95a cells in a standard $TCID_{50}$ assay. Briefly, serial two-fold dilutions (from 1:2 through 1:1024) of heat-inactivated sera in 96-well microtiter plates were added to a standard inoculum (10^2 $TCID_{50}$ units after incubation at 37 °C for 30 min) of the same virus strain, the Onderstepoort or Yanaka strain, diluted with media in quadruplicate. After incubation at 37 °C for 30 min, 5×10^4 B95a cells were added to each well. The plates were then incubated at 37 °C in 5% CO_2 for 7 days. The endpoint titer of each serum sample collected from dogs was expressed as the reciprocal of the highest dilution that protected 50% of the cells from viral cytopathic effect (CPE).

2.10. Virus isolation from peripheral blood mononuclear cells

Peripheral blood was collected from the jugular vein into heparinized tubes and separated by Ficoll-Paque (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) to obtain peripheral blood mononuclear cells (PBMCs). Red blood cells were lysed using ammonium chloride and washed twice in cold PBS. PBMCs were diluted in RPMI-1640 medium containing 2% FCS and co-cultivated with B95a cells for seven days. Viruses were detected by observing CPE.

2.11. Tissue processing

The cerebrum, spleen, urinary bladder, mesenteric and popliteal lymph nodes were collected aseptically from dogs euthanized at 14 dpi with either the Snyder Hill or Bunkyo-K strain and fixed in 4% paraformaldehyde. Aseptically collected tissues were homogenized from each dog and serially diluted in PBS at dilutions ranging from 10^{-1} through 10^{-5}. Total RNA was extracted and subjected to RT-PCR using primer pair 3F and 3R as described above. Paraformaldehyde-fixed tissues were processed for paraffin embedding. Consecutive sections of each tissue were mounted on slides, stained with hematoxylin and eosin (HE), and observed via light microscopy (Olympus Corporation, Tokyo, Japan).

3. Results

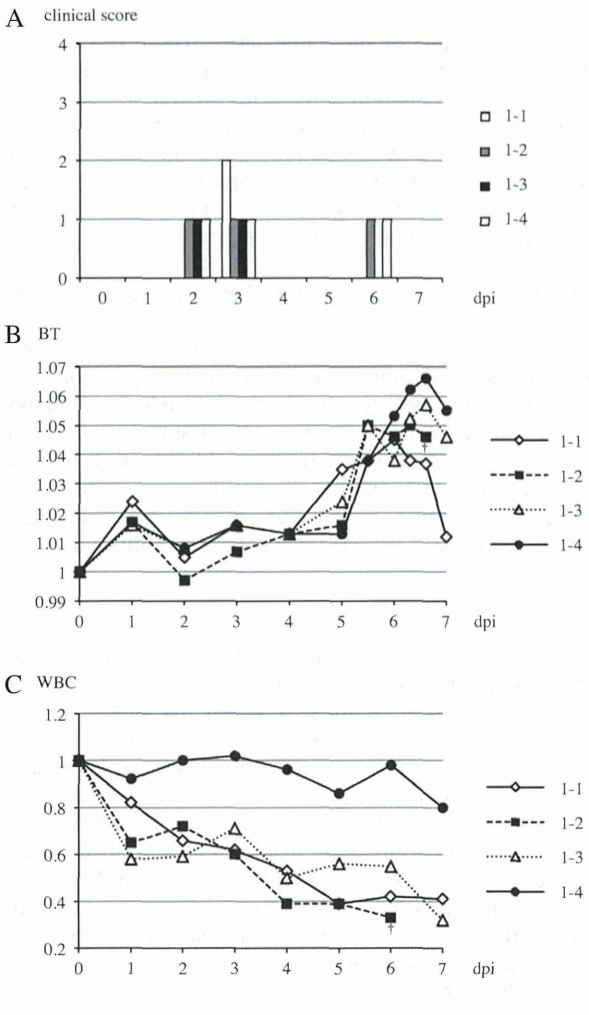
3.1. The Yanaka strain is avirulent in dogs

The Yanaka strain was first isolated in 1994 using B95a cells (Iwatsuki et al., 1997). Although six puppies inoculated intracerebrally with virus passaged three times in B95a cells did not develop clinical signs, all dogs developed antibody responses to CDV in sera (data not shown).

Previous reports demonstrated that CDV virulence was diminished when CDV adapted to cell culture (Hamburger et al., 1991). Therefore, to determine virulence of the Yanaka strain, the virus was passaged intracerebrally in a weanling dog, and brain or spleen homogenate was inoculated into two additional dogs. The inoculation induced no clinical signs of distemper including fever and weight loss in any of the dogs (data not shown), and no virus was detected in PBMCs (data not shown). These results demonstrate the Yanaka strain is avirulent.

3.2. The Bunkyo-K strain is virulent in dogs

The Bunkyo-K strain was isolated from the brain of a Japanese dog with canine distemper in 1995. Four dogs



Dog No.	Cerebrum	Spleen	Lung	Mesenteric lymph nodes
1-2	$10^{3.7}$	10^{3}	10^{2}	10^{4}
1-4	10^{4}	10^{3}	$10^{2.5}$	$10^{2.7}$

Fig. 1. Clinical signs and virus titers in dogs inoculated with the Bunkyo strain propagated in B95a cells. (A) Clinical signs, (B) body temperature [BT], (C) total white blood cell count [WBC], and (D) virus titers from dogs 1-2 and 1-4. Dog 1-2 was euthanized at 6 dpi, indicated by †.

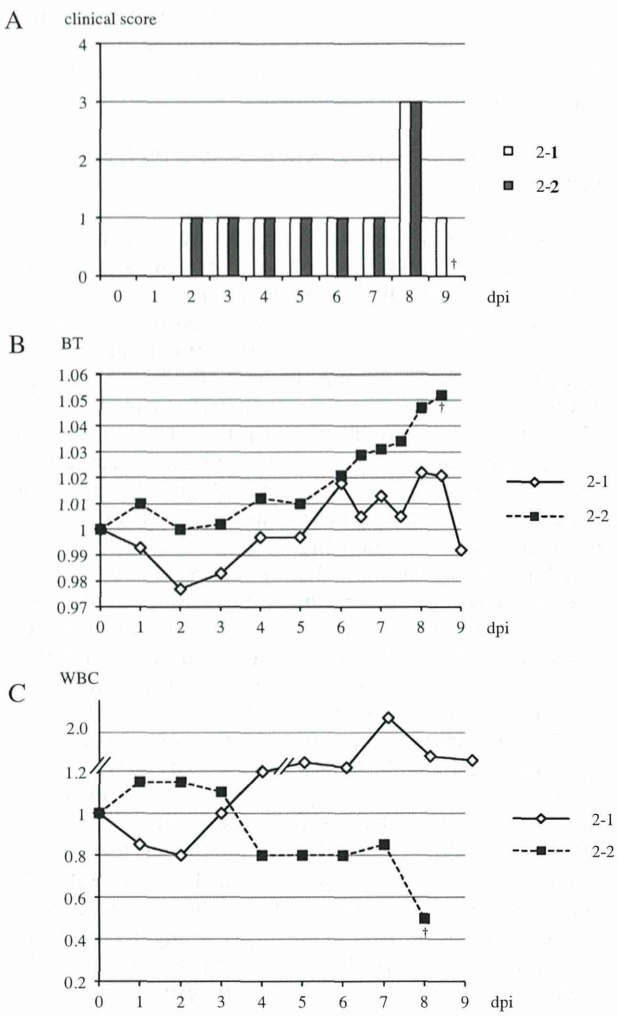


Fig. 2. Clinical signs in dogs inoculated with the Bunkyo-K strain passaged in dog brain. (A) Clinical signs, (B) body temperature [BT], and (C) total white blood cell count [WBC]. Dog 2-1 became moribund and was euthanized at 8 dpi, indicated by †.

were inoculated intracerebrally with the Bunkyo-K strain grown in B95a cells. As shown in Fig. 1A, mild gastrointestinal signs (diarrhea and hematochezia) were observed in all dogs at 2–3 dpi, and tremors occurred in two dogs at 6 dpi. All dogs showed elevated body temperature at 5–6 dpi, and three dogs had a 40% decrease in WBC (Fig. 1B and C). Virus titers in tissues collected at 6–7 dpi ranged from 10^2 to 10^4 $TCID_{50}$ (Fig. 1D). Therefore, Bunkyo-K replicates well in tissues of infected dogs and maintains virulence even after passage in B95a cells. To increase the virulence, we passaged the virus in dog brain. Brain homogenate of dog 2-2 in Fig. 1 was intracerebrally inoculated into two additional dogs. One dog developed clinical signs, including diarrhea starting on day 2, sneezing, rhinitis, and mild depression at day 8. This dog recovered on day 9 (Fig. 2). The other dog developed severe disease with fever, leukopenia from 4 dpi, diarrhea from 2 dpi, dyspnea, depression, anorexia, and became moribund at 8 dpi (Fig. 2).

These results demonstrate that the Bunkyo-K strain is virulent in dogs. In particular, severe clinical signs were observed in dogs inoculated with brain-passaged virus, suggesting the Bunkyo-K strain might be useful as a recently isolated challenge virus because of its high virulence in dogs.

3.3. Sequence analysis of the Bunkyo-K H gene

We previously described the nucleotide sequence of the H gene of the Yanaka strain as belonging to a recent prevalent group, Asia-1, that is divergent from the vaccine group (Iwatsuki et al., 1997). The Bunkyo-K strain showed 92.7–93.1% nucleotide identity to the America-1 CDV